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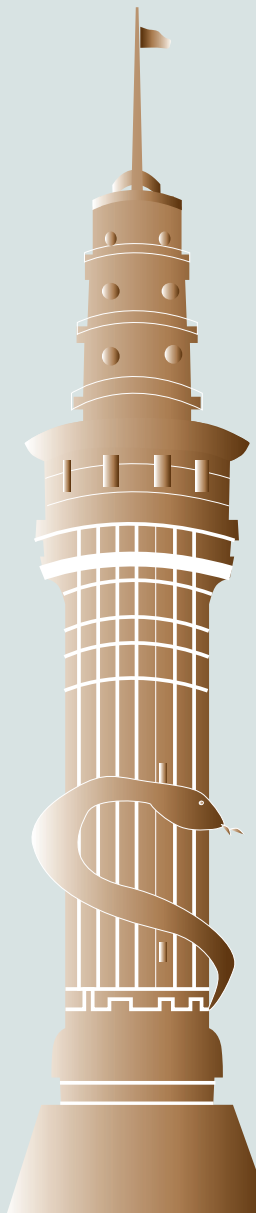

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İstanbul Journal of Pharmacy

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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.

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

The editorial and publication processes of the journal are shaped in accordance with the guidelines of the International Committee of Medical Journal Editors (ICMJE), World Association of Medical Editors (WAME), Council of Science Editors (CSE), Committee on Publication Ethics (COPE), European Association of Science Editors (EASE), and National Information Standards Organization (NISO). The journal is in conformity with the Principles of Transparency and Best Practice in Scholarly Publishing (doaj.org/bestpractice).

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- Grant information and detailed information on the other sources of support,
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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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All acronyms, abbreviations, and symbols used in the manuscript must follow international rules and should be defined at first use, both in the abstract and in the

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

main text. The abbreviation should be provided in parentheses following the definition.

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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)"

All references, tables, and figures should be referred to within the main text, and they should be numbered consecutively in the order they are referred to within the main text. Limitations, drawbacks, and the shortcomings of original articles should be mentioned in the Discussion section before the conclusion paragraph.

REFERENCES

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İstanbul Journal of Pharmacy complies with APA (American Psychological Association) style 6th Edition for referencing and quoting. For more information:

- 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.

If more than one citation is made within the same paranthesis, separate them with (;).

Samples:

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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CONTENTS

ORIGINAL ARTICLES

SHORT PAPER

Istanbul J Pharm 50 (2): 71-78
DOI: 10.26650/IstanbulJPharm.2019.0052
Original Article

Development and validation of HPTLC method for estimation of dofetilide in pharmaceutical dosage form and determination of its degradation profile by MS-MS method

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Cite this article as: Bhole, R., Chadar, K., Zambare , Y., & Bonde, C.G. (2020). Development and validation of HPTLC method for estimation of dofetilide in pharmaceutical dosage form and determination of its degradation profile by MS-MS method. *İstanbul Journal of Pharmacy, 50* (2), 71-78.

ABSTRACT

Background: Dofetilide is the class III antiarrhythmic drug used as a potassium channel blocker approved by US FDA in 1999 for the maintenance of sinus rhythm in individuals prone to atrial flutter and atrial fabrication. Currently there is no HPTLC-MS method reported for systematic characterization of degradation products dofetilide.
Methods: As per the ICH guidelines, the HPTLC method for the determination of dofetilide both in bulk and pharmaceutical formulation has been developed and validated. The degradation products were identified and characterized by using MS/MS. The Rf value was found to be 0.52±0.3. The degradation study was performed as per ICH guidelines (Q2R1). Isolation of the degradation product by HPTLC method and categorized by MS/MS method.
Results: The linearity of the method was found suitable over the range 100-600 ng/band with r^2 of 0.998. Dofetilide was subjected to stability studies, the drug was found to degrade under various stress conditions. The recovery was found in the range of 98-101%. HPTLC-MS/MS method showed a possible degradation mechanism of 7 degrading products. The degradation of the drug under various stress conditions indicates the storage conditions for the drug and drug product during its shelf life.
Conclusion: The HPTLC method developed for its linearity, range, precision studies, LOD and LOQ can be used for the routine quality control of the drug dofetilide in bulk drugs. The degradation pathway of a drug can help in the future to identify the impurities and for the impurity profiling of dofetilide.

Keywords: Stability indicating, HPTLC, HPTLC-MS/MS degradation studies, dofetilide

INTRODUCTION

Dofetilide (Figure 1) is the class III antiarrhythmic drug used as a potassium channel blocker approved by US FDA in 1999 for the maintenance of sinus rhythm in individuals prone to atrial flutter and atrial fabrication with a very potent dosage form in 0.25, 0.250 and 0.500 mg capsules of Dofetilide. (Wells, Khairy, Harris, Anderson, & Balaji, 2009; Krafte, & Volberg, 1994; Woolf, Miler, & Gosting, 1962). This dosage form is manufactured by Pfizer. (Wells et al., 2009; Aktas, Shah, & Akiyama, 2007; Al-Dashti, & Sami, 2001). The method refined by HPTLC was vali-

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dated for linearity range, interday and intraday precision, Limit of Quantitation (LOQ) and Limit of Detection (LOD) according to ICH Q2R1 guidelines. (2005: ICH Guidelines)

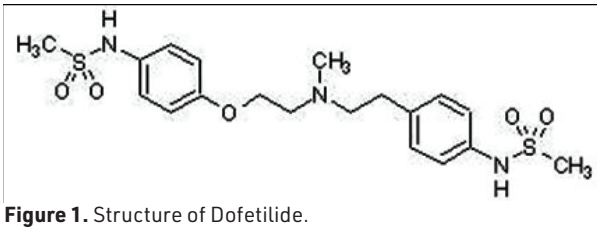

Figure 1. Structure of Dofetilide.

Dofetilide is a drug that can target potassium channels present in the cardiac region with good potency. (Bhole, Naksarkhre, & Bonde, 2019; Udin, Sung, Hunge, & Hu, 2019; Qile, Henriett, & David, 2019; Chi, Liu, & Wang, 2017; Kaddar, Pilote, Wong, Caillier, & Patoine, 2013; Mounsey, & DiMarco, 2007; Clusin, 2003), this can be an alternative therapy or considered as an aid for the currently available methods (catheter-based ablation and alternative pharmacological approach for the treatment of atrial arrhythmias.) According to ICH guidelines - Q1A (R2) the degradation of the drug under various stress conditions may help for the determination of stability of the drug. The forced degradation studies of drug substance or product should be evaluated for the development of stability-indicating methods. As per the prescribed guidelines, 20% degradation is within the acceptable range. As per the Literature review there are some methods reported for quantification of Dofetilide in pure form or in its pharmaceutical dosage form. (Udin et al., 2019; Chi et al., 2017; Bhole, Shinde, Chitlange, & Wankhede, 2015). On the other hand, high performance thin-layer chromatography (HPTLC) is a promising sustainable alternative to HPLC in some analysis. As HPTLC, separations has several advantages, it takes a short time for analysis. Moreover, it requires few nanoliter injection volumes. Furthermore, minimal use of solvent and no prior extraction steps compared to HPLC. However, There are very few HPTLC determination methods are available for the drug dofetilide as per literature. Some of these methods also report the degradation behavior under some stress conditions. As the solvent used in these methods are not very cost effective. It was also noted that there is an emerging need to perform a systematic characterization of degradation products by using MS-MS technique (Bhole, Biradar, & Bonde, 2018). So the current study was performed to develop selective and highly sensitive, specific, cost-effective and precise HPTLC method for determination of Dofetilide in presence of their degradation products and also in its dosage form. Moreover, the isolated stressed sample of Dofetilide was characterized by MS/MS method and also its possible degradation pathway was proposed.

MATERIALS AND METHODS

Chemicals and Reagents

Toluene, methanol, triethylamine, dofetilide

Instrumentations

Precoated aluminium plates with silica gel 60 F254 plates (Merck, Germany; supplied by Merck India, Mumbai, India). Camag Linomat V (Muttenz, Switzerland). The pressure requirement for the sample application is 4 µL. Dimension 360 mm x 510 mm x 410 mm (Width x Length x Height)]. Camag 100 µL syringe (Hamilton, Bonaduz, Switzerland). Camag twin trough glass chamber (10 x 10 cm and 10 x 20 cm). It has the dual-wavelength function (254 and 366 nm) [Dimension: 477 mm x 343 mm x 285 mm (Length x Width x Height)].

Chromatographic Conditions

4 µL of sample and standard solutions are applied on the TLC plate by using Camag Linomat V automatic sample applicator in the form of band (band with: 6 mm, and the distance is 5.6 mm between two bands) using micro-syringe. In twin trough glass chambers, the plates were saturated (10 minutes) with the mobile phase of composition toluene: methanol: triethylamine (7:2.5:0.5, v/v/v). Ascending development was performed up to a distance of 8 cm by placing the plates in the mobile phase. Later the development, the plates were dried in air and a densitometric scanning (slit dimensions: 5 × 0.45) was performed at 231 nm using Camag TLC scanner III operated in reflectance–absorbance mode (Bhole et al., 2018; Bonthagarala, 2003).

Analysis Formulation

Preparation of Standard Stock Solution

Accurately weighed 12.5 mg Dofetilide, transferred to 100.0 mL volumetric flask, and dissolved in AR grade 25 methanol by ultra-sonicating for 10 min and volume was made up to the mark using the methanol to give a stock solution of concentration 0.125 mg/mL or 125 µg/mL. Further dilution 8 ml stock solution to 10 mL (concentration100 µg/mL) to above solution of dofetilide was applied on the TLC plates in the range 0.1 to 0.6 µL i.e. 100 – 600 ng/band of dofetilide with the help of Hamilton syringe using LINOMAT-V automatic sample applicator. The plate was then developed in an optimized mobile phase. Different mobile phase combinations were tested and finally this ratio selected for the method development on HPTLC. The mobile phase ratio is methanol: toluene: triethylamine (7:2.5:0.5) was selected as it gives good resolution and peak symmetry for dofetilide. The Rf value for dofetilide was found to be 0.52 respectively (Fegade, Bhole, Patil, & Chaudhari, 2009; Rakibe, Tiwari, Mahajan, Rane, & Wakte, 2018).

Preparation of Sample Solution

(Each capsule contains 0.500 mg of Dofetilide) 25 capsules shells were opened and the powder was weighed equivalent to 12.5 mg (13.75 mg with excipients) of the Dofetilide sample into a 100 ml clean volumetric flask added about 40 ml of diluents and sonicated up to 10 min to completely dissolve and diluted up to the mark with diluents. The final concentration of the stock solution was 125 µg/ml. then further dilution. Further dilution 8 ml to 10 ml (concentration 100 µg/ml). (Jadhav, Nimbalkara, Mathad, & Mali, 2013).

Selection of Working Wavelength (λmax)

The UV spectrum of 4 µg/mL of Dofetilide in methanol, the spectrum was recorded by scanning in the range (200 nm to 400 nm). From the UV spectrum wavelength selected as 231 nm. The spectrum was shown in Figure 2 (Shivashankar, & Gandhimathi, 2005).

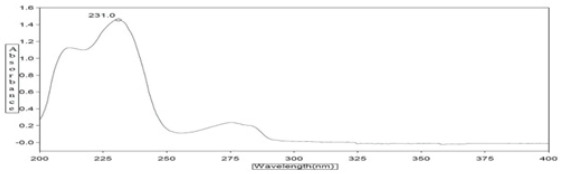

Figure 2. UV-VIS spectrum was found to be 231 nm for Dofetilide.

Method Validation

The method was validated in compliance with ICH guidelines.

Linearity

The linearity of the method was evaluated at the five equal-spaced concentrations (Ceresole, Moyano, Pizzorno, & Segall, 2007) by diluting the standard stock solution to give solution over the range of 100-600 ng/band of dofetilide. A calibration curve was constructed at six linear concentrations of dofetilide (0.1 to 0.6 µg/mL). solutions were injected into the chromatographic system, after getting the results plotted a graph concentration versus an area to evaluate correlation coefficient.

Acceptance criteria: Correlation coefficient Not less than 0.99.

Accuracy & Recovery

Accuracy solutions prepared into three levels (80%, 100%, and 120%). For each level the preparations were prepared individually. 80%, 100% & 120% Solutions were prepared with different drug weights with a constant weight of placebo in the manner of sample preparation.

Acceptance Criteria: % Recovery should be 98.0 to 102.0 Acceptance Criteria: % RSD for nine preparations recover values should be ≤ 2.0

Precision

Method precision, validation parameter investigated using the six individual sample preparations as reported above. Six samples were injected individually into the chromatographic system and calculated the % assay of individual samples. Repeatability and Intermediate precision (Intraday and Interday precision).

Acceptance Criteria: % Assay should be 95.0 to 105 & %RSD for six preparations assay should be ≤ 2.0.

Robustness

To evaluate the robustness of the proposed method, small but deliberate variations in the optimized method parameters were done. By introducing small changes in the mobile phase composition, mobile phase volume and duration of chamber saturation with the mobile phase, the effects on the Rf value of drugs were examined. The composition of the mobile phase was changed slightly (± 0.1 mL for the component). Robustness of the method was performed as per the standard guidelines.

Acceptance Criteria: System suitability should be within the acceptance criteria: System suitability should be within the acceptance criteria.

Limit of LOD and LOQ

The LOD and LOQ of the developed method were calculated by using the standard method

Acceptance Criteria: ≤ 2

Forced Degradation Studies

The stability of the drug was studied at various stress conditions as indicated by the ICH guidelines Q1A (R2) for acid hydrolysis with 0.1 M HCl, base hydrolysis with 0.1M NaOH, 3% hydrogen peroxide for oxidation, thermal degradation at 40°C, 60°C, 80°C, and photolysis. Accurately weighed the quantity of capsule powder equivalent to about 12.5 mg of Dofetilide was transferred separately to six different 10.0 ml volumetric flask, (flask no. 1, 2, 3, 4, 5 and 6). To flask no.1, 2 and 3, followed by the addition of 3.0 mL of 0.1 M HCl, 3.0 mL of 0.1 M NaOH, 3 mL water for neutral hydrolysis and 3% H_2O_2 respectively. The content of flask no. 1, 2, 3 and 4 were heated in a water bath at 80°C for 1 hrs 30 min, 30 min, 1 hr and 2 hrs 30 min respectively. The flask no. 5 containing powder kept in a hot air oven at 600°C for 10 min to study the effect of heat on the sample (heat degradation). Flask no. 6 containing powder was exposed to UV-radiations for 72 hrs to study the effect of light on the sample (photodegradation). The Whatman filter paper no 42 is used for filtration. From the filtrate, the 1.0 mL solution was diluted to 10 mL with the mobile phase. The diluted solution was analyzed similarly as described under the analysis of marketed formulation. The typical dendrogram was shown in Figure 3.

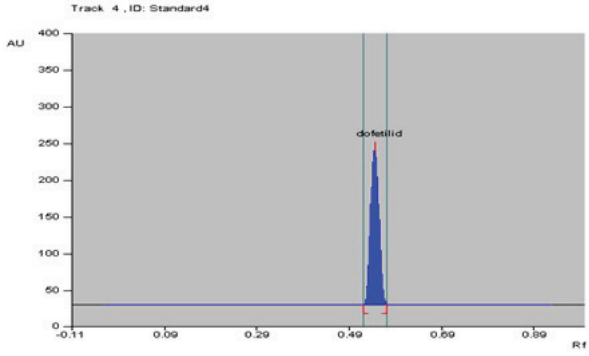

Figure 3. Typical densitiogram of Dofetilide retention factor: 0.52 (±0.03).

RESULT AND DISCUSSION

These studies were performed to develop a sensitive method, and an economically and less time-consuming HPTLC technique, which may be implemented for the determination of Dofetilide in pharmaceutical formulation. The HPTLC method was developed as per the stated method. Such as a large number of samples handle easier to scan the band and the detector response is higher. The chromatographic saturation is 10 minutes. Many trial and error methods were tried by using various solvents with altering polarity and in the diverse extent to obtain superior resolution and sharp peaks with acceptable Rf values (0.1–0.6 µg/band). Amongst the various mobile phase blend tested, mobile phase consisting of toluene: methanol: triethylamine (7:2.5:0.5 v/v/v) shows enhanced resolution and sharp

peaks with Rf values of 0.5205±0.05 of dofetilide. The linearity graph and linearity table are shown in Figure 4 and Table 1.

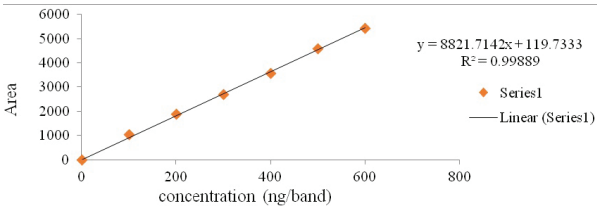

Figure 4. Linearity graph.

Table 1. Calibration parameter.

Parameters	Dofetilide
Linearity Range	100ng/band
Linearity Equation	Y=8221.742x+119.7333
Correlation Coefficient	0.998

Accuracy (Recovery)

To determine the accuracy of the said methods, accuracy studies were carried out as per ICH guidelines. The determination was performed in triplicate at each level was shown in Table 2.

Table 2. Recovery study.

Level of recovery	% Recovery*	S.D.	R.S.D.
80%	98.93	0.29	0.29
100%	99.37	0.45	0.46
120%	99.65	0.12	0.12

***Mean of three determinations**

Precision

The precision study was performed as per the standard method. The % RSD for intraday and interday precision is less than 2, indicating the precision of the method as shown in Table 3.

Table 3. Precision of developed method.

Precision parameter	% label claim	S.D.	%R.S.D.
Repeatability	98.03	1.12	1.14
Intraday	99.70	0.10	0.10
Interday	99.20	0.20	0.20

*Mean of three determinations

Robustness

The mobile phase composition changed within a range of ±1 ml. Moreover, the chamber saturation time was varied in the range of and ±2.5 min, respectively. The effect of these changes on both the Rf values and peak area was shown in Table 4.

Table 4. Result of robustness study

Robustness study			
Factor	**Level**	**Area**	**Rf**
Mobile Phase Composition (±0.1 ml)			
6.9:2.6:0.5	-0.1	3670	0.53
7:2.5:0.5	0	3660	0.52
7.1:2.4:0.5	+0.1	3675	0.55
%RSD		0.21	
Duration for Chamber saturation (±5 min)			
5 min	-5 min	3670	0.49
10 min	0 min	3675	0.52
15 min	+5 min	3690	0.57
Volume of mobile phase (±1 ml)			
9.0 ml	-1 ml	3699	0.50
10.0 ml	0 ml	3674	0.52
11.0 ml	+1 ml	3620	0.52

*Mean of three determinations

LOD and LOQ

Limit of detection and Limit of quantification were done separately and found to be 0.17 ng/band for dofetilide. LOQ was found to be 0.51 ng/band for Dofetilide.

Analysis of Formulation

Twenty-five capsules (Tikosyn) were weighed and crushed to obtain a fine powder. The average weight of the capsule was calculated. Accurately weighed the quantity of capsule powder equivalent to about 500 mcg of Dofetilide. The content of the drug was close to 100%. The result was summarized in Table 5 and 6.

Table 5. Marketed formulation

	Tikosyn		Label claim: 500 mcg	
Sr. No.	**Wt. of capsule powder (mcg)**	**Peak area***	**Amount found (mcg)**	**% label claim**
1.	550	3669	492	98.40
2.	550	3637	480	96.00
3.	550	3680	490	98.00
4.	550	3669	490	98.00
5.	550	3680	497	99.40
6.	550	3643	492	98.40

*Mean of three determinations

Table 6. Statistical validation for analysis of marketed formulation

Drug	Amount of drug found (mcg)	% Label claim*	S.D. (±)	R.S.D.
Dofetilide	490.16	98.03	1.12	1.14

* Mean of six determination

Forced Degradation Studies

Dofetilide was found to degrade in various stress conditions (acid, alkaline, and oxidation). Utmost degradation was observed in the said conditions, (alkaline, acid, neutral, heat, and photodegradation). Percentage assay of active substance along with Rf values of degradation products is summarized in Table 7 and Figures 5-10. (alkaline, acid, neutral, heat, and photodegradation stress conditions). Acid degradation shows two peaks.

Table 7. Result of degradation studies

Sr. No.	Stress Condition	Temperature and Time	% assay of active substance	Rf of degraded product
1	Acid (0.1 M HCl)	80°C for 1hr 30 min	89.96	0.03, 0.60
2	Alkali (0.1 M NaOH)	80°C for 30 min	86.96	0.07, 0.20 0.58
3	Neutral (H_2O)	80°C for 1 hr	91.89	0.07, 0.12, 0.16, 0.23
4	Oxide (3 % H_2O_2)	80°C for 2hr 30 min	91.64	0.08
5	Thermal	60°C for 10 min	91.25	0.08, 0.16, 0.23
6	Photo Degradation	24 hr	90.71	0.60, 0.64

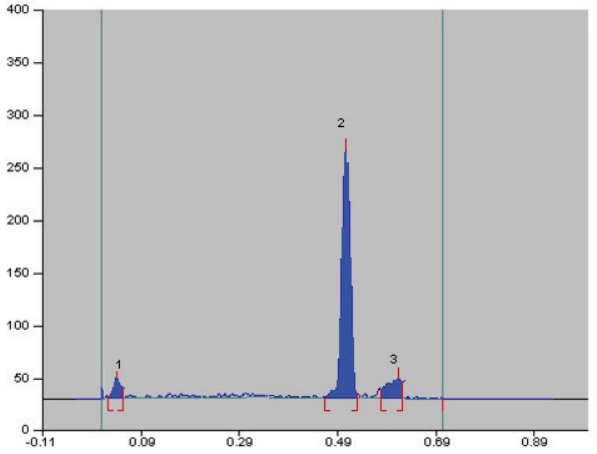

Figure 5. Densitogram of acid (0.1 M HCl) treated sample.

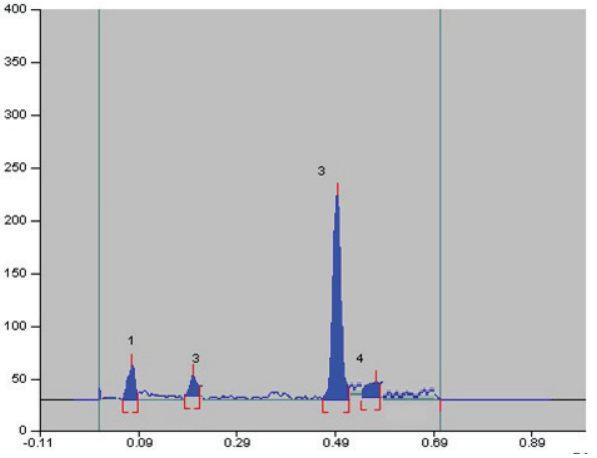

Figure 6. Densitogram of alkali (0.1M NaOH) treated sample.

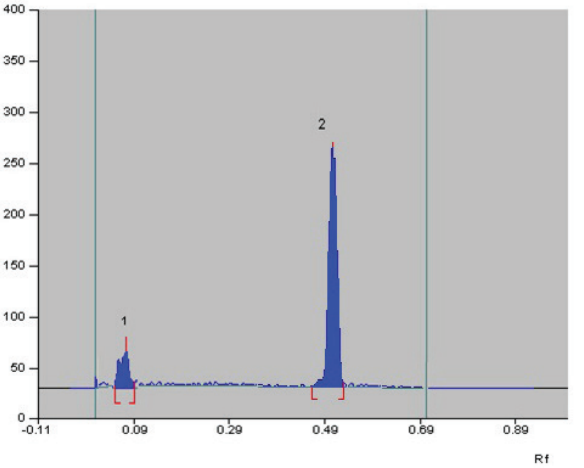

Figure 7. Densitogram of oxide (3% H_2O_2) treated sample.

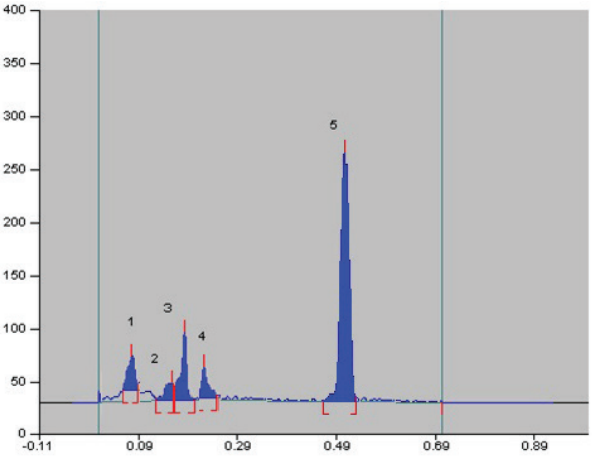

Figure 8. Densitogram of the sample exposed to neutral hydrolysis.

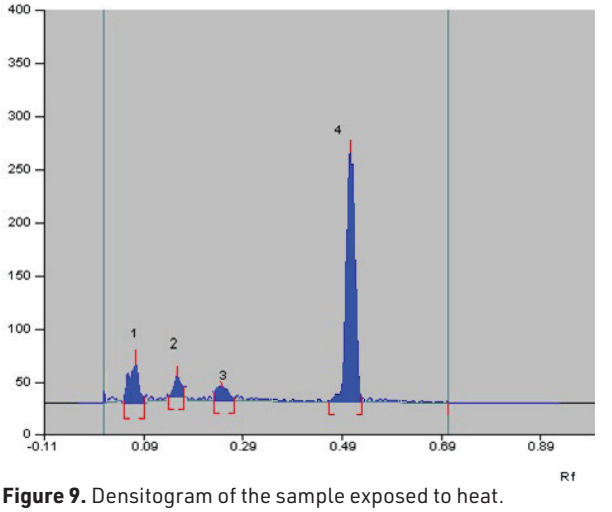

Figure 9. Densitogram of the sample exposed to heat.

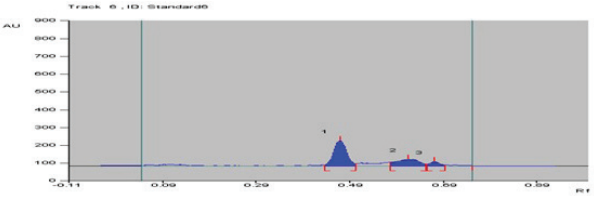

Figure 10. Densitogram of the sample exposed to photodegradation.

Separation, Characterization of Degradation Product by HPTLC and by MS/MS (Tandem Mass Spectroscopy) Method

Isolation of degradation product by using HPTLC method

An accurately weighed quantity of 10.0 mg of Dofetilide was transferred to10.0 mL of volumetric flask than add 3.0 ml of distilled water flask no.1 3.0 mL of 0.1 M acid in flask no 2, 0.1 M NaOH in flask no 3, 3% of H_2O_2 in flask no 4. The forced degradation study was carried out by exposing samples to the stress condition as 0.1 M HCl, 0.1 M NaOH, neutral, oxide for degradation, contents of the flask were reflux in a water bath at 80°C for 1 hr 30 min, 30 min, 1 hr and 2 hr 30 min respectively. After the respective time intervals, all the flasks were removed and allowed to cool. Then the samples were applied on the TLC plate with the sample volume of about 10 µL/ band, 4 band of degraded sample and 1 band of std. were applied. Then the TLC plate was allowed to develop under optimized chromatographic conditions for Dofetilide. After development of the plate these plates were kept under the UV chamber on the basis of Rf value of the std. and degradation product they are marked and that portion of TLC plate was cut and allowed it to extract into methanol. Then the IR spectra and MS-MS spectra was recorded for interpretation of the probable structure of the degradation product. The diagrammatic procedure was given below degradation of sample in acid, alkali, oxide and neutral stressed condition.

The said degradation products were then subjected to MS/MS studies by using positive electrospray ionization (ESI) mode (mass range of 50–1500 daltons). The drug (concentration of 6 µg/mL) was directly infused using a syringe pump into MS/MS in methanol: water (50:50 v/v) as a solvent system. This is for the optimization of mass parameters which inform about the molecular ion peak of the drug. These were further modified to get complete fragmentation of the drug. High purity nitrogen was used as the nebulizer as well as the auxiliary gas (Bhole, Naksarkhre, & Bonde, 2019; Bhole et al., 2018). Fragmentation of various precursor ions formed in MS/MS studies was achieved at different collision energies The seven degrading products were observed in the four neutral stress conditions, two in acidic stress condition, two in alkaline stress condition and one in oxidative stress condition. (Molecular formula C19H27N3O5S2; Molecular weight: 441.56) i.e. DP-1, DP-2, DP-3, DP-4, DP-5, DP-6, DP-7. The correlation of the degradation product in Dofetilide and its stress conditions were shown in Figures 11-16 (mass spectrum) and fragmentation pattern shown in Figures 17-20.

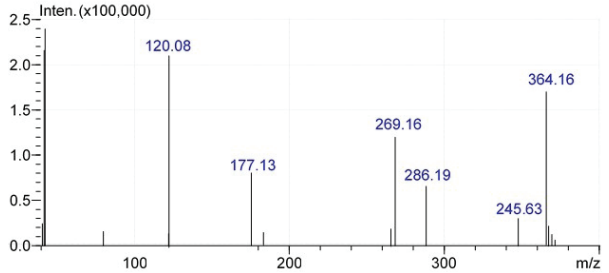

Figure 11. MS/MS spectrum of the DP-1(m/z 364.17).

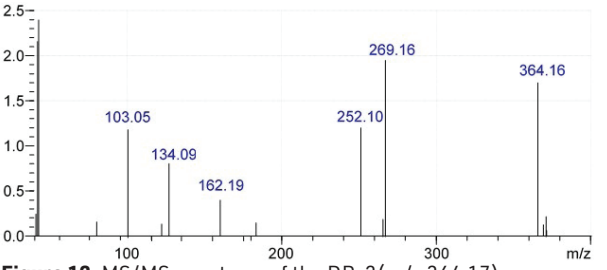

Figure 12. MS/MS spectrum of the DP-2(m/z 364.17).

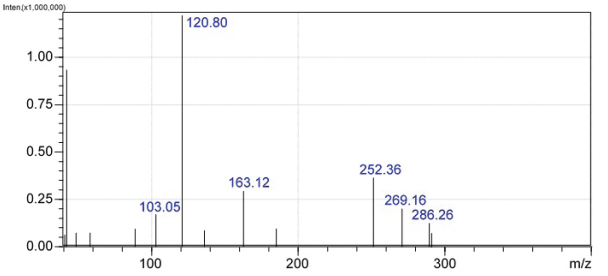

Figure 13. MS/MS spectrum of the DP-3 and DP-4 (m/z 285.18).

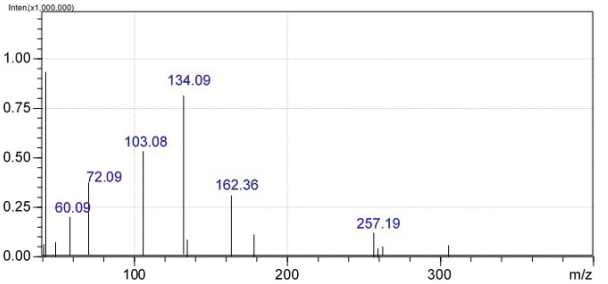

Figure 14. MS/MS spectrum of DP-5 (m/z 257.13).

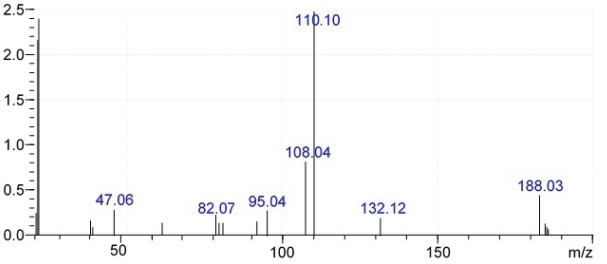

Figure 15. MS/MS spectrum of DP-6 (m/z 188.04).

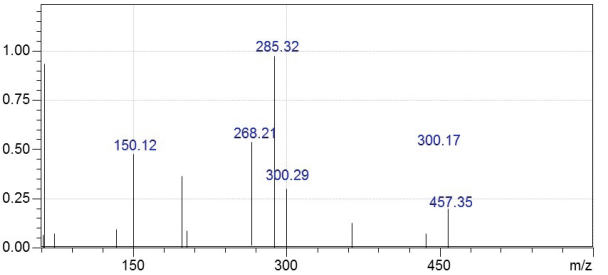

Figure 16. MS/MS spectrum of DP-7 (m/z 363.14).

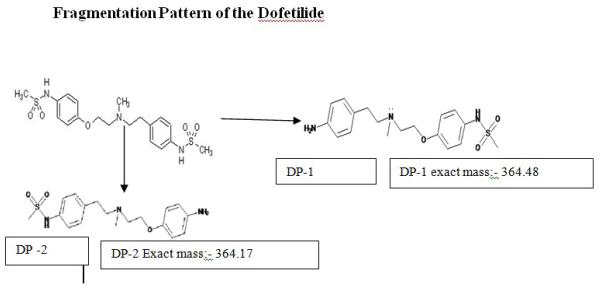

Figure 17. Possible degradation pathway in neutral condition.

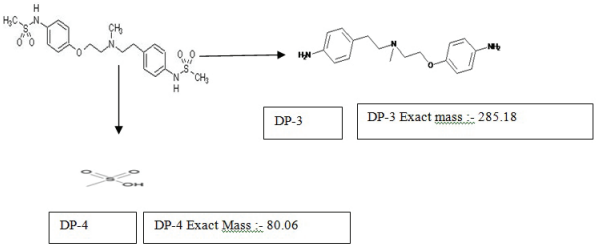

Figure 18. Possible degradation pathway in alkali stress condition.

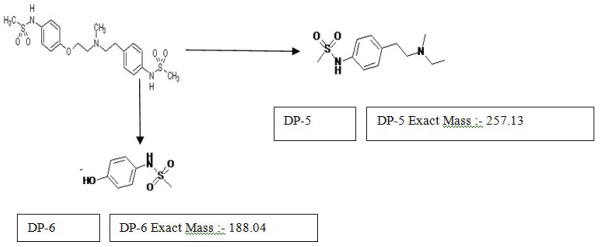

Figure 19. Possible degradation pathway in acid stress condition.

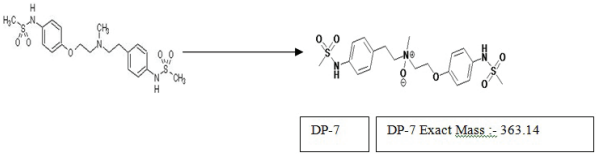

Figure 20. Possible Degradation pathway in oxidative stress condition.

CONCLUSION

The HPTLC method developed for its linearity, range, precision studies, LOD and LOQ can be used for the routine quality control of the drug dofetilide in bulk drugs. The degradation of the drug under various stress conditions indicates the storage conditions for the drug and drug product during its shelf life. Moreover, the degradation pathway of a drug can help in the future to identify the impurities and for the impurity profiling of dofetilide.

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Original Article

In vitro characterization of mucoadhesive polysaccharide polymers tablets fabricated using FTIR press

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ABSTRACT

Background and Aim: Buccal/sublingual drug delivery is gradually becoming one of the most experimented routes for alternate drug delivery. The major advantage of both routes is their high vascularity that allows a substantial permeation of drugs into systemic circulation. Mucoadhesive biopolymers are the mainstay of a transmucosal drug delivery system.
Methods: We formulated blank tablets of explored mucoadhesive biopolymers (sodium alginate, carboxymethyl cellulose, hydroxyl propyl methyl) cellulose using a combination of two at a time. The novelty of this script lies in the formation of tablets using the FTIR hydraulic press, as opposed toa conventional tablet punching machine, at two different pressures. The tablets were subjected to basic characterizations of polymeric interaction, hardness, and swelling behaviour.
Results: An interaction analysis using XRD revealed a good interaction between the polymers. HC-300 and AC-300 were found to be the hardest among the tablets formulated. In terms of swelling behaviour, AC-200 and HA-300 displayed the best swelling as compared to other combinations.
Conclusion: In the absence of a conventional tablet punching machine, we fabricated swellable biopolymeric tablets using the regular KBr hydraulic press that comes as an accessory with the FTIR instrument. These tablets can possible be used for delivering drugs through buccal mucosa.

Keywords: Biopolymer, FTIR press, tablets, drug delivery, transmucosal

INTRODUCTION

Buccal and sublingual mucosae have become sites of great interest for local and systemic drug delivery. They are gradually becoming the preferred routes since they can bypass the first-pass metabolism usually encountered through the oral route. Secondly, drugs that are administered parenterally encounter poor patient compliance (Vila, Tardelli, Chaud, Tubino, & Balcão, 2014). For those drugs, this route can completely bypass the pain and local site morbidity. The basic anatomy of buccal mucosa consists of an epithelial layer (stratified squamous epithelia) which overlays a layer of connective tissue known as lamina propria. The buccal epithelium is approximately 50 cell layers thick and non-keratinized. The buccal mucosa is approximately 500-800 µm thick with a surface area of around 50 cm^2. Numerous tiny blood vessels are present in lamina propria that drain into the jugular vein providing a direct entry point for drugs delivered through the buccal mucosal route. The presence of saliva is key to mucoadhesion and degradation of the formulation. The viscosity and mucoadhesion support rendered by saliva is attributed to the presence of glycoprotein mucin. The drug permeation via the buccal route takes place mainly by the paracellular and transcellular route (Barua et al., 2016; Boddupalli, Mohammad, Nath, & Banji, 2010). The sublingual area, currently the most widely utilized oral transmucosal

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site, is more vascular as compared to the buccal mucosa. Being thinner and non-keratinized, it provides better permeability to drugs (Sudhakar, Kuotsu, & Bandyopadhyay, 2006).

Patient acceptability has been one of the major advantages of buccal/sublingual mucosae apart from the technical advantage of bypassing the first-pass metabolism. Various formulations such as mucoadhesive tablets, sprays, gels, and patches have been formulated to date (Barua et al., 2016; Mura, Cirri, Mennini, Casella, & Maestrelli, 2016). The most recent work on mucoadhesive buccal tablets reports freeze-dried formulations for prilocaine and lidocaine as local anesthetics for dental procedures (Favacho et al., 2020). Mucoadhesive polymers have been the mainstay of drug delivery formulation through mucosal routes. Some widely explored polymers include hydroxypropyl methylcellulose (HPMC), sodium carboxymethyl cellulose (NaCMC), alginate (ALG), chitosan (CH), and xanthan gum (XG) (Shridhar, Manohar, & Bhanudas, 2013). Favacho *et al.* (2020) have used Pullulan as the mucoadhesive polymer to formulate the mucoadhesive tablet.

The novelty of the work presented in this script lies in the fabrication of biopolymeric tablets from ALG, NaCMC, and HPMC mucoadhesive biopolymers using the non-conventional simple in-house available FTIR KBr hydraulic press at two different pressures. Utilization of an FTIR press to the polysaccharide tableting was an interesting and ingenious idea that was further subjected to hardness testing, polymeric interactions, and swelling behaviour in simulated salivary fluid-based solid agar base. Our tablets displayed mutual interaction among polymers and significant swelling behaviour while significantly low hardness as compared to the regularly reported values.

MATERIALS AND METHODS

Materials

Sodium alginate (ALG; CAS No. 9005383) was procured from SRL Chemicals, India. Hydroxypropyl methyl cellulose (HPMC; CAS No. 9004653) was procured from Molychem, Mumbai, India while sodium carboxy methyl cellulose (NaCMC; CAS No. 9004324) was procured from Fisher Scientific, Mumbai, India. All other chemicals mentioned were procured from HiMedia, India.

Tablet formation

Since we did not have a regular tablet punching machine, for this study we utilized the KBr press machine that is an important associated part of the FTIR spectrophotometer. The KBr press was Metrex made (Figure 1). The blank or unloaded tablets were formulated in the combination mixture of (ALG + NaCMC), (ALG + HPMC) and (NaCMC + HPMC).

Hardness test

The tablets were subjected to hardness using the Pfizer hardness tester. Three tablets of each combination were tested, and an average was taken.

Ingredient interaction study

The interaction between ingredients was visualized by X-ray diffraction (XRD) using a PANalyticalX'Pert instrument (Malvern Panalytical, UK). The XRD graphs were plotted for each individual polymer and each combination formulation.

Figure 1. Camera image of the KBr press used to form tablets.

In vitro swelling study

Formulations targeted for specific routes need to be tested in simulated conditions. Therefore, the swelling behaviour of formulated tablets was analysed using a simulated salivary fluid. The simulated salivary fluid was formulated in accordance with the formula suggested by Koland *et al.* (Koland, Vijayanarayana, Charyulu, & Prabhu, 2011). Briefly, it consisted of disodium hydrogen phosphate (2.38 g/L), potassium dihydrogen phosphate (0.19 g/L), and sodium chloride (8 g/L). The pH was maintained at 6.75. Agar plates were formed by dissolving 2% agar (w/v) in the simulated salivary fluid. The tablets, after being weighed for the initial weight (0 hour), were kept in agar plates at 37°C in the incubator. The weight of the tablets was measured at an interval of one hour for five consecutive hours.

RESULTS

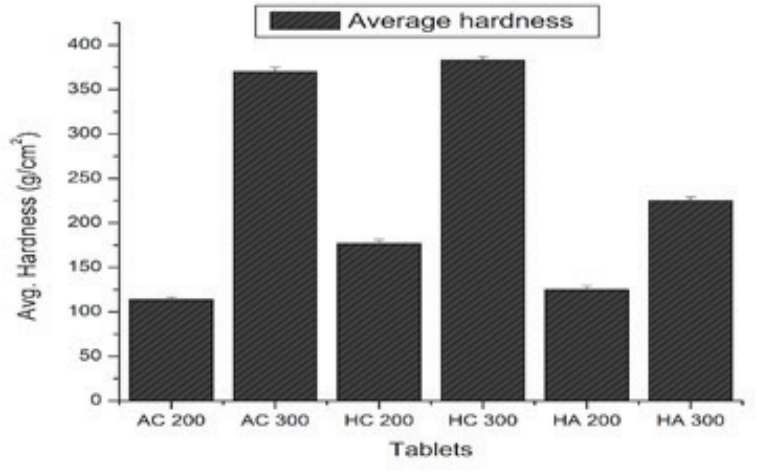
Tablet formulation

Tablets were formed at two different pressures of 200 psi and 300 psi. The tablets were eight mm in diameter with a thickness of 2±0.4 mm. The tablets were uniform in shape and size (Figure 2a).

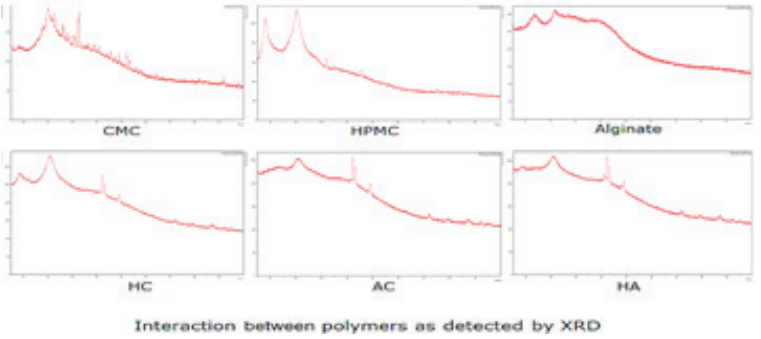
Hardness test

As stated in the previous section, since we did not have a regular tablet punching machine, the tablets formed from the hydraulic press of the FTIR spectrophotometer lacked the hardness reported usually. The mean hardness of each formulation is shown in Figure 2b. AC 300 and HC 300 displayed the best hardness among the fabricated tablets.

(2a)

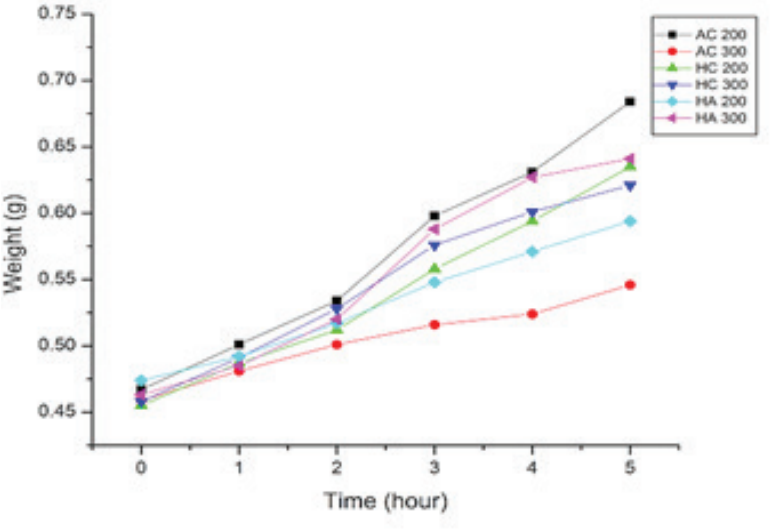


(2b)



(2c)

Figure 2. Camera image of tablets (2a); Hardness of tablets (2b); Interaction between polymers (2c).



(3a)

(3b)

Figure 3. Swelling behaviour of tablets (3a); Camera image before and after swelling (3b).

Interaction study

The ingredient interaction ensures bonding and stability of the formulation. Our tablets, on being subjected to XRD analysis, revealed a significant change in the peaks as compared to the peaks of individual polymers that divulge the presence of molecular interactions among polymers. The interaction results are shown in Figure 2c.

***In-vitro* swelling study**

The swelling of buccal tablets is an important parameter since a drug will be released upon swelling of the formulation. Swelling results in weakening of interactions among polymers would lead to sustained release of the drug. This study revealed that the best swelling is shown by tablets namely AC 200 and HA 300. The graph representing the swelling behaviour of tablets displayed in Figure 3a. Figure 3b shows a comparative image of tablets before and after the swelling study.

DISCUSSION

Buccal/sublingual mucosal routes are gradually becoming one of the most explored alternative routes for drug delivery. These sites are easily accessible and highly vascular. Drugs that display extensive first-pass metabolism orally or that are administered at a very high dose are usually considered for this route. The delivery systems for these routes consist of mucoadhesive polymers as the primary carriers. The first and foremost requisition for polymers to be considered for being a carrier in such a delivery system is being mucoadhesive. Secondly, the biopolymers should be biocompatible or non-immunogenic to the specific site in particular and the human system in general. Biopolymeric tablets provide an easy and compatible way to deliver drugs through the buccal/sublingual route since they stay for long and are considered good for a sustained drug release (Sudhakar et al., 2006).

This study was aimed at proving that the formation of tablets using three established mucoadhesive biopolymers and a FTIR hydraulic press could be a feasible alternative to a tablet punching machine. The tablets formed underwent basic essential characterizations such as hardness, interaction, and swelling behaviour. The study revealed that AC 200 and HA 300 tablets displayed the best swelling while HC 300 and AC 300 displayed the best hardness. We agree that we have not used any tablet binder that is commonly applied, but the use of FTIR press can result in tablet formation using pressure and these tablets displayed swelling without significant fragility in a simulated salivary fluid. It must be understood that for transbuccal/sublingual drug delivery, the formulation cannot be kept for more than 2 hours (for patient compliance related to eating and drinking) if the formulation is not fast disintegrating and degradable. Since this is a preliminary study, better optimization is definitely required to formulate tablets for transbuccal/sublingual drug delivery so that the maximum amount of drug can be delivered within 2 hours through the buccal mucosa. The usefulness of the FTIR hydraulic press should be considered as a feasible alternative for tablet formation. Further modifications and characterizations of these biopolymeric tablets can be extended to formulate a better oral transmucosal drug delivery system.

Peer-review: Externally peer-reviewed.

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Original Article

Effects of Duraseal® and Fibrin Glue on healing of normal and ischemic colon anastomosis

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ABSTRACT

Background and Aims: Anastomotic leaks represent a major complication of colorectal surgery. This study, involving a rat model of normal and ischemic colon anastomosis, aims to compare the effects of Duraseal® with those of Fibrin Glue (FG).
Methods: Fifty adult male Wistar Albino rats were divided into six groups; normal colon anastomosis, ischemic colon anastomosis, FG on normal colon anastomosis, Duraseal® on normal colon anastomosis, FG on ischemic colon anastomosis, Duraseal® on ischemic colon anastomosis. After scarification, bursting pressure were measured and samples were collected for histopathological examination and hydroxyproline assays.
Results: While the mean bursting pressure was statistically higher in groups treated with Duraseal® when compared to controls ($p<0.05$), no significant differences between Duraseal® and FG were detected ($p>0.05$). The mean hydroxyproline level was significantly lower in the Duraseal® groups than in the FG groups ($p<0.05$). However, significant differences between Duraseal® and control groups were found only in ischemic colon anastomosis ($p<0.05$). Histopathological examinations did not show any differences in wound healing.
Conclusion: Considering the advantages associated with the use of Duraseal®, we may assume that it may play role in gastrointestinal surgery with respect to prevention of anastomotic leaks. However, data is limited, and further studies are warranted to better define its place in surgery.

Keywords: Duraseal, surgical anastomosis, fibrin glue, rat, ischemia

INTRODUCTION

Anastomotic leaks represent a major complication of traditional or laparoscopic colorectal surgery that are associated with increased morbidity, risk of reoperation, prolonged hospitalization, and reduced quality of life (Raptis, Pramateftakis, & Kanellos, 2018). Although the reported rates of anastomotic leaks vary between 1% and 24%, this figure is approximately 5% in experienced centers (McArdle, McMillan, & Hole, 2005; Raptis et al., 2018; ; Vakalopoulos et al., 2017b), while it may increase up to 30% to 40% in ischemia, where wound healing is poor, and in patients with inflammatory bowel disease (Wu et al., 2015). Anastomotic leaks following curative surgery for colorectal cancer have been shown to have an adverse impact on the overall survival (McArdle et al., 2005).

Systemic factors influencing anastomotic healing include age, nutritional status, cigarette smoking, chemoradiation, and diabetes, while local factors include the ischemia at the site of anastomosis as well as the surgical technique utilized (Raptis et al., 2018).

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Until now, a variety of surgical techniques, drugs, and adhesion barriers have been investigated in experimental studies of colon anastomosis in an attempt to identify effective means of leakage prevention. In addition to agents such as 5-fluorouracil and hydrocortisone that have been shown to negatively affect the anastomotic healing, others, including tacrolimus and iloprost, have exerted positive effects (Raptis et al., 2018). In recent years, tissue adhesives have been increasingly used for the prevention of upper gastrointestinal leaks (Fullum, Aluka, & Turner, 2009). Their effects have also been tested in a number of studies involving lower gastrointestinal procedures, although they are not used in clinical practice. While fibrin glue (FG) is one of the most frequently investigated agents in experimental studies of colonic anastomosis (Aghayeva et al., 2017; Daglioglu, Duzgun, Sarici, & Ulutas, 2018; Raptis et al., 2018; Senol et al., 2013; Torres-Melero, Motos-Mico, Lorenzo-Linan, Morales-Gonzalez, & Rosado-Cobian, 2016; Vakalopoulos et al., 2017a), Duraseal® has also been subject to some research (Karagoz Avci et al., 2011; Wu et al., 2015).

In this study involving a rat model of normal and ischemic colon anastomosis, we compared the effects of Duraseal® with those of FG, which has been previously shown to have positive effects on the healing of colonic anastomosis in a number of studies.

MATERIALS AND METHODS

Tissue adhesives in current clinical practice can be classified into four categories based on their chemical structure: cyanoacrylates (CA), FG, polyethylene glycol (PEG) adhesives and, biological adhesives, which contain albumin and/or gelatin (Vakalopoulos et al., 2017a). In this study, FG was compared with Duraseal®, which is a modified PEG.

Duraseal®

Duraseal® (Confluent Surgical, Inc., Waltham, MA) is an FDA-approved surgical sealant that is generally used to prevent cerebrospinal fluid leaks in cranial and spinal surgery as well as for improved anastomotic safety in cardiovascular surgery (Jeon et al., 2017; Nishimura, Kimura, & Morita, 2012; Osbun et al., 2012; Pereira, Grandidge, Nowak, & Cudlip, 2017; Strong et al., 2017).

This hydrogel system consists of two solutions; the first contains modified PEG and very low concentrations of FD&C Blue #1 dye, while the second solution contains a low molecular weight, water soluble trilysine amine at very low concentrations. When sprayed onto the tissues, these two solutions react and form cross-links within a few seconds, leading to the formation of a strong hydrogel (90% water) without measurable increases in local temperature and without requiring the application of any external source of energy (Preul, Bichard, & Spetzler, 2003). FD&C Blue #1 dye, on the other hand, provides a measure of the extent and thickness of the application (Preul et al., 2003), and diffuses out of the wound site to be finally excreted via the renal route without being incorporated into the hydrogel structure (Preul et al., 2003).

The hydrogel formed by the above-described reaction leads to the formation of a barrier impermeable to fibroblasts, and remains on site for 4 to 8 weeks. Subsequently, it is broken down into water soluble PEG molecules, and is excreted primarily through the kidneys.

Some advantages of Duraseal® include storage at room temperature, absence of a requirement for heating or external source of energy, easy preparation, good mechanical strength and elasticity, usability in moist conditions, good adaptation to irregular surfaces, good tissue adhesion, and clear visibility due to the blue dye content.

Care should be practiced when using Duraseal® in patients with severe impairment of kidney or liver function, pregnant women, patients with immune suppression or autoimmune conditions, and in individuals allergic to FD&C Blue #1 dye. Also, concomitant use with other tissue adhesives or hemostatic agents should be avoided, and it should not be used in patients who have active infection at the site of surgery.

Fibrin glue

FG is a biological adhesive derived from human fibrinogen concentrates, and has been reported to provide strong tissue adhesion for wound healing, in addition to hemostatic properties at the wound site (Raptis et al., 2018; Senol et al., 2013; Wu et al., 2015).

Fibrin adhesives contain thrombin and aprotinin, and mimic the final step of the coagulation cascade (Karagoz Avci et al., 2011), leading to the conversion of fibrinogen to fibrin with the effect of thrombin. On the other hand, Factor XIII is responsible for the formation of a stable clot thanks to the formation of covalent bonds between fibrin monomers. In order to prevent excessive and sudden fibrinolysis, aprotinin is added into fibrin adhesives. Fibrin adhesives trigger the clotting cascade on the site of application, resulting in the conversion of fibrinogen to fibrin and formation of a gel-like adhesive.

Fibrin glues have positive effects on wound healing, reduce hematoma formation due to their hemostatic effects, and stimulate the migration of macrophages that are involved in the maturation of fibroblasts and in angiogenesis (Karagoz Avci et al., 2011).

Contraindications to the use of fibrin glues include arterial or severe venous bleeding and hypersensitivity to bovine proteins or to any of the ingredients. Data on their use during pregnancy or breastfeeding is insufficient.

In this experimental study, Beriplast® P combi-set (Farma-Tek, Istanbul, Turkey) was used as the fibrin glue.

Design of the study

This experimental study was performed at Istanbul University, Cerrahpasa Medical Faculty, Experimental Medicine Research Institute after approval of the Institutional Review Board.

Fifty adult male Wistar Albino rats 10-12 weeks of age and weighing 200-250 g, were obtained from Istanbul University Cerrahpasa Medical Faculty Experimental Animals Research Laboratory. The rats were cared for in accordance with the "Guide for the Care and Use of Laboratory Animals" prepared by the In-

stitute of Laboratory Animals Resources and published by the National Institute of Health; maintained in colony cages (five rats per cage) under controlled conditions of temperature (28°C), light (10 h light: 14 h dark) and humidity (50°F 5%). The rats were not permitted ad libitum access to standard lab chow and tap water starting from 12 hours before the surgery until the end of the experimental procedures to decrease fecal contamination.

The rats were placed under general anesthesia using intraperitoneal administration of 50 mg/kg Ketamine HCl (Ketalar® vials, Eczacibasi). After the site of surgery was shaved, skin was cleansed with povidone iodine. Experimental animals were categorized into six groups based on the procedure and type of adhesion barrier to be applied (Table 1). Beriplast P® and Duraseal® were prepared in accordance with the instructions of the manufacturers.

Table 1. Procedures and adhesion barriers applied to the groups.

Group	Procedure	Adhesion barrier	Number of subjects (n)
Group 1	Segmenter colon resection + end-to-end anastomosis	-	5
Group 2	Segmenter colon resection + end-to-end anastomosis at ischemic colon segment	-	5
Group 3	Segmenter colon resection + end-to-end anastomosis	Fibrin Glue	10
Group 4	Segmenter colon resection + end-to-end anastomosis	Duraseal®	10
Group 5	Segmenter colon resection + end-to-end anastomosis at ischemic colon segment	Fibrin Glue	10
Group 6	Segmenter colon resection + end-to-end anastomosis at ischemic colon segment	Duraseal®	10

In all animals, the abdomen was accessed with a four cm standard midline incision. After the descending colon was released, a 0.5 cm segment was resected. End-to-end anastomosis was performed by 6-8 interrupted sutures using 5/0 polypropylene suture material. In Group 1, no procedures were carried out on the anastomotic line, while FG was applied on anastomosis in Group 3, and Duraseal® in Group 4. In Groups 2, 5, and 6, the free ends of the colonic segments were devascularized up to a distance of 0.5 cm from the end, followed by anastomosis with the same method to allow for the formation of ischemic colonic anastomoses (Portilla-de Buen et al., 2014). In Groups 5 and 6, FG and Duraseal® were applied on the anastomosis, respectively. In all groups, for the closure of the midline incision, fascia and skin were closed separately using 3/0 silk sutures.

The rats were sacrificed at postoperative day four using high dose ether inhalation. Then, the bursting pressure were measured at anastomosis sites, and samples were collected for histopathological examination and hydroxyproline assays.

Bursting pressure

Bursting pressures were measured in mmHg. Adhesions around the anastomoses were not released after opening the abdominal cavity, as these were thought to reflect an effect on anastomotic healing.

A catheter was advanced 1 cm distally into the left colon through the anal canal of the rats and was fixed using 3/0 silk sutures. The rats were sunk into a bowl filled with water. Air insufflation was performed at a stable speed of 6 ml/min, and the bursting pressure was measured using a sphygmomanometer. Bursting pressure was defined as the highest reading at the sphygmomanometer with simultaneous visualization of air bubbles in the water.

After bursting pressure measurements, the anastomoses were released from the surrounding adhesions. A 1-cm segment encompassing the proximal and distal parts of the anastomotic line was removed. One part of the segment was fixed in 10% formaldehyde for histopathological examinations, and the other was wrapped into aluminum folios for hydroxyproline assays and was stored at -22°C.

Hydroxyproline quantification

Hydroxyproline is a part of collagen that was demonstrated to be positively correlated with the amount of collagen formation and healing of colonic anastomosis. Hydroxyproline quantification was performed at the Biochemistry Laboratory, Cerrahpasa Medical Faculty, Istanbul University. After weighing, the colonic samples were treated with the modified Bergman and Loxley method for quantification of hydroxyproline, which was expressed as mg/g in wet tissue (Karagoz Avci et al., 2011; Lee et al., 2005).

Histopathological examination

Half of the 1 cm colon segment removed after bursting pressure measurements that included the line of anastomosis was fixed in 10% formaldehyde. Then, cross-sections obtained from colonic segments were embedded in paraffin blocks as to expose all layers of the colon. Samples were stained with hematoxylin and eosin. Inflammatory cells, neutrophils, extent of neovascularization, fibroblastic activity, and collagen fibrils were examined microscopically to assess the healing (Ersoy et al., 2016).

Statistical evaluation

All the values were expressed as the mean ± standard deviation (SD). The data of the bursting pressure and the hydroxyproline content were analyzed by ANOVA (Analysis of Variance) test. Post-hoc analyses were performed with the Tukey test. Values were considered as significant when $p<0.05$.

RESULTS

Although the surgical procedures and anesthesia were well tolerated by the animals, one rat in each of Groups 1 and 6, and two rats in each of Groups 3, 4, and 6 died in their cages before postoperative day 4. However autopsy in these eight rats showed no signs of macroscopic anastomotic leak or peritonitis.

None of the rats sacrificed at postoperative day four using high dose ether inhalation had macroscopic leaks. All bursts occurred in the line of anastomosis during the measurement of bursting pressures. Average bursting pressures in the study groups are shown in Table 2.

Table 2. Mean bursting pressure and Hydroxyproline levels.

Experimental group	Mean bursting pressure (mmHg)	Mean Hydroxyproline level (mg/g wet tissue)
Group 1	98.75±15.47	1.85±2.13
Group 2	87.00±16.80	12.14±8.44
Group 3	115.62±23.21	13.61±15.94
Group 4	129.37±20.07	0.21±0.04
Group 5	107.77±22.33	8.72±9.10
Group 6	122.50±19.45	0.29±0.15

values are given as mean ± SD

Rats undergoing Duraseal® and FG treatment were found to have significantly higher mean bursting pressures both in ischemic and normal colon anastomoses as compared to rats in the other groups ($p<0.05$). Although the mean bursting pressure in the Duraseal® groups (Groups 4 and 6) were higher than in the FG groups (Groups 3 and 5), the difference was not statistically significant ($p>0.05$).

The mean hydroxyproline levels were significantly lower in the Duraseal® groups (Groups 4 and 6) than in the FG groups (Groups 3 and 5) ($p<0.05$). When the Duraseal® groups (Groups 4 and 6) were compared with the control groups (Group 1 and 2), the difference was significant only between the ischemic colonic anastomosis groups (Groups 2 and 6) ($p<0.05$).

Comparison of inflammatory cells, neutrophils, neovascularization, fibroblastic activity, and collagen fibers showed no significant differences between groups. However, despite similar collagen content between the Duraseal® and the control groups, these groups were found to have irregular collagen alignment.

DISCUSSION

Anastomotic leaks are a major complication of colorectal surgery that lead to increased morbidity and mortality (McArdle et al., 2005; Raptis et al., 2018; ; Vakalopoulos et al., 2017b). Although the reported rates of anastomoses range between 1% and 24%, this figure may rise up to 30% to 40% in the presence of conditions that lead to poor wound healing, such as ischemia (McArdle et al., 2005; Raptis et al., 2018; Vakalopoulos et al., 2017b; Wu et al., 2015). Due to the recent increase in the use of cytoreductive surgery together with hyperthermic intraperitoneal chemotherapy (HIPEC), concerns have been expressed regarding the effect of chemotherapeutic agents on anastomotic healing, with a consequent emphasis on the prevention of such leaks (Raptis et al., 2018).

Although a variety of surgical techniques, drugs, and adhesion barriers have been utilized in experimental studies of colonic anastomoses, no ideal algorithms for the prevention of anastomotic leaks have been established until now (Aghayeva et al., 2017; Daglioglu et al., 2018; Demiryas et al., 2019; Raptis et al., 2018; Senol et al., 2013; Torres-Melero et al., 2016; Vakalopoulos et al., 2017a). Advances in technology have allowed the introduction of adhesion barriers in a wide spectrum of procedures (Vakalopoulos et al., 2017a). Despite the confirmed efficacy of adhesion barriers, the ideal molecule, particularly for the lower gastrointestinal system, has not been defined (Daglioglu et al., 2018; Fullum et al., 2009; Raptis et al., 2018; Senol et al., 2013; Torres-Melero et al., 2016; Vakalopoulos et al., 2017a, 2017b; Wu et al., 2015). An ideal barrier should have certain characteristics such ease of preparation, low cost, sterility, pliability, biochemical inertness and harmlessness, and minimal or no inflammatory properties as well as causing no adhesions or infections.

Duraseal® is an FDA-approved synthetic hydrogel that is commonly used in cranial and spinal surgery, and in this study, its effects on ischemic and normal colonic anastomoses have been investigated and compared with an established product, i.e. FG, in the current study.

The efficacy of FG has been shown in many previous studies (Fullum et al., 2009; Raptis et al., 2018; Senol et al., 2013; Vakalopoulos et al., 2017a; Wu et al., 2015). While experimental and retrospective studies have shown negative effects of HIPEC on colon anastomoses, others have reported positive effects for the fibrin glue on colorectal anastomosis following HIPEC (Aghayeva et al., 2017; Piso et al., 2019; Raptis et al., 2018; Torres-Melero et al., 2016). Buen et al. (Portilla-de Buen et al., 2014) showed a positive effect of the fibrin glue on bursting pressure in an ischemic left colon anastomosis model, while

Fullum et al. (Fullum et al., 2009) suggested a possible reduced risk of leakage with the use of FG in the anastomosis and stapler lines, following laparoscopic Roux-en-Y gastric bypass. In another experimental model, FG provided improved anastomotic safety in anastomoses performed in both clean abdominal wounds as well as in the presence of peritonitis (Senol et al., 2013). Despite these reported positive effects of FG on anastomosis, a major drawback is its aprotinin content, which may be associated with certain complications such as renal failure, myocardial infarction, and anaphylaxis (Zoegall, 2008). Furthermore, FG requires storage in cold temperatures, and absence of a dye precludes estimation of the extent of application. Also, the surgical site should be dry for effective use of FG.

Duraseal® adhesion barrier is a practical synthetic hydrogel free of infection-risk. Some of its advantages over FG include storage in room temperature, easy preparation, good pliability, suitability for moist conditions, good tissue adhesion, and blue stain showing the extent of the application.

Several previous studies have compared Duraseal® with FG. In an experimental rat study by Avci et al. (Karagoz Avci et al., 2011), it was not significantly different from the fibrin glue in duodenal perforation, while it showed no superiority over the conventional repair. In the study by Wu et al. (Wu et al., 2015) the effects of FG, CA, and Duraseal® were compared in the presence of experimental colitis, and a lower bursting pressure was found in the control group, than in the CA and Duraseal® groups. Conversely, in two separate experiments by Vakalopoulos et al. (Vakalopoulos et al., 2017a; 2017b) involving colonic anastomoses and suture-free colonic repair, Duraseal® did not show superiority over the fibrin glue.

In the current study, although the bursting pressure was statistically higher in groups treated with Duraseal® adhesion barrier when compared to controls, no significant differences between Duraseal® and fibrin glue could be detected ($p>0.05$). Hydroxyproline was significantly lower in the Duraseal® groups. Histopathological examinations did not show any differences in wound healing.

Thus, although Duraseal® offered certain advantages such as ease of use, reduced risk of side effects, and less restrictive use as compared to fibrin glue, it was found to have a negative impact on the hydroxyproline level. However, increased bursting pressure in the Duraseal® groups, higher than in the controls, suggests that it may still hold some promise in gastrointestinal surgery.

CONCLUSION

Although Duraseal® was clinically superior in normal and ischemic colon anastomosis in comparison with other approaches, it failed to provide biochemical superiority. Considering the advantages associated with the use of Duraseal® adhesion barrier, we may assume that it may play a role in gastrointestinal surgery with respect to prevention of anastomotic leaks. However, the data is limited, and further studies are warranted to better define its place in such surgery.

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Original Article

Evaluation of excipients effects on the impurity profile of lyophilized hydroxocobalamin formulation

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ABSTRACT

Background and Aims: The aim of the research work was to compare excipient effects and develop a stable pharmaceutical product in the form of lyophilized powder. Mannitol, lactose monohydrate, EDTA and glycine are widely used in pharmaceutical formulations and food products.

Methods: The production method consists of mainly four parts: raw material weighing process, preparation of bulk solutions, lyophilization and analytical determination. A total of five formulations were prepared and lyophilized to the stability study. To improve the stability of hydroxocobalamin formulations (F1-F5), they were evaluated with different excipients (mannitol, lactose monohydrate, EDTA and glycine) during the stability period. Stability studies were performed to check impurity and assay of hydroxocobalamin.

Results: The rate of impurity and assay results were compared to F1-F5 formulations. As a result of impurity and assay analysis for F3 and F4, the formulations were found to be within limit. Both of them were determined to the best formulations for impurity of hydroxocobalamin.

Conclusion: The research proposes a new stable formulation and proper storage conditions for lyophilized hydroxocobalamin parenteral solutions. The impurity problem of lyophilized hydroxocobalamin formulation was optimized with lactose monohydrate and lactose monohydrate + EDTA combination.

Keywords: Drug formulation, excipient, lyophilization, hydroxocobalamin, stability

INTRODUCTION

Cyanocobalamin and hydroxocobalamin are best known as a water-soluble vitamin, one of the B-vitamins involved in energy production and cellular functions (Kennedy, 2016; Edelmann, Chamlagain, Santin, Kariluoto, & Piironen, 2016). Many chemical and physical factors could have a negative effect on the stability of these compounds. Both of these two vitamins are prone to degradation in liquid environments, particularly when exposed to light (Monajjemzadeh, Ebrahimi, Milani & Valizadeh, 2014). B group vitamins are sensitive to factors such as: heat, light, moisture, oxidizing and reducing agents, acids and or bases (Shchavlinskii, Neiman, Lazareva, & Orlov, 1995; Ahmad, Ansari & Ismail, 2003; Kondepudi, 2016; Schnellbaecher, Binder, Bellmaine, & Zimmer 2019). Hydroxocobalamin is a derivative of cyanocobalamin and the cyano functional group attached to Co^{+3} in the tetrapyrrolic corrin macrocyclic ring in cyanocobalamin is replaced by a hydroxyl group in hydroxocobalamin (Ahmad, Ahmed, Anwar, Sheraz & Sikorski, 2016).

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Lyophilization (Freeze-drying) is a well-known method for formulating liquid or parenteral products to get stablity of active pharmaceutical ingredients during shelf life. In the lyophilization process, the water content of the final product is reduced to a low-level that does not support chemical reactions related impurity (Mishra, Saini, & Maurya, 2017).

Excipients (inactive compounds) are the components of a pharmaceutical formulation to achieve the stability and efficacy of the final product. They are added to increase the solubility of bulk, improve stability, enhance drug delivery and targeting, and modify drug safety or pharmacokinetic profile (Mehmood & Farooq, 2015). According to statistical data, about 67% of the lyophilized marketed products of active molecules contain excipients in their formulation (Baheti, Kumar, & Bansal, 2010). Bulking agents are well-known excipients for a lyophilized product. Bulking agents in lyophilized formulations provide an adequate cake structure.

Although poor stability of hydroxocobalamin has been previously reported (Ahmad et al., 2014), some lyophilized products are commercialized in the pharmaceutical market (www.drugbank.ca). In spite of the increasing impurity problem of hydroxocobalamin formulations, there have been limited studies on stability of vitamins in literature.

Therefore, the aim of this study was to optimize impurity related hydroxocobalamin and proper choice of excipients for lyophilized vitamin drug products. In addition, lyophilized formulations were designed and made ready using some bulking, chelating and buffering agents to improve stability of formulations. In this respect, our study can be the first to investigate this pharmaceutical research.

MATERIAL AND METHODS

Materials

Hydroxocobalamin was produced from Interquim; EDTA, hydrochloric acid and glycine were purchased from Merck, lactose monohydrate was produced from Meggle, and mannitol was produced from Roquette. Vials were produced from Mefar (Istanbul, Turkey). 0.2 µm filter was purchased from Sartorius. The pharmaceutical grade sample of hydroxocobalamin hydrogen chloride was purchased from Ferrer. The lyophilized powder for injection containing hydroxocobalamin, diclofenac potassium and betamethasone sodium phosphate sample was produced by the World Medicine Pharmaceutical Industry and Trade Inc. (Istanbul, Turkey). Citric acid sodium salt was supplied from Acros, disodium hydrogen phosphate and methanol was purchased from Merck. The water (0.05 µc) was produced by the Sartorius Stedim Biotech system as HPLC grade.

Methods

The manufacturing method consists of mainly four parts: raw material weighing process, preparation of bulk solutions, lyophilization and analytical determination. All production steps are detailed below. The excipients that we used in our formulation are classic excipients used in pharmaceutical product formulations. In stability analysis, batch size was 100 vials for each formulation. Our finished product was produced using conventional production equipment.

Method of preparation for formulation trials

1. Take water for injection (WFI) into a proper production tank by 80 percentage of total volume (20-25°C).
2. Add excipient (bulking or chelating agents) to wfi slowly under continuous mixing at 600 rpm and mix it until it is completely dissolved.
3. To the solution add hydroxocobalamin under continuous mixing at 600 rpm and mix it until it is completely dissolved
4. Check pH and if it is necessary adjust pH with 1 M diluted HCl solution
5. Add water for injection up to total volume and mix the solution
6. Filter bulk solution through 0.2 µm filter
7. Lyophilization

Formulation trials

Lyophilized hydroxocobalamin powder for injection formulation development study is shown in Table 1. In order to obtain an optimum impurity profile for the final product with different excipients, the F1-F5 formulation trials were evaluated. A composition of each formulation is presented below. All samples

Table 1. Detailed formulation trials.

		Formulation Trials				
Function	**Ingredients**	**F1 (mg/vial)**	**F2 (mg/vial)**	**F3 (mg/vial)**	**F4 (mg/vial)**	**F5 (mg/vial)**
API	Hydroxocobalamin	13,00	13,00	13,00	13,00	13,00
Bulking agent	Mannitol	87,00	87,00	-	-	-
Chelating agent	EDTA	-	0,25	-	0,25	-
Bulking agent	Lactose Monohydrate	-	-	150,00	150,00	-
Bulking agent	Glycine	-	-	-	-	22,50
pH agent	1M HCl	q.s	q.s	q.s	q.s	q.s
Solvent	Water for injection	q.s to 1,50 ml	q.s to 1,50 ml	q.s to 1,50 ml	q.s to 1,50 ml	q.s to 1,50 ml
	pH adjusting	5,58	5,50	5,48	5,50	5,51

Table 2. Lyophilization set parameter for cycles.

Process	Temperature °C	Gradient/min	Holding/min	Vacuum/ mbar
Freezing	-40	60	400	-
1st Drying	-20	60	200	0.120
	-10	50	300	0.120
	0	50	250	0.120
	5	40	80	0.100
2nd Drying	20	60	120	0.090
	30	40	230	0.090

were loaded into the lyophilizer and the system was started by setting parameters. The set parameters for lyophilization cycle are recorded in Table 2. After the cycles were completed, the vials were removed from the lyophilizer and were stored at room temperature for analytical determination.

Lyophilization

Vials filled with hydroxocobalamin are put in the lyophilizer (Tofflon-Lyo 0,5 L), homogenously distributed and the system is started (Table 2).

Preparation of formulations for stability test

Stability studies were performed for long-term (25°C ± 2°C/60% RH ± 5% RH), intermediate term (30°C ± 2°C/65% RH ± 5% RH) and accelerated (40°C ± 2°C/75% RH ± 5% RH) test conditions using ICH guidelines [Q1A (R2)]. Samples were stored in stability rooms until the end of the stability period for assay and impurity analysis. These studies were investigated to increase the rate of chemical degradation of lyophilized product using HPLC analysis. Each stability evaluation was performed at least 3 times.

HPLC chromatographic conditions of hydroxocobalamin related substances

The HPLC method was carried out on Kromasil 100 C8 (250 mm × 4.6 mm, 5 µm) column with 20 µL injection volume at a wavelength of 351 nm on a Waters Alliance E2695 separation module equipped with a Waters 2489 photodiode array (PDA) detector and 2998 UV detector, an Empower-pro data handling system (Waters Corporation, Milford, MA, USA). Column and sample temperatures were 25°C. The separation was employed using isocratic elution, and the flow rate was maintained at 1.5 mL/min. Buffer solution was prepared by dissolving 16.7 g citric acid monosodium salt and 8.1 g disodium hydrogen phosphate in 1000 mL purified water. Mobile phase was prepared by mixing the buffer solution and methanol at the ratio of 805:195 (v:v) and filtering through 0.45 µm filter (Atici & Yazar, 2015; Atici, Yazar, Agtas, Ridvanoglu, & Karlıga, 2017).

Preparation of standard solution

10.3 mg hydroxocobalamin HCl equivalent to 10.0 mg hydroxocobalamin was weighed into a 100 mL amber volumetric flask, dissolved in an ultrasonic bath for 5 minutes adding approximately 40 mL mobile phase and then completed to volume with mobile phase. 1.0 mL of this solution was transferred into a 10 mL amber volumetric flask and completed to volume with mobile phase and filtered through 0.45 µm PTFE filter (Eldawy, Mabrouk, & El-Barbary, 2002).

Preparation of sample solution

1 vial content equivalent to 10.0 mg hydroxocobalamin was dissolved with mobile phase and transferred into 10 mL amber volumetric flask, washing it carefully, and diluted to volume. Filtered through 0.45 µm PTFE filter.

RESULT AND DISCUSSION

F1 to F5 were formulated with different excipients. Lyophilization was carried out with an optimized recipe and setting parameter (Table 2). After lyophilization process, the vials obtained from the five different formulations cake structure were intact and elegant. Hydroxocobalamin stability studies were performed with different excipients for 3-months in different storage conditions. The lyophilized samples were analyzed for assay and impurity parameters after reconstitution with solvent at the end of the stability period. The analytical data relating to the samples are reported in Tables 3 and 4.

Table 3. Assay results of hydroxocobalamin formulations during 3-months stability period.

Formulation	Limit (mg/vial)	25°C ± 2°C/60% RH ± 5% RH Assay (mg/vial)	30°C ± 2°C/65% RH ± 5% RH Assay (mg/vial)	40°C ± 2°C/75% RH ± 5% RH Assay (mg/vial)
F1	9-14,3	11,04	10,96	10,35
F2	9-14,3	10,93	10,84	9,70
F3	9-14,3	12,19	11,82	11,99
F4	9-14,3	14,11	14,05	13,76
F5	9-14,3	7,59	6,78	7,01

Table 4. Total impurity results of hydroxocobalamin formulations during 3-months stability period.

Formulation	Limit (%)	25°C ± 2°C/60% RH ± 5% RH Total Impurity (%)	30°C ± 2°C/65% RH ± 5% RH Total Impurity (%)	40°C ± 2°C/75% RH ± 5% RH Total Impurity (%)
F1	Max 10	8,72	11,21	16,19
F2	Max 10	9,58	10,44	15,28
F3	Max 10	6,59	6,96	7,14
F4	Max 10	5,56	5,57	5,71
F5	Max 10	28,66	30,03	32,10

The assay of hydroxocobalamin is shown during the stability period in Table 3. The presence of lactose monohydrate and lactose monohydrate + EDTA in formulation (F3-F4) positively affected the assay of the vitamin in storage conditions. In addition, the assay results of F1 and F2 were in the limits under all stability conditions. Additionally, based on HPLC analysis, the assay of hydroxocobalamin of F5 decreased to 6,78 mg/vial and 7,01 mg/vial in the intermediate term and accelerated stability period (Table 3). According to the F5 stability results, the active compound is not stable in glycine solution form. Due to the negative effect of glycine, the amount of hydroxocobalamin decreased by about 30% in each stability condition.

Based on impurity analysis, the formulation of F3 and F4 are compatible with an active substance and total impurity limits are lower than the other three formulations during the stability period. The results of 3-month impurity test for F5 formulation in 40 °C is the highest among all formulation and storage conditions. Moreover, both F1 and F2 formulations exceed the impurity limit. According to the data obtained from the formulation of F3 and F4 impurity analysis, excipients of lactose monohydrate and EDTA improve hydroxocobalamin stability and formulation impurity. Meanwhile, glycine negatively altered impurity and stability of formulation (Table 4).

It is observed that the highest rate of impurity is determined at about 30% in each of the three stability conditions with F5. F1 and F2 both have acceptable impurity values, and can be stabilized in stability condition (25°C) but they exceed limits in other conditions for impurity. According to analysis results, degradation of hydroxocobalamin and rate of impurity are related to each other, it is clear that there is a correlation between F5 impurity and assay results.

The HPLC method was validated according to ICH guidelines [Q2 (R1)]. The validation parameters included system specificity, suitability, linearity, accuracy, precision (system, method and intermediate precision) and robustness.

In specificity test, no another peak was observed in dilution and placebo solution chromatograms at the retention time of hydroxocobalamin, which were all separated from each other and found spectrally pure (purity angle < purity threshold). Linearity test standard solutions were prepared at concentration levels ranging from LOQ to 140% of the specification level. The results are given in Table 5.

The accuracy was determined by measuring recovery at known concentrations of the hydroxocobalamin (80%, 100% and 120%) and analyzed. 95% Confidence interval limits of recoveries were calculated (Table 5).

Table 5. Results of validation parameters.

Parameter		Results
Linearity	Range	LOQ-140.0%
	Equation	y=12117723.7107x - 333.0610
	Correlation coefficient	r^2=1.0000
Accuracy	Range	80.0%-120.0%
	Average	99.72
	95% Confidence Interval Limits	99.29-100.15
Precision	System Precision	RSD=0.31%
	Method Precision	Total impurity RSD=0.95%
	Intermediate precision	Total impurity RSD=7.02%

System precision was conducted with six repeated injections of standard solutions prepared at 100% concentration and RSD of peak areas was found below 10.0%. Method precision was performed by preparing 6 sample solutions described in section preparation of sample solution. Relative standard deviations (RSD) were calculated and the results were found below 10.0%. Intermediate precision was studied by different analysts and with different devices. Each analyst prepared 1 standard solution and 6 sample solutions. All the results were compared and RSD was calculated (Table 5).

To validation of the developed method, parameters were verified and standard solutions were tested. Column temperature by ±2˚C, mobile phase ratio by ±5.0 ml was changed and column with different lot number was used. Variations (%) were calculated and no significant difference was found between initial and altered conditions.

Solution stability was also evaluated by monitoring the peak area response. Standard and sample solutions were analyzed right after its preparation 6, 24, and 48 hours after at 5˚C and 25˚C. Results were compared and % variations were calculated and all results were below 10.0%.

According to literature, mannitol, trehalose, sucrose, lactose, glucose, and dextran glycine, are the most commonly used bulking agents for lyophilized products (Cappola, 2000). Also, the moisture ratio of lyophilized powder showed better stability with mannitol than with lactose as bulking agents (Korey & Schwartz, 1989). Lactose is a well-known reducing sugar. Although it may undergo Maillard Reaction with an amine group leading to instability of the formulation and it tends to increase impurity (Frank, 2004), we reported that hydroxocobalamin is much more stable with lactose than other excipients according to our formulation studies. Heathgote et al. demonstrated that hydroxocobalamin forms a complex with glycine more easily than with other amino acids (Heathgote, Moxon, & Slifkin, 1970). We detected that impurity level is the highest formulation with glycine. Glycine is an organic compound that contains amine and carboxyl groups. Related functional groups may lead to Maillard Reaction with hydroxocobalamin as well as degradation of the active ingredient. In contrast, the formulations included lactose monohydrate and EDTA enhance hydroxocobalamin stability. In the previous study, Herman et al. reported that the rate of decomposition of sodium methylprednisolone succinate with both mannitol and lactose as excipients, and marked that formulation which is mannitol as excipient showed a faster degradation in comparison to with lactose (Herman, Sinclair, Milton, & Nail, 1994). It is well-known that mannitol is a crystallization of the bulking agent unlike lactose. It is hypothesized that the product stability with lactose is better than mannitol. Crystallization of mannitol consists of δ-mannitol and mannitol hemihydrate during lyophilization. Release of water molecules from hemihydrate structure during stability period may cause degradation and instability of humidity sensitive drug products (Liao, Krishnamurthy, & Suryanarayanan, 2007; Gressl et al., 2017). Dubost et al. established the connection between a cyclic peptide drug and mannitol interaction in a lyophilized formulation. The results showed that degradation takes place with mannitol induced oxidation in a lyophilized injection (Dubost et al., 1996) Many stability and impurity problems during development and commercialization may be encountered in matching the inappropriate ingredients in pharmaceutical dosage forms (Carstensen, Osadca, & Rubin, 1969). Excipients that may have different functional groups interact with active pharmaceutical ingredients. These compounds, even in trace amounts, can adversely affect the stability and efficacy of formulation. Excipient related functional group associated with drug-excipient interaction such as Schiff base formation in formulation. Oxidation, hydrolysis, photolysis, polymerization and isomerization reactions are well known active compound-active compound interactions or active compound-excipient interactions (Fatima, Mamatha, Qureshi, Anitha, & Rao, 2011). These chemical drug-excipient interactions are important in drug impurity problems and incompatibilities in drug formulation (Hotha, Roychowdhury, & Subramanian, 2016; Vranic, 2004). Unwanted chemicals in formulation, called impurity profile, take place in some chemical interactions. For this reason, impurity profile of drug formulation is important for the efficacy and safety of the final products (Tegeli et al., 2011).

To the best of our knowledge, there is little research that has been done using lactose monohydrate, EDTA and glycine as stabilizing additives in hydroxocobalamin mixed parenteral solutions. Herein, we report novel lyophilized hydroxocobalamin formulations.

CONCLUSION

The present research study was designed to develop a lyophilized dosage form of a hydroxocobalamin formulation during the stability period. Based on the physicochemical properties of hydroxocobalamin and excipients, the impurity parameter was optimized with lactose monohydrate and lactose monohydrate + EDTA combination formulations. We also showed that the hydroxocobalamin vitamin is incompatibility with glycine for pharmaceutical research. The finding of this research proposes a new stable formulation and proper storage conditions for lyophilized hydroxocobalamin parenteral solutions. Thus, the most important aspect of this study is being the first scientific report specifically for hydroxycobalamin related pharmaceutical literature.

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Original Article

Spectrophotometric determinations of most commonly used statins in pharmaceutical preparations with 2,3-dichloro-5,6-dicyanobenzoquinone

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ABSTRACT

Background and Aims: In this study, simple, accurate and precise spectrophotometric methods were developed for the determination of five 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase inhibitors in pure and pharmaceutical dosage forms.

Methods: In the developed methods, atorvastatin fluvastatin, pitavastatin, rosuvastatin and simvastatin were used in this class of drugs called statins. The methods were based on the charge-transfer reaction of n-electron donor drugs with ϖ-acceptor 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). All variables such as temperature, time, reaction medium, amount of reagent were examined for optimal formation of complexes to be given by the drugs with DDQ and optimal conditions were determined.

Results: The linearity ranges for atorvastatin, fluvastatin, pitavastatin, rosuvastatin and simvastatin were found to be 0.5-50, 1-6, 2.5-50, 5-25 and 5-50 µg/mL, respectively.

Conclusion: The proposed methods were successfully applied to both the pure and the pharmaceutical dosage forms.

Keywords: HMG-CoA reductase inhibitors, spectrophotometric determination, charge-transfer reaction, DDQ, pharmaceutical preparations

INTRODUCTION

3-Hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase inhibitors, called statins, are the most effective drugs used to prevent hypercholesterolemia and related diseases. Statins show therapeutic effects by competitively inhibiting HMG-CoA reductase, an enzyme that catalyzes the conversion of HMG-CoA to mevalonate, an early rate limiting step in cholesterol biosynthesis in the body. These agents are highly effective in reducing total cholesterol and low-density lipoprotein levels in several forms of hypercholesterolemia (Tobert, 2003; Jones et al., 2003; Caslake et al., 2003; Antal et al., 2017).

Regarding the statins that form part of this study, simvastatin is a semi-synthetic product, while the others are fully synthetic compounds (Sweetman, 2005; Antal et al., 2017).

Atorvastatin calcium (AT) (3R,5R)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propan-2-ylpyrrol-1-yl]-3,5-dihydroxyheptanoic acid, calcium salt, Fluvastatin sodium (FL) (E,3R,5S)-7-[3-(4-fluorophenyl)-1-propan-2-ylindol-2-yl]-3,5-dihydroxyhept-6-enoic acid monosodium salt, Pitavastatin calcium (PT) (E,3R,5S)-7-[2-cyclopropyl-4-(4-fluorophenyl)quinolin-3-yl]-3,5-dihydroxy-

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hept-6-enoic acid monocalcium salt, Rosuvastatin calcium (RS) (E,3R,5S)-7-[4-(4-fluorophenyl)-2-[methyl(methylsulfonyl) amino]-6-propan-2-ylpyrimidin-5-yl]-3,5-dihydroxyhept-6-enoic acid calcium salt and Simvastatin (SM) [(1S,3R,7S,8S,8aR)-8-[2-[(2R,4R)-4-hydroxy-6-oxooxan-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl]-2,2-dimethylbutanoate (Figure 1) are the most commonly used statins in the treatment of hyperlipidemia (Lennernas & Fager, 1997; Terata et al., 2003; Sweetman, 2005; Antal et al., 2017). Several analytical methods such as spectrophotometric (Wang & Asgharnejad, 2000; Erk, 2002; Krishna & Sankar, 2007; Stanisz & Rafa, 2008; Saminathan, Sankar, Anandakumar, & Vetrichelvan, 2009; Gupta, Mishra, & Shah, 2009; Ashour, Bahbouh, & Khateeb, 2010; Moussa, Mohammed, & Youssef, 2010; Kokilambigai, Seetharaman, & Lakshmi, 2017), high performance liquid chromatographic (HPLC) (Carlucci, Mazzeo, Biordi, & Bologna, 1992; Ochiai, Uchiyama, Imagaki, Hata, & Kamei, 1997; Vuletic, Cindric, & Kouznjak, 2005; Sankar, Babu, Kumar, & Krishna, 2007; Gomes et al., 2009; Kaila, Ambasana, Thakkar, Saravaia, & Shah, 2010; Abdallah, 2011; Kumar, Nisha, Nirmal, Sonali, & Bagyalakshmi, 2011; Kokilambigai et al., 2017), HPTLC (Yadav et al., 2005; Sane, Kamat, Menon, Inamdar, & Mote, 2007) and electroanalytical techniques (Antal et al., 2017) have been used for the determination of these drugs, either simultaneously or alone, both as bulk drugs and as formulations (Figure 1).

As a result of our article, scanning studies, the spectrophotometric determination of these five statins based on the charge transfer reaction with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) are not available.

When we consider other analytical methods used in drug active substance analysis, spectrophotometric methods are advantageous because of the need for a simple and cheap device and a fast measurement capacity. The accuracy and precision of the methods are also well suited for the identification of drugs in both pure and dosage forms. With this information, we aimed to develop spectrophotometric determination of five statins using DDQ reagent. DDQ has been intensively used as the reagent for visible-spectrophotometric methods of a number of n-electron donor drugs (Foster, 1969; Melby & Patai, 1970; Rao, Bhat, & Dwivedi, 1972; Kovar, Mayer, & Auterhoff, 1981; Kovar & Abdel-Hamid, 1984; Hussein, Mohamed, & Abdel-Alim, 1989; Saleh, 1998; Abdellatef, 1998; Abdel-Gawad, Issa, Fahmy, & Hussein, 1998; Saleh, Askal, Darwish, & El-Shorbagi, 2003). In these methods, the blue-purple colored DDQ^{-} radical anion formed by the interaction of the drug substances in the appropriate solvent with the reagent was measured in the visible region.

MATERIALS AND METHODS

Apparatus

In this study, measurements were made using a Shimadzu UV-160 A spectrophotometer. A 1 cm glass cell was used for the measurements.

Reagents and solutions

AT and its pharmaceutical dosage form, Ator film tablet® (20 mg of per tablets) were provided by Sanovel pharmaceuticals

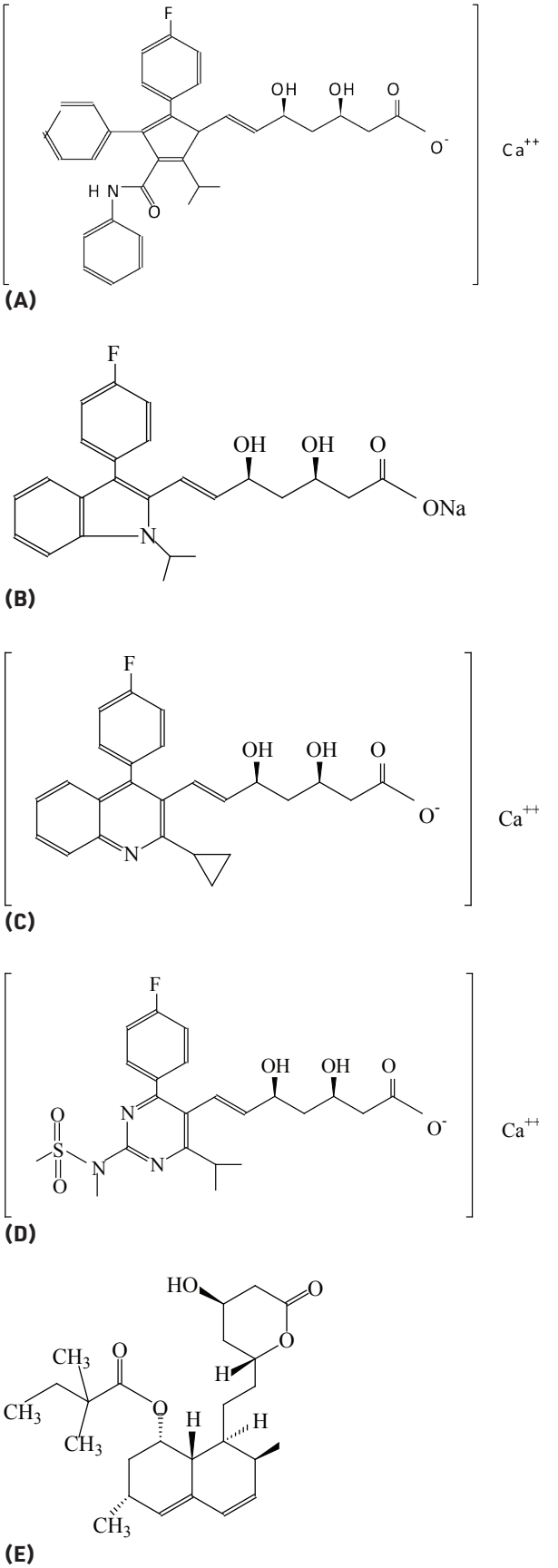

Figure 1. Chemical structure of statins, (A): AT, (B): FL, (C): PT, (D): RS, (E): SM.

(Istanbul, Turkey). FL and its pharmaceutical dosage form, Lescol capsul® (40 mg of per capsul) were obtained from Novartis pharmaceuticals (Istanbul, Turkey). PT and its pharmaceutical dosage form, Livalo film tablet® (2 mg of per tablets) were provided by Kowa pharmaceuticals (Japan). RS and its pharmaceutical dosage form, Crestor film tablet®, (20 mg of per tablets) were obtained from Astra Zeneca pharmaceuticals (Istanbul, Turkey). SM and its pharmaceutical dosage form, Zocor film tablet® (20 mg of per tablets) were obtained from Nobel pharmaceuticals (Istanbul, Turkey). DDQ was purchased from Merck (Darmstadt, Germany). In this study, analytical-reagent grade chemicals and solvents were used.

Stock solutions were prepared separately as follows: 2.0 mg/mL RS and 1.0 mg/mL SM solutions (equivalent to the same milligram of drug base) were prepared by dissolving in acetonitrile. 1.0 mg/mL FL solution (equivalent to the same milligram of drug base) was prepared by dissolving in a methanol-acetonitrile mixture (1: 9). 2.0 mg/mL. AT and PT solutions (equivalent to the same milligram of drug base) were prepared using dimethylsulfoxide as solvent.

1, 2 and 3 mg/mL. DDQ solution was prepared in acetonitrile (for RS, FL and SM) and in dimethylsulfoxide (DMSO) (for AT and PT). These solutions were freshly prepared every day.

Choice of solvent

During the selection of the most suitable solvent in the reactions and measurements, different solvents such as acetone, acetonitrile, chloroform, 1,4-dioxane, DMSO, ethanol, methanol and methylene chloride were tested. As a result of solvent selection studies, the most suitable solvents were determined to be acetonitrile for fluvastatin, rosuvastatin and simvastatin and DMSO for atorvastatin and pitavastatin.

Reagent concentration

The effect of DDQ concentration (%, w / v) on the efficiency of the reaction was investigated. Therefore, a constant concentration of statin was selected and various concentrations (%, w / v) of DDQ solution were added thereto. Measurements were made at the end of the same waiting period.

Reaction time and temperature

In order to find the optimum reaction temperature, the reactions were carried out at room temperature, 50, 60, 70 and 80 °C and the absorbance values were read.

Similarly, in order to find the optimal reaction time, the reactions were determined by monitoring the absorbance values for 10, 20 and 30 minutes at the temperature at which the highest absorbance was obtained for each substance.

Stoichiometry of the reaction

The molar ratios of drug-DDQ for each statin were examined according to Job's continuous variation method (Job, 1928).

General procedure

Appropriate volumes of stock solutions or drug solutions were taken up in a 5 mL volumetric flask, 1 mL of DDQ solution was added and the volume was adjusted to the volume with acetonitrile or DMSO. The reaction mixtures were allowed to stand for 20 min at 70°C for SM, 30 min at 80°C for FL, 20 min at 80°C for RS, 20 min at 90 for AT and PT. Then the absorbance of the formed complexes was measured at 459 nm (for FL, RS and SM) and 469 nm (for AT and PT) against reagent blank obtained in a similar way .

Assay

Ten capsules or tablets were weighed separately and the average capsule/tablet weight was calculated. The tablets or capsule contents were powdered using a mortar. The amount of powder, equivalent to one tablet weight per drug, was accurately weighed and transferred to a 100 mL volumetric flask. Then the appropriate amount of acetonitrile for RS and SM, acetonitrile-methanol mixture (9: 1) for FL or DMSO for AT and PT was transferred to the flask. The mixtures were shaken mechanically for five minutes and sonicated in an ultrasonic bath for 30 min. Then they were filled to the volume with the above solvents and filtered through a filter. A suitable portion of the filtrate was determined as defined in the General Procedure.

Method validation

Validation studies were planned according to the International Conference on Harmonization guidelines (ICH, 2005).

The selectivity of the method was performed with a mixture of common tablet excipients such as cellulose, glucose, lactose, starch, talc, magnesium stearate, titanium dioxide etc.

The calibration graph was created in accordance with Beer's laws with absorbance values (5 determinations per level) measured at 5 concentration levels of each statin.

The limits of quantitation (LOQ) and limits of detection (LOD) were calculated using the following formulas:

$LOQ = 10SDa / b$

$LOD = 3SDa / b$

(SDa is the standard deviation of the intercept and b is the slope)

The interday and intraday precision were studied by analysis of standards for same day and five different days (each n=5).

The accuracy of the proposed method was determined by standard addition method. Three different concentrations of standard statin solutions were added over different amounts of sample solutions and these mixtures were analysed. The recovery percentage of the standard added to the test samples was calculated using the following formula:

$$\text{Recovery \%} = [(C_t - C_u) / C_a] \times 100$$

Where C_t is the total concentration of the analyte; C_u is the concentration of the analyte present in the formulation; and C_a is the concentration of the analyte added to the formulation.

The robustness of the recommended method was evaluated by examining the effect of small changes in reaction conditions such as reaction time (5±0.5 min) and additional reagent volume (1.0±0.05 mL).

The proposed methods were examined by determining statins in drug dosage forms for applicability of the methods.

RESULTS AND DISCUSSION

AT FL, PT, RS and SM are the drugs most commonly used to prevent hypercholesterolemia and related diseases. , A current rise in cardiovascular disease has increased the need for these drugs and has led many pharmaceutical companies to add statins to their products. This has also increased the need for simple, accurate and precise methods for the determination of these substances.

Since statins are very popular drugs, there have been many studies to date on their analysis in pharmaceutical preparations. However, visible spectrophotometric determination of these drugs in pharmaceutical preparations based on charge transfer reaction with DDQ has not yet been published. Therefore, visible spectrophotometric analyses using DDQ reagent were developed for the determination of these drugs in pharmaceutical preparations. The developed methods are based on the formation of ion pair complexes of these p-acceptor drugs with DDQ, the n-electron donor. For many years, π –acceptors have been known to produce charge transfer complexes and radical anions with various electron donors (Foster, 1969; Melby & Patai, 1970; Rao et al., 1972; Kovar et al., 1981; Kovar & Abdel-Hamid, 1984; Hussein et al., 1989; Saleh, 1998; Abdellatef, 1998; Abdel-Gawad et al., 1998; Saleh et al., 2003; Antal et al., 2017).

Blue chromogens were obtained from the reaction of statins with DDQ in acetonitrile or DMSO. Those obtained in acetonitrile or DMSO from these chromagens showed maximum absorption at 459 and 469 nm, respectively (Figure 2).

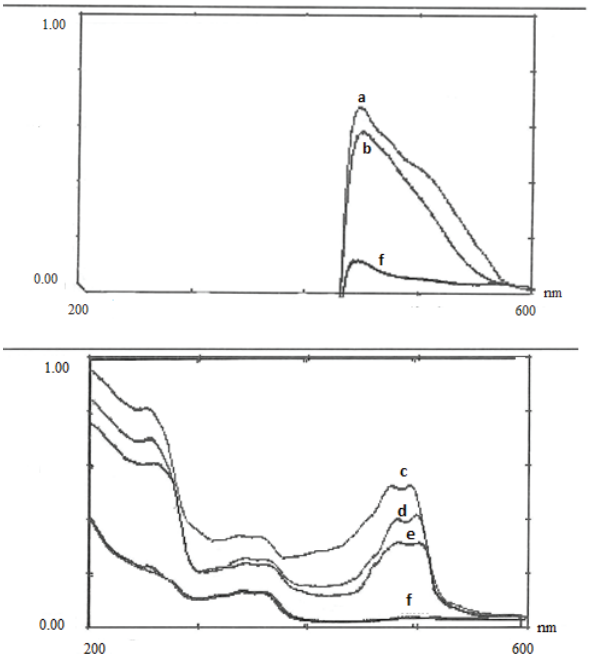

Figure 2. Absorption spectrum of charger transfer complex between drugs and DDQ reagent a: PT; 20 µg mL^{-1}, b: AT; 40 µg mL^{-1}, c: FL; 2.5 µg mL^{-1}, d: RS; 10 µg mL^{-1}, e: SM; 10 µg mL^{-1}, f: reagent blank.

In order to determine the optimum conditions, the effect of different parameters on chromogens was examined.

Choice of solvent

In solvent selection studies, a number of solvents were tested as mentioned above in the experimental section. The most suitable solvent was found to be acetonitrile for FL, RS and SM, and DMSO for AT and PT.

Reagent concentration

In the reagent quantification experiments, various concentrations of the DDQ solution (by volume) were studied by adding to a constant statin concentration. 1.0 mg/mL (w/v) DDQ solution was found to be sufficient for the quantitative determination of PT, 2.0 mg/mL (w/v) DDQ solution was found to be sufficient for the quantitative determination of SM and RS; 3.0 mg/mL (w/v) DDQ solution was found to be sufficient for the quantitative determination of FL.

Reaction time and temperature

The optimum reaction temperature and time was determined for each drug by following the absorbance values at 50, 60, 70, 80 and 90 °C, at 10, 20 and 30 minutes. The reaction temperature and time appropriate for each substance are shown in Table 1 (below).

Table 1. The results of optimum temperature and time

Statins	Optimum Temperature	Optimum Time
SM	70 °C	20 min
FL	80 °C	30 min
RS	80 °C	20 min
AT	90 °C	20 min
PT	90 °C	20 min

Stoichiometry of the reaction

Using the equimolar drug and DDQ solution, the reaction stoichiometry was examined separately for each drug. The mole ratios (drug/reagent) were found 1:1 for SM and for PT (one molecule of reacts with one molecule of DDQ), 5:1 for RS and AT (five molecules of reacts with one molecule of DDQ) and 1:2 for FL (one molecule of FL with two molecules of DDQ).

Method validation

In method validation studies, there was no interaction from the additions and excipients, e.g. lactose, glucose, fructose, magnesium stearate and starch.

Linear relationships between the absorbance and the concentration were found to be in the ranges of 0.5-50, 1-6, 2.5-50, 5-25, 5-50 µg/mL for AT, FL, PT, RS and SM, respectively. The regression equation parameters of the developed methods are shown in Table 2.

LOD values of the methods were calculated to be 1.13, 0.33, 1.84, 1.61 and 0.27 µg/mL; and additionally, LOQ values of the methods were calculated to be 3.77, 1.10, 6.14, 5.37 and 0.90 µg/mL for AT, FL, RS, PT and SM, respectively.

Table 2. The results of validation parameters for proposed methods.

Parameter	Statins				
	AT	FL	RS	PT	SM
Linearity range[a] (mg mL^{-1})	0.5-50.0	1.0-6.0	5.0-25.0	2.5-50.0	5.0-50.0
Regression equation*	A=0.0106C-0.192	A=0.1264C-0.0341	A=0.0423C-0.0586	A=0.0132C-0.1941	A=0.0191C-0.0141
Slope ± SD	0.0106± 0.0012	0.1264± 0.0026	0.0423±0.002	0.0132±0.0043	0.0191±0.0002
Intercept ± SD	-0.192± 0.0059	-0.0341± 0.0078	-0.0586±0.005	-0.1941± 0.0055	-0.0141±0.0012
Correlation coefficient, r	0.9993	0.9998	0.9992	0.9985	0.9998
LOD (mg mL^{-1})	1.13	0.33	1.84	1.61	0.27
LOQ (mg mL^{-1})	3.77	1.10	6.14	5.37	0.90

[a]Average of six determinations
[b]n=6 correspond to replicate analysis for each level
*Results of six different days
**$A = a + bC$ (where C is the concentration of drug in mg mL^{-1}, A is the absorbance at λ_{max}).

In the precision study, the RSD values were found te be 0.14-1.69% for intra-day precision and 0.25-1.88% for inter-day precision. The obtained results indicate good repeatability and reproducibility as outlined in Table 3.

Accuracy studies were conducted using the standard addition method and the obtained results are shown in Table 4. The average recovery percentage obtained was between 99.7-101.7%, which showed good accuracy of the methods.

In studies on the robustness of the proposed methods, small changes in the procedure variables such as reaction time (5±0.5 minutes), and added reagent volume (1.0±0.05 mL) were examined and it was found that the methods were not affected by these changes.

The proposed methods were applied to the quantification of the pharmaceutical preparations of these drugs and the results obtained are shown in Table 5. The results obtained by the methods were satisfactorily accurate and precise with excellent % recovery and RSD values.

Table 3. The results of precision studies by obtained with proposed method.

Statins	Amount taken (mg mL^{-1})	Intraday RSD[a] (%)	Interday RSD[a] (%)
AT	2.5	1.60	1.88
	10.0	1.69	1.83
	30.0	1.26	1.40
FL	1.0	1.11	1.42
	3.0	1.05	1.28
	6.0	0.43	0.64
RS	5.0	0.85	1.54
	10.0	0.25	0.74
	25.0	0.33	0.58
PT	2.5	1.13	1.33
	25.0	0.14	0.33
	50.0	0.20	0.25
SM	5.0	0.89	1.59
	25.0	0.36	0.40
	50.0	0.34	0.47

[a]Relative standard deviation

Table 4. The results of recovery studies obtained with standard addition method.

Statins	Amount taken (mg mL^{-1})	Amount added (mg mL^{-1})	Total amount found[a] (mg mL^{-1}) (Mean± S.D[b])	Recovery (%)	RSD (%)
AT[1]	10.0	10	19.984±0.402	99.7	1.84
		20	30.09±0.558	100.5	1.63
		30	39.73±0.698	101.2	1.80
FL[2]	1.0	1.0	2.022±0.013	101.1	0.65
		2.0	3.036±0.027	100.9	0.91
		4.0	5.094±0.017	101.7	0.30
RS[3]	5.0	2.5	7.54±0.041	100.6	0.43
		5.0	10.11±0.039	100.8	0.54
		15	20.44±0.227	101.2	0.42
PT[4]	10.0	2.5	12.53±0.032	100.3	0.26
		15	25.13±0.083	100.4	0.24
		35	45.16±0.075	100.3	0.14
SM[5]	10.0	5.0	15.12±0.054	100.6	0.42
		15.0	25.24±0.108	100.8	0.53
		35.0	45.19±0.111	100.5	0.19

[1]Ator tablet®, containing 20 mg of AT per tablets
[2] Lescol tablet®, containing 40 mg of FL per tablets
[3] Crestor tablet®, containing 20 mg of RS per tablets
[4] Livalo tablet®, containing 2 mg of PT per tablets
[5] Zocor tablet®, containing 20 mg of SM per tablets
[a]Six independent analyses.
[b]Standard deviation

Table 5. The results analysis of drugs in tablets.

Statins	Mean[a] ± S.D[b]	Recovery (%)	RSD (%)
AT[1]	19.69 ±0.49	99.45	2.52
FL[2]	39.97±0.4	100.279	1.000
RS[3]	19.902±0.0482	99.40	0.2420
PT[4]	1.97±0.05	99.25	2.56
SM[5]	20.002±0.62	99.91	3.1

[1]Ator tablet®, containing 20 mg of AT per tablets
[2]Lescol tablet®, containing 40 mg of FL per tablets
[3]Crestor tablet®, containing 20 mg of RS per tablets
[4]Livalo tablet®, containing 2 mg of PT per tablets
[5]Zocor tablet®, containing 20 mg of SM per tablets
[a]Six independent analyses.
[b]Standard deviation

The obtained results were statistically compared by the student's *t*-test (for accuracy) and the variance ratio *F*-test (for precision) with the results obtained by the official methods for AT (USP, 2009), FL (USP, 2007) and SM (USP, 2007), reference methods for RS (Gomes et al., 2009) and PT (Kumar et al., 2011). It was observed that the values of t- and F-tests obtained at 95% confidence level did not exceed the theoretical table value and there was no significant difference between the methods compared (Table 6).

CONCLUSION

This study aimed to develop validated spectrophotometric methods, which are also fast, simple and economical, to analyse AT, FL, RS, PT and SM in pharmaceutical preparations. The methods developed are based on the charge transfer reaction of these drugs with DDQ reagent.

The sensitivity of the proposed methods is almost the same (Gupta et al., 2009; Moussa et al., 2010; Kokilambigai et al., 2017), or more sensitive, when compared with some previously published methods (Erk, 2002; Stanisz & Rafa, 2008; Saminathan et al., 2009; Ashour et al., 2010; Kokilambigai et al., 2017). Additionally, the proposed methods are faster than some previously published methods (Saminathan et al., 2009; Ashour et al., 2010; Kokilambigai et al., 2017).

Furthermore, the proposed methods are cheaper than the HPLC techniques (Carlucci et al., 1992; Ochiai et al., 1997; Vuletic et al., 2005; Sankar et al., 2007; Gomes et al.,

Table 6. Statistical evaluations of the results obtained by proposed and reference methods for the assay of drugs in pharmaceutical preparations (*n*=5)

Statistical value	AT		FL		RS		PT		SM	
	Proposed method[a]	Ref. Method[48]	Proposed Method[b]	Ref. Method[47]	Proposed Method[c]	Ref. Method[21]	Proposed Method[d]	Ref. Method[47]	Proposed method[e]	Ref. Method[30]
Mean*± SD	19.69±0.49	20.01±0.345	39.97 ±0.4	39.93 ±0.367	19.902±0,0482	20.31±0.375	1.97±0.05	2.028±0.04	20.002±0.62	19.88±0.256
Recovery (%)	99.45	100.05	100.279	99.82	99.40	101.55	99.25	101.4	99.91	99.4
RSD (%)	2.52	1.72	1.0	0.919	0.2420	1.846	2.56	1.923	3.1	1.287
t-test of significance**	1.8945		0.8685		1.387		1.747		0.3982	
F-test of significance**	0.559		0.45767		0.659		0.79957		1.10596	

[a]Ator tablet® (20 mg AT), [b]Lescol tablet® (40 mg FL), [c]Crestor tablet® (20 mg RS), [d]Livalo tablet® (2 mg PT), [e]Zocor tablet® (20 mg SM),
* Five independent analyses. **p = 0.05, t = 2.228, F = 5.05

2009; Kaila et al., 2010; Abdallah, 2011; Kumar et al., 2011; Kokilambigai et al., 2017).

The methods described can safely be used to determine statins in pharmaceutical formulations without intervention from excipients and can easily be applied in quality control laboratories for routine analysis of these drugs in raw materials and pharmaceutical formulations.

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Original Article

Evaluation of in vitro anti-cancer effects of *Styphnolobium japonicum* root extract in human colon (HT-9), brain (U-87), and prostate (PC-3) cancer cell lines

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ABSTRACT

Background and Aims: *Styphnolobium japonicum* (L.) Schott. *(Sophora japonica)* is a medicinal plant applied for various diseases, in the traditional medicine field. The evaluation of methanol extract of *S. japonicum* root derived from the Pharma Grade plant drug, was performed in terms of various *in vitro* biological activities.

Methods: The LC-MS analysis was used for the chemical characterization of the methanol extract. The anti-cancer activity was evaluated in colon (HT-9), brain (U-87), and prostate (PC-3) cancer cells by Cell Titer Glo viability assay (Promega) and western blot analysis of PARP (Poly ADP-ribose polymerase) cleavage.

Results: The relative amounts of matrine and oxymatrine in the extract were found as 0.49±0.006 mg/mL and 0.27±0.016 mg/mL, respectively. The *S. japonicum* extract showed 53.17±0.97 mg of gallic acid (GA)/g corresponding to the total phenolic amounts, resulting in relatively moderate antioxidant activity (1.94±0.23 and 2.79±0.15 mg/mL) on the *in vitro*2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS•) and 2,2-diphenyl-1-picrylhydrazyl (DPPH•) assays. Treatment with 10 mg/mL *S. japonica* root extract for 24h resulted in a significant decrease in cell viability. The cell viability of U-87, HT-29, and PC-3 cancer cell lines was determined as 35±2.21%, 14±2.11%, and 46±5.67%, respectively. The extract showed 5.104, 5.012 and 0.555 mg/mL IC_{50} values for HT-29, U-87, and PC-3 cell lines, respectively. Particularly, the IC_{50} value of PC-3 cancer cell line was significantly lower than the healthy human fibroblast cells. In further, the apoptosis in *S. japonicum* root extract treated PC-3 cells was detected through flow cytometry analysis of Annexin V positive cells and western blot analysis of PARP cleavage.

Conclusion: It can be concluded that the methanol extract in determined doses induces the apoptosis of the PC-3 cancer cells, without any significant cytotoxic effect on healthy human fibroblast cells. In addition, the LCMS analysis showed the presence of matrine and oxymatrine, which are known for their anticancer activity. To the best of our knowledge, these are the first preliminary results indicating the possible use of *S. japonicum* root extract. Thus, the methanol extract can be further studied for its therapeutic potential of primarily prostate and other cancer types.

Keywords: Cytotoxicity, *Styphnolobium japonicum (Sophora japonica)*, antioxidant, cancer cell lines, western blotting, flow cytometry analysis

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INTRODUCTION

Cancer is a serious health burden and is responsible for the second leading cause of death worldwide. A World Health Organization (WHO) report in 2003 stated that cancer rates could further increase by 50% to 15 million during the year 2020 (Verma & Singh, 2020). Traditional medicinal plants and their natural components have been under special scientific research interests during recent years. Application of aromatic and medicinal plants in phytotherapy is typically due to their numerous biological activities such as antiviral, antibacterial, anticarcinogenic, and antioxidant properties. (Nasrollahi, Ghoreishi, Ebrahimabadi, & Khoobi, 2019). Naturally derived anticancer chemotherapeutic products being currently used in cancer management such as vincristine, vinblastine, irinotecan, etoposide, paclitaxel, camptothecin, and epipodophyllotoxin occupy a crucial position because of their limited side effects and anti-multidrug resistance (Cragg & Newman, 2005; Nobili et al., 2009).

Styphnolobium japonicum (Sophora japonica) is a plant known as Chinese Scholar Tree which also grows in Asian countries such as Korea, and Japan. It belongs to the *Fabaceae* family and is used in Traditional Chinese Medicine. Monographs of the herbal drug have been published in the Materia Medica as well as the European Pharmacopoeia. Fruits, roots, and bark preparations are commonly used to treat haemorrhoids, haematuria, arteriosclerosis, headache, hypertension and as well as a haemostatic agent in Korean traditional medicine (He et al., 2016; Kim & Yun-Choi, 2008).

More than 150 chemical compounds have been characterised from *S. japonicum* such as isoflavonoids, flavonoids, alkaloids, triterpenes, and other compounds (He et al., 2016). Especially the roots are rich in quinolizidine alkaloids. Matrine and oxymatrine are the characteristic constituents of the root extract and were reported for their diverse biological and pharmacological activities (Pelletier, 1991). There are studies on the sedative, inotropic, antipyretic, antitumor effects, antinociceptive activity among others (Ding, Liao, Huang, Zhou, & Chen, 2006; Higashiyama et al., 2005; Ma et al., 2008). Clinically, oxymatrine is known to be more active than matrine. In previous studies, it has been recorded that oxymatrine can regulate cardiac arrhythmias. Matrine is used against eczema, psoriasis, and neurodermatitis in combination with other anti-inflammatory combinations (Ting, Ruwei, Guoyong, Meizhen, & Songhua, 2002). In previous studies, matrine showed *in vitro* activities in cervical cancer research (Zhang, Jiang, Yan, Liu, & Zhang, 2015). In another study, it was found that matrine inhibited the growth of MCF-7 breast cancer cells with MTT assay. It was determined that the MCF-7 cell cycle changed 48 hours after the administration of matrine and is more effective in S-G0-G1 phases in this cell cycle (Shi, Shen, Fang, Xu, & Hu, 2015).

The aim of the present study is to detect possible anticancer activity of the methanol extract of *S. japonicum* root. For this aim, in the present study, the extract was analysed by LC techniques to confirm its matrine and oxymatrine content. *S. japonicum* root methanol extract was evaluated in respect to its *in vitro* cytotoxic, apoptotic and antioxidant activities. To analyse cell death, upon treatment with *S. japonicum* root extract, the viability was measured *in vitro* in various cancer cell lines including colon (HT-9), brain (U-87), and prostate (PC-3) cancer cells in comparison with healthy human fibroblasts. Furthermore, apoptosis was analysed by Annexin V/PI staining and western blot detection of PARP cleavage as a final downstream biochemical indicator of apoptosis (Fischer, Jänicke, & Schulze-Osthoff, 2003; Kaufmann, Desnoyers, Ottaviano, Davidson, & Poirier, 1993; Tewari et al., 1995).

MATERIAL AND METHODS

Materials

The standard chemicals were provided from Sigma Chemical Co. (USA) and the HPLC-grade solvents were obtained from Merck.

Plant material and extraction

The Pharma Grade *Styphnolobium japonicum* root was acquired from Germany. For the extraction procedure, the roots were ground to a powder (100 gr), and then macerated with methanol (3x 100 mL) for 48 h. The extract was filtered, and the filtrate was concentrated using a rotary evaporator (Heidolph, Germany). The prepared extract (21g) was stored at 4°C until the experiments.

Antioxidant activity

DPPH• scavenging assay

The antioxidant capacity of the extract was detected using DPPH• by its capability to bleach the stable radical (Blois, 1958). The reaction mix contained 100 µM DPPH• in crude extract and methanol. After 30 min, absorbances were measured at 517 nm by using a UV–Vis spectrophotometer (UV-1800, Shimadzu, Japan) at 25±2°C and the radical scavenging activity (RSA) was determined as follows:

$$\text{DPPH• RSA \%} = [(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test sample}}) / \text{Absorbance}_{\text{control}})] \times 100$$

ABTS• scavenging assay

The other method for determining antioxidant capacity of the extract was the ABTS• method (Re et al., 1999). ABTS• solution (7 mM) was mixed with potassium persulfate (2.45 mM). The mixture was kept for 12-16 h in the dark at 25±2°C. To regulate its absorbance at 734 nm, this mixture was diluted. To calculate the absorbance of the extract, 990 µL ethanol was used instead of ABTS• in the control. Trolox was used as the positive control standard (Okur et al., 2018). The outcomes were signified as IC_{50} as follows:

$$\text{ABTS• RSA \%} = [(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test sample}}) / \text{Absorbance}_{\text{control}})] \times 100$$

Total phenolic content of the extract

Folin-Ciocalteu method was used for determination of total phenolics content. Folin-Ciocalteau's reagent (0.25 mL) and Na_2CO_3 (0.2 mL) were mixed with the extract (5 mL) and allowed to incubate at 45°C for 15 min. The absorbance was determined at 765 nm at 25±2°C. The total phenolic content was measured from a linear calibration curve ($R^2 = 0.9892$) (Spanos & Wrolstad, 1990).

LC-MS analysis

The LC analyses were studied on a Shimadzu (LC 2040c, Japan). LC was run on an Agilent C18 column (4.6 x 250 mm x 5 μm, Zorbax, Agilent, Japan) and the column temperature was kept at 40°C. The mobile phase was methanol/water/diethylamine (50:50:0.07, v/v/v) at pH 10.5. The flow rate was 0.8 mL min^{-1}. Injection volume was 50 μL and total run time was 22 min for each test sample.

An MSD mass spectrometer system (Shimadzu 2020, America) was equipped with electrospray ionisation (ESI) source for the mass analysis and detection. MS data were acquired in the positive mode with selective ion monitoring (SIM). The drying gas (nitrogen) flow rate was 12.0 L min^{-1} and gas temperature was maintained at 300°C (Wu, Chen, & Cheng, 2005). Matrine and oxymatrine were analysed by matching their retention times and mass spectra against those of the standards analysed under the same conditions.

Preparation of stock solutions

For the preparation of calibration curves of matrine and oxymatrine, 2 mg of each compound was dissolved in water and filtered. By diluting the stock solutions, three different (0.7, 0.4, 0.1 mg/mL) concentrations of matrine and oxymatrine were prepared. Each concentration was applied triplicate to the system. The regression equation of the calibration curve was obtained as 0.9927.

Cell culture

HT-29; colon cancer, U-87-GBM; brain cancer (ATCC, #HTB-14), (ATCC, #HTB-38), PC-3; prostate cancer (ATCC, #CRL-1435) and human primary dermal fibroblast cells (HDFa) (ATCC, #CRL-PCS-201-012) were purchased from ATCC (U.S.). Then the cells were grown and expanded in DMEM (Gibco) medium with 10% fetal bovine serum (Gibco), 1% antibiotics (penicillin/streptomycin) at 37°C in a 5% CO_2 incubator. The cells were then removed from the flask with Trypsin/EDTA 0.25% (Gibco) and seeded at a density of $5x10^3$ cells/well into 96 black well plates (Corning) for cell viability assays.

Cell viability assay

Extracts were dissolved in methanol to prepare stock solutions, and serial dilutions were made using 1% methanol as a final concentration to normalise measurements. After seeding into 96 well plates, cells were incubated at 37°C in 5% CO_2 for 48 h. Then the culture medium was discarded, and cells were treated with 1, 5 and 10 mg/mL of *S. japonicum* extract as triplicates. After 24 h of treatment, Cell Titer Glo reagent (Promega) was added into each well and the percentage of viable cells was determined by reading the luminescence signal by SpectraMax i3x Multi-Mode Detection Platform (Tomani et al., 2018).

Western blotting

For western blot sample collection, cells were seeded at $2x10^5$ cells/well into 6 well plates. Then the next day, cells were incubated at 37°C in 5% CO_2 for 24 h. Then the culture medium was discarded and cells were treated with 0 mg/mL (control) or 1 mg/mL of *S. japonicum* root methanol extract. (Control wells were treated with an equal amount of extract solvent; DMSO). After 24 h of treatment, protein lysates were obtained from each well using Ripa lysis buffer (Thermo Fischer Scientific; #89900).

Equal amounts of protein samples were run on SDS-PAGE and Bio-Rad semi-dry western blotting protocol was applied. As for primary antibodies, anti-cPARP (CST; #9542), and anti-β-actin (CST #4970) were used. As for secondary antibodies, anti-rabbit (CST; #7074), and anti-mouse (GenDEPOT; #W3903) were used.

Flow cytometry analysis

After seeding into 100 mm × 20 mm culture dishes, cells were incubated at 37°C in 5% CO_2 for 24 h. Then the culture medium was discarded, and cells were treated with 0 mg/mL (control) and 1 or 5 mg/mL, of *S. japonicum* extract. (Control wells were treated with an equal amount of extract solvent; DMSO). After 24 h treatment with *S. japonicum* extract, Annexin V-FITC/Propidium Iodide (PI) early apoptosis double staining protocol was applied according to manufacturer's instructions (CST #6592 Annexin V-FITC Early Apoptosis Kit). Then, the percentages of apoptotic cells were determined by flow cytometry analysis.

Statistics

Statistical comparisons were performed by unpaired *Student's t-test* assuming equal variance. Differences were considered as statistically significant at $0.001<p^*< 0.005$; $p^{**}< 0.0005$; and $p^{***}< 0.0001$. Data are the mean ± standard error (SE).

RESULTS AND DISCUSSION

According to our results, the *S. japonicum* methanol extract showed relatively less antioxidant activity against DPPH (IC_{50}=2.79±0.15 mg/mL) and ABTS (IC_{50}=1.94±0.23 mg/mL) radicals compared to the standards ascorbic acid and trolox, respectively. The total phenolic content (TPC) of the *S. japonicum* MeOH extract was measured by using the Folin-Ciocalteu technique and calculated as a gallic acid (GA) equivalent amount. The *S. japonicum* extract showed 53.17±0.97 mg of GA/g corresponding to the total phenolic amounts, resulting in relatively moderate antioxidant activity on the *in vitro* ABTS• and DPPH• assays.

Our results also indicate that the *S. japonicum* root extracts have remarkable antioxidant activity. In previous antioxidant activity studies on *S. japonicum* extracts, it was observed that *S. japonicum* extracts showed different and varying results. It can be concluded that this may be due to the differences in the locations and extraction methods of the plant material (Mihaylova & Schalow, 2013; Tang, Li, Hu, & Lou, 2002). The results suggest that TPC is present in a relatively good amount in the extract. Based on the data obtained from performed experiments, a high correlation was found between the total phenolic content and antioxidant activity for methanol extract of *S. japonicum*.

As shown in Figures 1-3, matrine and oxymatrine standards and crude methanol extract were analysed and quantified by LCMS. According to the results obtained from LCMS analysis,

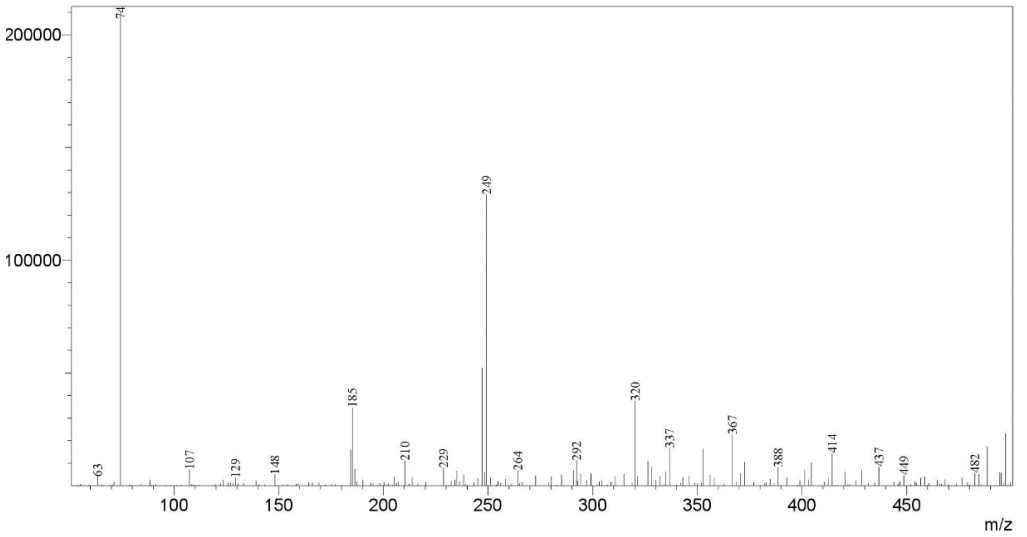

Figure 1. MS Chromatogram of Matrine Standard.

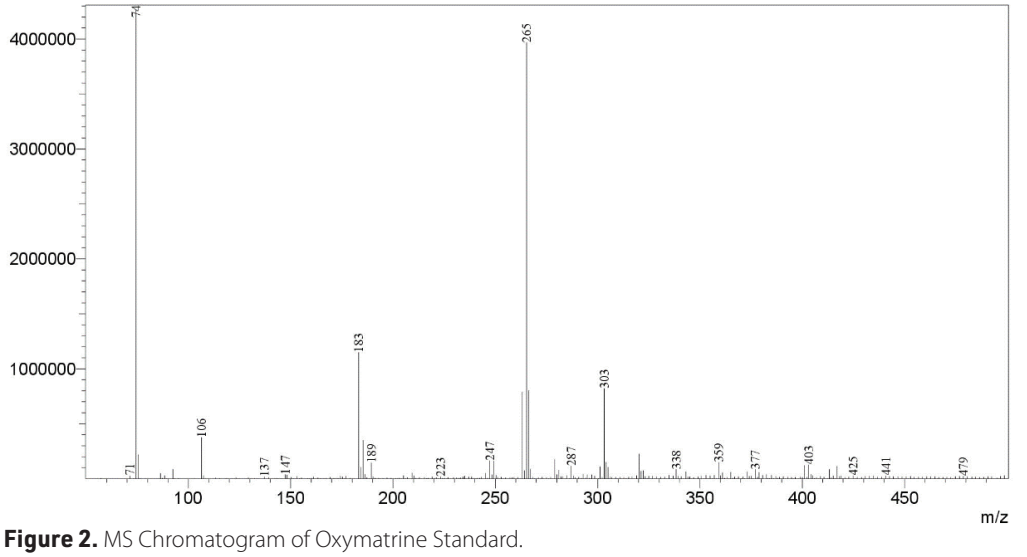

Figure 2. MS Chromatogram of Oxymatrine Standard.

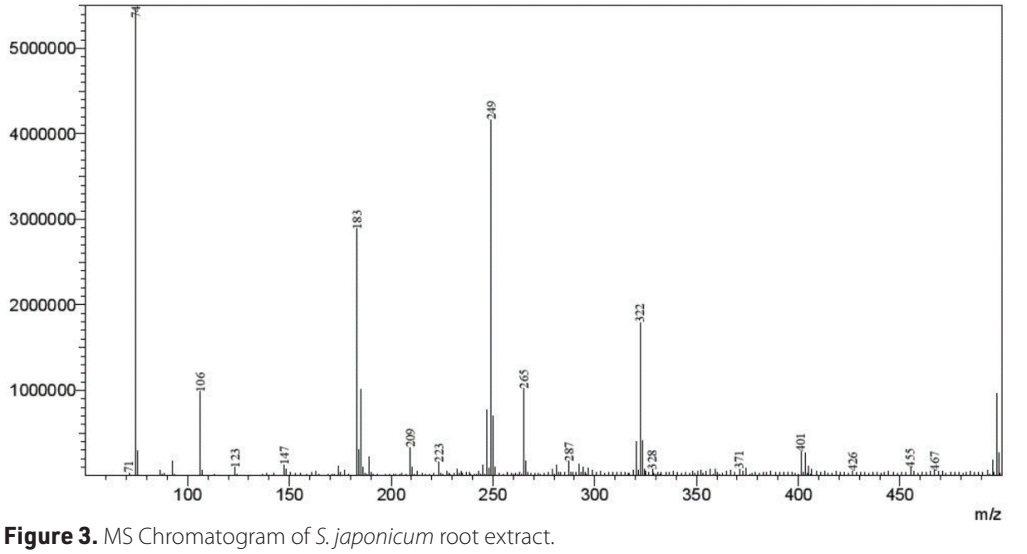

Figure 3. MS Chromatogram of *S. japonicum* root extract.

matrine (0.49±0.006 mg/mL) and oxymatrine (0.27±0.016 mg/mL) were determined in *S. japonica* root methanol extract. Therefore, it can be asserted that matrine and oxymatrine alkaloids in the extract are responsible for the anticancer activity of the extract. It can be thought that the extract shows a significant cytotoxic effect against the tested cell lines due to these alkaloids (Li et al., 2011; Ma et al., 2008; Shi et al., 2015; Yu et al., 2009; Zhang et al., 2015 .

In previous studies, *S. japonicum* leaf and bud extracts have been investigated for their anti-cancer activity against breast and colon cancer cell lines (Abdelhady, Kamal, Othman, Mubarak, & Hadda, 2015; Lee et al., 2015). The obtained results were quite significant in terms of anti-cancer activity. Besides, matrine and its derivatives are molecules for which their anticancer activity is well documented (Li et al., 2011; Yu et al., 2009); matrine is the main alkaloid of *S. japonicum* root extracts. In past studies, matrine and oxymatrine have been assessed for their remarkable anticancer activity. Matrine and its derivatives have been reported as antineoplastic agents since they can inhibit proliferation and induce apoptosis of cancer cells. Besides, matrine could synergistically improve the efficacy of chemotherapy when it is used in combination with other anticancer drugs (Rashid, Xu, Muhammad, Wang, & Jiang, 2019).

Cancer is defined as uncontrolled or abnormal growth and proliferation of cells as a result of errors in DNA division during cell division (Hassanpour & Dehghani, 2017). In this present study, the effect of *S. japonicum* root MeOH extract on the viability of various cancer cell lines was investigated. The cytotoxic effects of the root of *S. japonicum* methanol extract was tested on HT-29, U-87 and PC-3 cell lines by measuring metabolically active cells using a luciferase-based assay, Cell Titer Glo (Promega). According to the obtained results, the cell viability was decreased at varying concentrations (1 mg/mL, 5 mg/mL and 10 mg/mL) in a concentration-dependent manner for all tested cancer cell lines. The results are in accordance with previous studies (Chang et al., 2013; Coussens et al., 2018; Huang et al., 2018; Rodenhizer, Dean, Xu, Cojocari, & McGuigan, 2018). Previous studies have shown the superior effects of matrine on the growth of HT29 cell lines, and the expression of the related proteins. MTT assay indicated that matrine considerably inhibited the HT29 cells proliferation *in vitro* in a dose- and time-dependent manner. MTT assay was used to study the inhibitory effects of matrine on the proliferation of HT29 cells; the treatment of cells was performed using different concentrations (2–32 mg/mL) for 24, 36 and 48 h. Consequently, when the matrine dose increased, the proliferation of the HT29 cells was significantly suppressed *in vitro* in a dose- and time-dependent way. In conclusion, matrine has strong antitumor activity against HT29 cells and can act as an alternative agent to treat colon cancer (Chang et al., 2013). Huang et al. studied the efficacy of matrine on prostate cancer lines (DU145 and PC3 cell lines). The results showed that matrine and GADD45B overexpression synergistically inhibited the proliferation, migration, and invasion of prostate cancer cells. Additionally, the apoptosis of prostate cancer cells was also synergistically enhanced by matrine and GADD45B overexpression (Huang et al., 2018).

Treatment with 10 mg/mL *S. japonicum* extract resulted in a 35±2.21% viability of cells on U-87 cells, while PC-3 cells displayed 46±5.67% cell viability, respectively. Additionally, the extract showed the lowest viability against the HT-29 colon cancer cell line as 14%. Cell viability of *S. japonicum* root extract-treated HT-29, U-87 and PC-3 cell lines at 24 hours are given in Figure 4 A-C. Accordingly, the tested methanol extract showed 5.104 and 5.012 mg/mL IC_{50} values for HT-29, and U-87 cell lines, respectively (Figure 4A and Figure 4B) while the IC_{50} value of PC-3 cell line was determined as 0.555 mg/mL (Figure 4C). Furthermore, among all the cancer lines tested, at the lowest dose of 1 mg/mL of extract treatment, only the PC-3 cell line showed significant cell death with a decreased viability to 64% (Figure 4C).

To analyse the anti-cancer effects of *S. japonicum* root extract, as a healthy control, human primary fibroblast cells were included in cell viability experiments. As a result, fibroblasts showed 1.320 mg/mL IC_{50} value which is approximately two-fold higher than the IC_{50} of PC-3 cancer line and therefore, fibroblasts did not show a significant decrease in cell viability when treated with 1mg/mL (Figure 4D).

This data indicated the PC-3 cancer cell line could be treated with *S. japonicum* root methanol extract while the same dose is not cytotoxic to the healthy cells. Then, further analysis of the cell death was followed with biochemical verification of cell death and staining of apoptosis in PC-3 prostate cancer cells upon treatment with *S. japonicum* root extract for 24h. For detection of apoptosis, we stained the extract-treated PC-3 cells (0, 1 and 5 mg/mL) with Annexin V and Propidium Iodide (PI) then analysed by flow cytometry. According to our results, 1 mg/mL and 5 mg/mL extract treatments showed increased apoptosis in PC-3 with 16.3% and 99.6% respectively while untreated cells showed 1.89% apoptotic cells (Figure 5). This data indicates that *S. japonica* root extract treatment causes enhanced apoptotic cell death following increased doses.

Poly (ADP-ribose) polymerases (PARPs) are enzymes that can catalyse the transfer of ADP-ribose to target proteins. They take an important role in various cellular processes, including transcription, replication, recombination, and DNA repair. Caspase mediated apoptosis occurs through the cleavage of several key proteins required for cellular functioning and survival (Fischer et al., 2003) and PARP-1 cleavage by caspases is considered to be a hallmark of apoptosis (Kaufmann et al., 1993; Tewari et al., 1995). One of the main biochemical indicators of cell death is the cleavage of PARP (Boulares et al., 1999). For this reason, we analysed cPARP levels of extract-treated PC-3 cells (1mg/mL), as well as DMSO treated control cells (DMSO was added instead of an equal volume of the extract). The results indicated significantly increased cPARP (89kda) levels in the extract-treated PC-3 cells ($p=0.0002$) (Figure 6). This reveals that cell death is triggered upon treatment and possibly by the matrine and oxymatrine compounds residing in *S. japonicum* extract.

In conclusion, the results revealed that *S. japonicum* root methanol extract could trigger apoptosis in cancer cells and it is

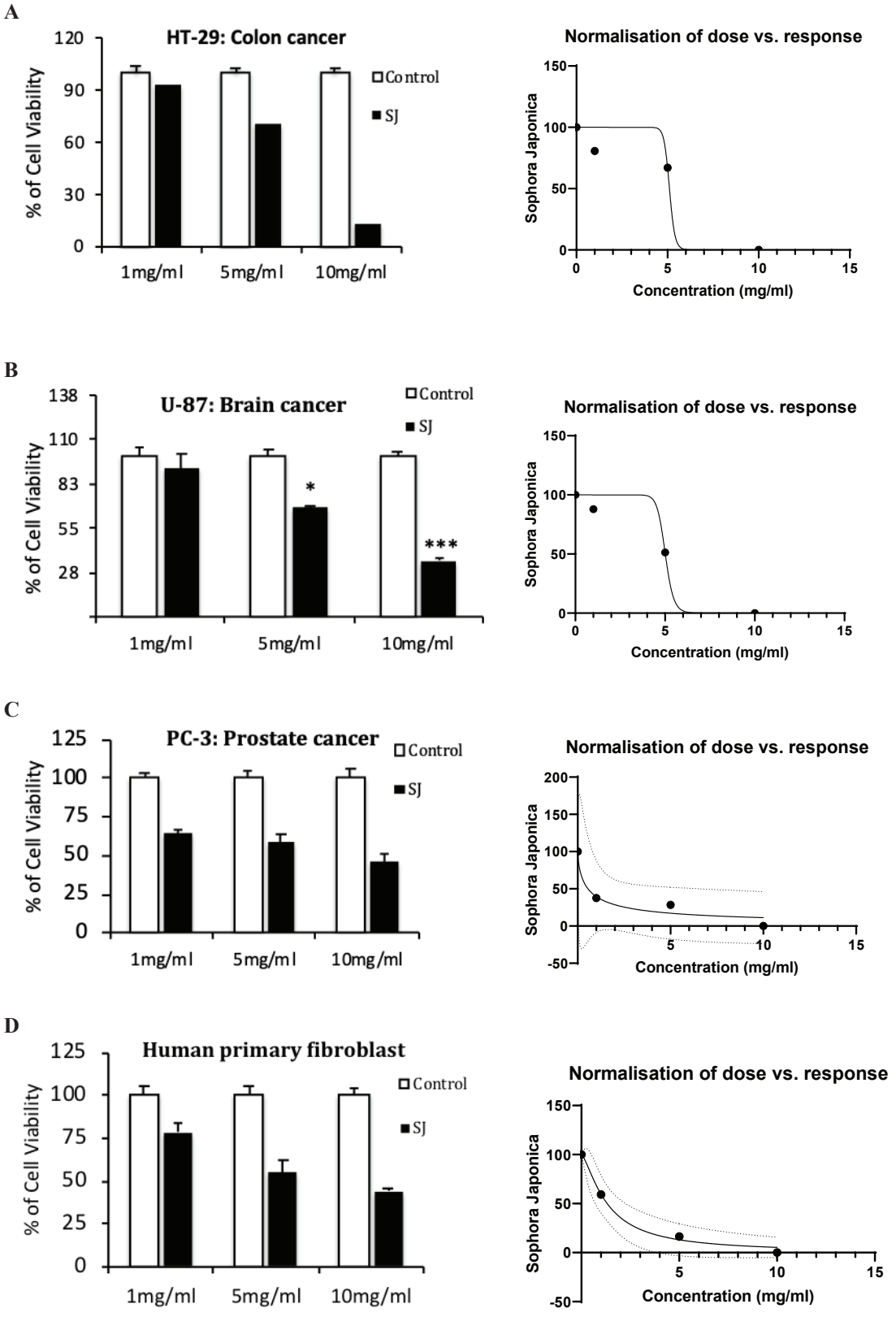

Figure 4. In vitro anti-cancer effects of *S. japonicum* root extract in various cancer cell lines and relative IC_{50} graphs. Effects of *S. japonicum* root extracts in cell viability were tested on A) HT-29 (colon cancer), B) U-87 (brain cancer; glioblastoma) C) PC-3 (prostate cancer), cell lines and D) Human primary fibroblasts (healthy control) and their IC_{50} calculations were plotted. Data are expressed as ± SE and considering the differences, statistical significance was determined as $0.001 < p^* < 0.005$; $p^{**} < 0.0005$; and $p^{***} < 0.0001$.

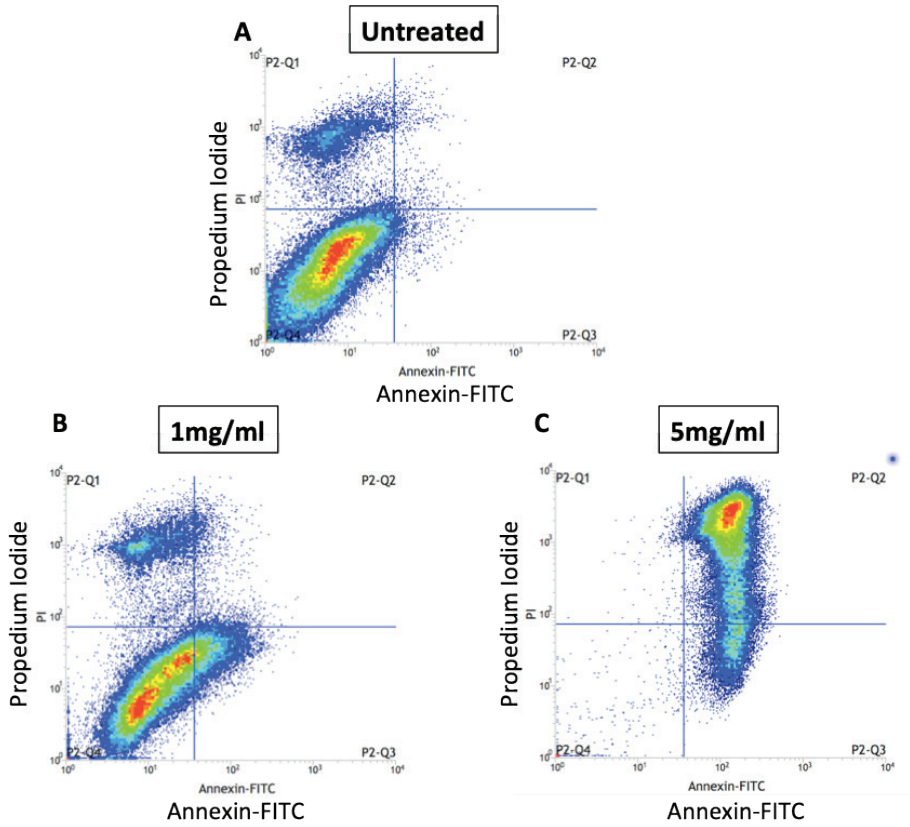

Figure 5. Flow cytometry analysis of cell death in *S. japonicum* root extract-treated PC-3 cancer cell line. Plots indicate Annexin V and Propidium Iodide (PI) stainings of A) Untreated, B) 1mg/mL and C) 5mg/mL extract-treated PC-3 cells.

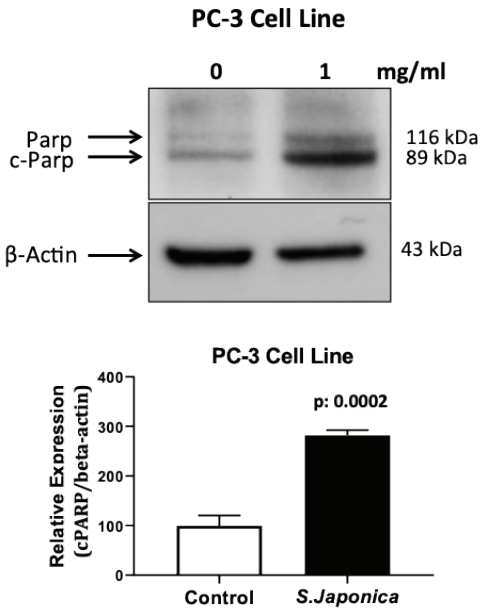

Figure 6. Western blot analysis of cell death in PC-3 prostate cancer cells treated with 1mg/mL *S. japonicum* root extract for 24h. Cleaved PARP (cPARP) is a biochemical marker of cell death and plot indicates significantly increased cPARP levels in *S. japonicum* root extract-treated PC-3 cells as compared to DMSO treated control group (p**= 0.0002). Experiments were performed as triplicates and band densities were calculated using ImageJ analysis tool.

highly cytotoxic to the PC-3 prostate cancer cell line while cells of healthy tissue (human fibroblasts) were not affected at the same concentrations.

To sum up, the obtained methanol extracts from *S. japonicum* roots can be recognised as a potential anticancer candidate, especially against prostate cancer while sparing healthy tissue. The obtained results are quite remarkable and the *in vitro* and *in vivo* anti-cancer effectiveness of *S. japonicum* extracts can be further studied, in detail. Therefore, this study can be considered as the first alternative report focusing on a pharma-grade *S. japonicum* root extract in the cancer field. The preliminary data could be used to demonstrate the potential of the *S. japonicum* root and can lead the way to future studies.

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Original Article

Determination of volatile compounds in green tea and black tea from Turkey by using HS-SPME and GC-MS

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ABSTRACT

Background and Aims: The tea plant, *Camellia sinensis* (L.) O.Kuntze is cultivated in the temperate parts of the world because of its economical value and has been known for ages. According to the processing method, tea can be classified mainly into three types; green, oolong and black tea. The composition of tea obtained from *C.sinensis* heavily depends on geographical location, harvesting time, storage condition and manufacturing process. So the purpose of this paper is to study the volatile composition of green and black tea volatiles manufactured from *C.sinensis* cultivated in North Anatolia.
Methods: The volatiles in green and black teas were extracted by using HS-SPME and analysed by GC-MS/FID analysis. The major compounds in each tea were identified.
Results: Totally, twenty-one and sixteen compounds were separated from green tea and black tea samples, respectively. The main components of green tea aroma were cis-3-hexenyl hexanoate (11.26%), n-octanol (8.55%) and n-decanal (8.02%), while the phenylacetaldehyde (11.26%) and n-decanal (9.93%) were found abundately in black tea aroma.
Conclusion: As aroma is a strong determinant for consumer demands and decisive in tea product speciality, a suitable selection of raw material and location, modification of the manufacturing process could be a reasonable approach to enrich the target aromatic profile of tea products.

Keywords: Tea volatiles, headspace-solid phase microextraction, gas chromatography-mass spectrometer

INTRODUCTION

The tea plant, *Camellia sinensis* (L.) O.Kuntze is a member of Theaceae family and evergreen shrub native from tropical to temperate regions in Asia and also cultivated in the temperate parts of the world because of its economical value and has been known for ages. It is not grown widely in Anatolia, but in the north part it is planted for a hot drink made from its leaves.

According to the processing method, tea can be classified mainly into three types; green tea, oolong tea, and black tea. Fermentation is the cornerstone in the process of manufacturing and the degree of fermentation affects the final product. Green tea is subjected to little or no fermentation, oolong tea is a semi-fermented final product. Black tea is the final product of full fermentation (Pripdeevech & Wongpornchai, 2013; Feng et al., 2019).

Tea volatiles play a determinative role in flavour and affect the consumer preference. It is clear that the composition of tea obtained from *C.sinensis* heavily depends on geographical location, harvesting time, storage condition and manufacturing process. Besides

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phenolic compounds, the aroma is a critical parameter to define the tea quality (Das, Kim, Hong & Eun, 2019; Li et al., 2019). Aroma is also included in the defense system of *C. sinensis* against herbivore attaction (Dong et al., 2011; Zeng et al., 2017).

It has been reported that characterization of different kinds of tea depends on the balance of aroma compounds in the tea. Tea aroma can basically have a green, floral, roasted, or nutty odor depending on volatile compounds present in tea samples (Wang, You & Chen, 2002).

Volatile compounds have been studied in detail and reported to include degradation products of glycosides, carotenoids, amino acids, and carbohydrates (Chaturvedula & Prakash, 2011; Pripdeevech & Wongpornchai, 2013). Oxidation and degradation steps of these precursors have been reported to be accelerated by tea endogenous enzyme (Feng et al. 2019).

During the distillation process, a high temperature is employed which is the reason for changes in the aroma composition. Recent studies on tea volatiles have shown that the complex chemical profile of tea is due to non-volatiles such as tea pigments and lipids (Yang, Baldermann & Watanabe, 2013; Zheng, Li, Xiang & Liang, 2016). The selection of a proper extraction method is required to figure out tea volatiles. The headspace-solid phase microextraction (HS-SPME) method is a rapid, effective, and non-solvent technique, and able to link both extraction and concentration steps (Lee, Chambers, Chambers IV, Adhikari & Yoon, 2013; Lau et al., 2018 (a;b)). It is the preferable method applied in tea analysis.

So, the purpose of this paper is to study the volatile composition of green tea and black tea extracts manufactured from *C.sinensis* cultivated in North Anatolia by using HS-SPME methodology and GC/MS-FID analysis.

MATERIALS AND METHODS

Plant material

All commercial samples of green tea and black tea were supplied from a company in North Anatolia, Turkey.

Headspace-solid phase microextraction (HS-SPME)

For each extraction, 200 mg of a sample was placed in a sealed 20 mL vial with PTFE-coated silicone septum. Extraction of volatiles was carried out using a Merck Supelco SPME fibre coated with 100 µm polydimethylsiloxane. It was exposed for 30 minutes to the headspace while maintaining a temperature of 70°C. Then, the SPME fibre was immediately inserted into the injection port of GC- MS and left for three minutes for desorption of the analytes. Finally, the fibre was baked out for two minutes in a GC-MS injector after each extraction and desorption cycle to reduce the contamination.

Gas chromatography-mass spectrometer/ flame ionisation detector (GC-MS/FID) analysis

The GC analysis of volatiles was carried out using the Shimadzu GC-17A/QP5000 system equipped with a flame ionisation detector (FID) and mass selective detector (MSD). The column used was a Supelcowax-10 capillary column (30 m × 0.32 mm i.d., 0.25 film thickness), and the gas carrier was helium at a rate of 1.8 mL/min. The oven temperature program consisted of a two minute hold at 40°C, followed by a 2°C/minute ascent to 220°C, and a 30 minute hold at 220°C. The injector and MS transfer line temperature were maintained at 200°C and 250°C, respectively. The FID temperature was 300°C. The analytes were detected after electron impact ionization (70 eV) in the SCAN-mode from m/z 50 to 550. Identification of volatile compounds was performed by comparing spectra and retention times of the standards. The components were identified based on the comparison of their relative retention time to a C8–C32 n-alkanes mixture and mass spectra with those of NBS75K, Wiley 7, NIST MS search 2.0 library data of the GC-MS system, literature data, and standards of the main components. The results were also confirmed by comparison of the compounds elution order with their relative retention indices on WAX columns. All analysis were performed in triplicate.

RESULT AND DISCUSSION

Unlike distillation, HS-SPME is able to quantify the high volatiles of tea samples that are more important for aroma perception (Du et al., 2014; Yang et al., 2013). So the purpose of this study is to determine the volatile profile of green and black tea by using HS-SPME coupled with GC-MS. The aroma profile of both samples are shown in Table 1.

Totally, twenty-one and sixteen compounds were separated from green tea and black tea samples, respectively and their structures were determined by MS and retention index data. We detected a total of six aldehydes, four ketones, eight alcohol, two esters, and one other compound representing 75.76±1.19% of the green tea volatile oil. The major identified constituents of green tea aroma were cis-3-hexenyl hexanoate (11.26±0.44%), n-octanol (8.55±0.38%), and n-decanal (8.02±0.31%). Four aldehydes, five ketones, one ester, and six alcohols representing 65.11±1.24% totally were found in black tea volatile oil. Phenylacetaldehyde, that has a honey-like odour, was determined as a major compound in black tea aroma with a value of 11.26±0.52%. Furfural, 1-penten-3-ol, nonanol, n-hexanal, cis-3-hexenal, and cis-3-hexenyl hexanoate were found in only green tea aroma and 1-penten-3-one was found to be included in black tea aroma with a value of 0.34±0.05%. Similar to green tea, n-decanal (9.93±0.32%) which is possibly one of the lipid degradation products and 2,6,6-trimethyl-2- hydroxy cyclohexanone (8.10±0.42%) originated from carotenoid degradation were found abundantly in black tea samples.

According to previous reports, the major compounds in the aroma are formed from carotenoids, lipids, glycosides, and amino acids/carbohydrate (Ho, Zheng &, Li, 2015; Feng et al., 2019; Tan et al., 2019). In general, the linalool and hexanal contents play a key role in the quality of green teas (Kato & Shibamoto, 2001; Pripdeevech & Wongpornchai, 2013).

The flavor and volatile composition of Kangra orthodox black tea extracted by simultaneous distillation extraction (SDE) and hydrodistillation were compared. The major volatiles identified were E-2-hexenal, pentene-3-ol, Z-3-hexenol, linalool, linalool oxides, geraniol, methyl salicylate, and 3,7-dimethyl-1,5,7-oc-

Table 1. Chemical composition of volatile oils of green tea and black tea.

Compound	LRI*	RI^{lit}	Green tea (%)	Black tea (%)
1-penten-3-one[b]	1024	1024	-	0.34±0.05
trans-2-hexenal[b]	1061	1061	3.09±0.15	0.32±0.04
n-hexanal[a]	1097	1095	0.31±0.07	-
cis-3-hexenal[b]	1135	1135	0.95±0.12	-
1-penten-3-ol[b]	1175	1173	0.49±0.02	-
Pentanol[a,b]	1252	1256	1.19±0.23	0.31±0.06
n-hexanol[a]	1262	1271	2.45±0.14	0.95±0.05
2,6,6-trymethyl-2- Hydroxycyclohexanone[a,b]	1282	1288	7.06±0.55	8.10±0.42
cis-3-hexenol[a]	1386	1386	3.37±0.27	2.12±0.35
n-nonanal[a,b]	1390	1396	6.66±0.39	8.09±0.65
trans-2-hexenol[b]	1409	1419	4.63±0.22	4.92±0.31
β-ionone[a]	1463	1462	2.45±0.11	3.33±0.31
Furfural[a]	1473	1474	1.15±0.05	-
n-decanal[a,b]	1502	1504	8.02±0.31	9.93±0.32
n-octanol[b]	1565	1569	8.55±0.38	3.15±0.28
cis-3-hexenyl hexanoate[b]	1642	1642	11.26±0.44	-
Phenylacetaldehyde[b]	1648	1648	5.40±0.38	11.26±0.52
Nonanol[a]	1663	1663	0.62±0.10	-
Benzylacetate[a,b]	1682	1686	1.32±0.11	2.92±0.09
α-ionone[b]	1863	1860	3.72±0.23	5.20±0.32
Geraniol[b]	1865	1864	0.62±0.05	2.27±0.11
Geranyl acetone[a]	1867	1867	2.45±0.23	1.90±0.21
		Alcohols	*21.92±0.59*	*13.72±0.56*
		Aldehydes	*24.43±0.66*	*29.60±0.89*
		Esters	*12.58±0.45*	*2.92±0.09*
		Ketones	*15.68±0.65*	*18.87±0.65*
		Others	*1.15±0.05*	-
		TOTAL	**75.76±1.19**	**65.11±1.24**

*LRI: Linear retention indices (WAX column) calculated against n-alkanes. % calculated from FID data with standart. [a]Compounds listed in order of elution from a WAX column. [b]Identification of components based on standard compounds; All values are mean ± standart deviation of triplicates; RI^{literature}: https://pubchem.ncbi.nlm.nih.gov

tatrien-3-ol and the SDE technique was reported as more efficient than hydrodistillation (Rawat et al., 2007; Pripdeevech & Wongpornchai, 2013).

Chen et al. studied key aroma compounds of Hanzhong black tea infusion and compared it with other black tea samples. They mentioned that the growing altitude area of *C. sinensis* affects the aroma profile of black tea samples (Chen et al., 2019).

In addition to climatic and geographical conditions, the manufacturing process deeply affects aromatic precursors and glycosidase enzyme content and leads to a great variation of the aromatic profile of tea (Lee et al., 2013; Zheng et al., 2016; Ravichandran & Parthiban, 1998). The volatile compounds of both green tea and black tea were reported to be varied within storage duration (Wang et al., 2002; Choi, Jung & Yun, 2016; Zheng et al., 2016). The off-flavour of green tea was mentioned as the most important problem during preservation, because of light and storage conditions like packaging material, moisture, and temperature (Horita, 1987; Katsuno et al., 2014). Tontul et al. have studied the impact of the shooting period and shading degree on two different tea clones in Turkey and reported that the volatile aroma of green tea samples could be different from each other by a few identical compounds but with considering the shading treatment in general, there were no significant differences in aroma profiles (Tontul et al., 2013).

Many studies have been interested in the volatiles of tea. Common volatile alcohols were mentioned such as hexanols, benzyl alcohols, linalool, and terpineol. Aldehydes commonly found were heptenal, (E)-2-hexanal, pentanal, and nonanal (Das et al,

2019). Le et al. (2013) studied the volatile profile of 24 green tea samples. Linalool and hexanal were found in almost all green tea samples but, the green teas from Africa had 1-penten-3-ol, 2-penten-1-ol, and benzaldehyde. Nonanal was reported to be present generally in green teas from Southeast Asia and India. Additionally, Northeast Asia samples were found to contain nonanal, benzene ethanol, and jasmone (Lee et al., 2013). Both sensory factors and aromatic compounds of black tea and instant teas manufactured by using freeze-dried, spray-dried, and decaffeinated methods were determined to show variations on the basis of processing method (Kraujalyte, Pelvan & Alasalvar, 2016).

CONCLUSION

It is very clear that tea aroma formation depends on many factors especially; raw material, harvesting location, manufacturing process, and storage condition. Tea is consumed all over the world using different preparation methods. So aroma is a strong determinant for consumer demands and decisive in tea product speciality. The HS-SPME extraction method is known as solvent-free, low cost, and rapid extraction procedure, and it is particularly used to quantify the high volatile profile of tea samples that are believed to be decisive in consumer preference. In overall evaluation, a suitable selection of raw material and location, modification of the manufacturing process could be a reasonable approach to enrich the target aromatic profile of tea products.

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Original Article

Nootropic herbal formulations for the treatment of Alzheimer's disease: *In vivo* pharmacological assay and molecular docking studies

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ABSTRACT

Background and Aims: The main aim of the study was to enhance the cognitive function of the brain by nootropic herbal formulations in animal models. Polyphyto herbal formulations were known to enhance the cognition and memory function by several pathways such as anti-oxidative, anti-inflammatory, and cell signaling pathways. In this study, six formulations were prepared by mixing specified plant parts and were coded as NHF1, NHF2, NHF3, NHF4, NHF5, and NHF6.
Methods: The potency of the formulations was assessed by *In vivo* (photo actometer, rod walking test, pole climbing test, and Ellman's acetylcholinesterase test) studies.
Results: NHF1 and NHF5 exhibited greater activity than the standard drug donepezil *in vivo* (Ellman's acetylcholinesterase test) analysis. NHF1 and NHF5 formulations containing plant parts were further investigated against several published literatures for the identification of chemical constituents and those chemical constituents were subjected to molecular docking and *in silico* ADME prediction studies to figure out the possible compounds responsible for the cholinesterase inhibition activity.
Conclusion: In conclusion, the computational studies also reveal that presence of chemical constituents such as sarsasapogenin (13.13 nM), racemosol (16.26 nM), and beta-sitosterol (30.47 nM) having binding energy (-10.75 kcal/mol), (-10.63 kcal/mol), (-10.25 kcal/mol), might be directly responsible for the nootropic activity.

Keywords: Herbal, nootropic, acetylcholinesterase, Alzheimer, autodock 4.2.6, sarsasapogenin, SwissADME

INTRODUCTION

Alzheimer's disease is a progressive neuronal damage that leads to shrinkage of the brain, which is characterized by the presence of plaques of amyloid beta and tangles of tau protein (Waldemar et al., 2007). It is the most common cause of dementia accounting for 60 - 80% in elder people. Alzheimer's disease has no therapeutic treatment, however, certain medications are available for symptomatic relief and improvement of cognition. In fact, the prescribed medications have serious side effects as well as pharmacokinetic limitations (De la Monte, 2012; Dos Santos Pisoni et al., 2010)

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Natural compounds are known to be one of the best sources for treating most of the clinical problems and they continue to inspire as the best alternatives (Musthaba et al., 2010). Many pathological conditions related to the central nervous system, in one way or the other causes the loss of memory. Alzheimer's and dementia are major known conditions for loss of memory (Aggleton, J. P., Pralus, A., Nelson, A. J., & Hornberger, M., 2016; McKhann et al., 2001). However, the nootropic herbal formulations can be used to enhance the cognition and improve memory function without producing any side effects (Shibnath, K., Madhav, N. V. S., & Sarkar, C. N., 2016). Nootropic herbs mainly enhance memory function by certain ways either by increasing blood circulation to the brain, which further improves brain activity, or showing anti-oxidative and anti-inflammatory activity, which results in the prevention of neurodegeneration. Some other herbs such as *Bacopa monnieri* have been found to act by inhibiting the acetylcholinesterase inhibition pathway to enhance memory (Murray, A. P., Faraoni, M. B., Castro, M. J., Alza, N. P., & Cavallaro, V., 2013).

Acetylcholinesterase is an enzyme which is involved in many physiological conditions in the central nervous system. Its main function is to convert acetylcholine into thiocholine and acetate, which results in a decrease of acetylcholine levels in the presynaptic region of neurons. This gradual decrease in acetylcholine levels leads to many pathological conditions such as Alzheimer's disease and dementia (Da Silva Goncalves, Franca, & Vital de Oliveira, 2016). There are many herbal phytochemicals which are known to inhibit the acetylcholinesterase without any side effects (Rashed, Cardoso Sucupira, Moita Neto, & Feitosa, 2013). Recently, many drugs successfully completed clinical trial investigation (Ghribia, Ghouilaa, Omrib, Besbesb, & Janneta, 2014).

The nootropic herbal formulations (NHFs) used in this study are composed of various herbal plant parts mixed in different ratios to achieve the desired effect (Kulkarni, Girish, & Kumar, 2012). The current study was concentrated on investigating the safety and efficacy of nootropic herbal formulations to enhance cognition, as well as to identify the natural compounds responsible for the acetylcholinesterase inhibition activity that are present in the NHF1 and NHF5 formulations through a computational study. In the study, different combinations of herbal formulations made from herbal plant parts such as *Aloe vera, Areca catechu, Asparagus racemosus, Avena sativa, Bacopa monnieri, Curcuma longa, Cinnamomum zeylanicum, Convolvulus pluricaulis, Glycine max, Hibiscus rosasinensis, Juglans regia, Lactuca sativa, Mentha piperita, Phyllanthus emblica, Piper nigrum, Ribes nigrum, Terminalia arjuna, Vigna mungo, Zingiber officinale* were incoporated. Specific plant parts from the mentioned plants were combined with a specific quantity to achieve the desired formulation, and all the six formulations were named as NHF1, NHF2, NHF3, NHF4, NHF5, and NHF6.

These prepared formulations were evaluated by *In vivo* (photo actometer, rod walking test, pole climbing test, and Ellman's acetylcholinesterase test) studies. The best formulations containing plant parts were further analyzed against several published literature studies for the identification of chemical constituents. Those selected chemical constituents were subjected to molecular docking and ADME prediction studies to figure out the possible compounds responsible for the cholinesterase inhibition activity.

In fact, the chemical constituents (Sarsasapogenin (Kashyap, Muthusamy, Niranjan, Trikha, & Kumar, 2020; Sy et al., 2016), Recemosol (Sivanandam, 2007), beta-sitosterol (Ayaz et al., 2017; Zeng et al., 2019)) which were found to be active in this study were also evaluated separately in several other studies stating their potential for treating symptomatic relief in Alzheimer's models. In other studies, the active molecules (Sarsasapogenin (Wang et al., 2018; Yang et al., 2018), recemosol, beta-sitosterol) were also modified synthetically to improve the activity, and achieved greater results in treating the Alzheimer's related symptoms in mice models. Therefore, this study provides evidence that these safer and pharmacologically active nootropic formulations can be an alternative drug therapy for symptomatic relief, and prevent clinical patients from progressing to the Alzheimer's disease.

MATERIALS AND METHODS

Plant materials and preparation of formulation

A variety of plant parts, as shown in Table 1, were collected from the local source, and were identified and authenticated

Table 1. Different plants and its parts used in this study.

S.No	Scientific Name	Family	Plant parts
1	*Aloe vera*	Xanthorrhoeaceae	Leaves
2	*Areca catechu*	Arecaceae	Fruit
3	*Asparagus racemosus*	Lilliaceae	Roots
4	*Avena sativa*	Poaceae	Fruit
5	*Curcuma longa*	Zingiberaceae	Rhizome
6	*Cinnamomum zeylanicum*	Lauraceae	Bark
7	*Convolvulus pluricaulis*	Convolvulaceae	Herbs
8	*Glycine max*	Fabaceae	Seed
9	*Hibiscus rosa sinensis*	Malvaceae	Flower
10	*Juglans regia*	Juglandaceae	Fruit
11	*Vigna mungo*	Fabaceae	Seed
12	*Mentha piperita*	Labiatae	Leaves
13	*Phyllanthus emblica*	Euphorbiaceae	Fruit
14	*Piper nigrum*	Piperaceae	Seed
15	*Ribes nigrum*	Grossulariaceae	fruit
16	*Zingiber officinale*	Zingiberaceae	Rhizome
17	*Bacopa monnieri*	Plantaginaceae	Herbs
18	*Terminalia arjuna*	Combretaceae	Bark
19	*Lactuca sativa*	Asteraceae	Leaves

by Sri Venkateshwara University, Thirupathi. The plant parts were kept for air dry under a shady place and grounded, and then passed over #100 sieves to prepare the specified formulations, as shown in Table 2.

Table 2. The composition of nootropic herbal formulations.

Formulation	Crude Powder	Quantity (g)
NHF1	*Cinnamon zeylanicum*	1.5
	Vigna mungo	1.5
	Avena sativa	2
	Asparagus racemosus	2
	Areca catechu	3
NHF2	*Mentha piperita*	2.5
	Ribes nigrum	2.5
	Aloe vera	2.5
	Glycine max	2.5
	Piper nigrum	2
NHF3	*Convolvulus pluricaulis*	2.5
	Zingiber officinalis	2.5
	Vigna mungo	3
	Avena sativa	2
	Asparagus racemosus	2.5
NHF4	*Lactuca sativa*	2.5
	Hibiscus rosasinensis	3
	Zingiber officinalis	0.5
	Convolvulus pluricaulis	1.5
	Curcuma longa	1
NHF5	*Phyllanthus emblica*	2
	Mentha piperita	2
	Hibiscus rosasinensis	3
	Terminalia arjuna	1
	Asparagus racemosus	1
NHF6	*Hibiscus rosasinensis*	2.5
	Convolvulus pluricaulis	2.5
	Bacopa monieri	3

NHF: Nootropic herbal formulation

Experimental animals

Albino Wistar rats belonging to the adult age group of male sex and weighing about (180±20 g) were procured and kept in polypropylene cages in a laboratory under ambient temperature with a regular day/night cycle. All the animals were randomized into two per cage and acclimatized for one week in the animal facility under standard conditions following OECD guidelines. A standard pellet diet and water were given *ad libitum,* and all the experiments were conducted in the day time (9.30 AM to 5.00 PM). The study protocol was approved by the Institutional ethical committee (1015/C/06/CPCSE9).

Phytochemical analysis

All the formulations which were selected for the activity were subjected to phytochemical analysis. Phytochemicals were extracted using 10 mL methanol and dried to achieve residue. To the obtained residue, dilute HCl was added, shaken well and filtered, the obtained filtrate used for analysis of alkaloids using the Dragendorff's, Mayer's, Hager's and Wagner's tests, for glycosides, the Legal's, Liebermann's, Foam, Haemolytic, and Borntrager's tests, for flavonoids, the Shinoda test, and for Steroids, the Liebermann's tests were performed (Odebiyi, & Sofowora, 1978; Trease, & Evans, 1996).

Acute and sub-chronic toxicity studies

To assess the acute (24 hours) and sub-chronic (14 days) toxicity of a nootropic herbal formulation, a single dose was given orally in pellet form to the randomized male Wistar rats, which were procured and kept in standard conditions following OECD guidelines. All NHF1, NHF2, NHF3, NHF4, NHF5 and NHF6 formulations were administered orally at a dose of 300, 1000 and 2000 mg/kg to a group of rats, which were fasted for 6 hours. All the animals were allowed free access to food and water under standard conditions (Chinedu, Arome, & Ameh, 2013). Six animals were observed for abnormal behavior and percentage of the mortality rate for a period of 14 days. The control group was treated with normal saline and the test group was treated with standard drug donepezil. After the observation period, blood was collected from all the animals for hematological observations. All the six formulations NHF1, NHF2, NHF3, NHF4, NHF5, and NHF6 were observed to be safe for administration.

Experimental methods

Actophotometer

The locomotor activity (horizontal activity) can be easily measured using an actophotometer which operates on a photoelectric cell which is in circuit with a counter. When the beam of the light falling on the photocell was cut off by the animal, a count was recorded and displayed digitally. The actophotometer contains a circular or square arena in which the animal moves. The animals were tested for the activity before and after the administration of the formulation (Reddy, & Kulkarni, 1998).

Rod walking test

The ability of the rat to balance on a stationary, horizontal rod and walk on it to come in one end of the rod measures cognitive study and learning activity. Animals were placed in the center of a rod measuring 100 cm long, 20 mm in diameter and positioned 50 cm above the table surface, latency to transfer to its one end was recorded. All the rats were tested three times to observe the holding time or transfer latency on different groups (Dunham, & Miya, 1957).

Pole climbing test

The pole climbing study was performed by incorporating a Cook's pole climbing apparatus; this experiment was utilized to understand the learning and its retention in response to stimuli applied by the instrument. The apparatus contains a chamber (25x25x25 cm) which was made of a stainless steel grid floor for the experimental area. In the center, a pole hangs, measuring 2.5 cm in diameter, which helps the rat to avoid shock by climbing it. Initially, a rat was placed in the experimental chamber and allowed to habituate the area for 45 sec. A simultaneous

conditioned stimulus and unconditional stimulus, i.e., buzzer signal and electric shock respectively were applied for 45 sec. The animal avoids the shock by climbing the pole after an alert from the conditional stimuli associated learning. Each rat was subjected on the first day and 24 hours later to 05 trails maximum. The transfer latency and escape latency were noted during the study period (Cook, & Weidley, 1957; Soman, Mengi, & Kasture, 2004).

In vivo acetylcholinesterase estimation

collection of brain samples

All the animals were euthanized by cervical decapitation 90 min after the last dose on the 15th day. The brain was removed carefully using forceps, weighed and homogenized in a glass homogenizer containing sterile normal saline. The supernatant which was obtained after centrifugation (Remi, Hyderabad, India) at 3000 rpm for 10 min was used for the analysis of cholinesterase activity using 3 replicas (Thomsen, Kewitz, & Pleul, 1988).

Ellman acetylcholinesterase activity

In vivo acetylcholinesterase activity was measured using a modified Ellman's method. About 0.5 mL of the supernatant which was obtained from the result of centrifugation was pipetted out into an 8 mL of freshly prepared DTNB solution (10 mg DTNB in 100 mL of Sorenson phosphate buffer) having pH 8.0. The above solution was divided into two equal parts and 2 drops of eserine solution were added to only one part. Then, 1 mL of substrate solution (75 mg of acetylcholine iodide per 50 mL of distilled water) was added to both tubes and incubated for 10 min at 30°C. The eserine containing solution was used for zeroing the colorimeter (Insif electronics, Hyderabad, India). The resulting yellow color was due to the reduction of DTNB by certain substances in the brain homogenate and due to non-enzymatic hydrolysis of the substrate. After the instrument (Shimadzu, Hyderabad, India) was calibrated, the absorbance change per minute of the sample was read at 420 nm (Ellman, Courtney, Andres, & Featherstone, 1961).

Computational methods

Based on the *in vivo* pharmacological evaluation, it was found that NHF1 and NHF5 had better activity than the standard drug. Therefore, these formulations were considered for further evaluation to figure out activity responsible chemical constituents using computational techniques. The main phytochemicals present in the plants which belong to NHF1 and NHF2 were downloaded from the NCBI-PubChem database in .sdf format.

Protein and ligand preparation

The crystal structure of acetylcholinesterase bearing PDB ID: 4M0E_A was downloaded from the RCSB PDB website (https://www.rcsb.org/). All the cocrystal ligands and water molecules were removed from the protein. Finally, the hydrogen atoms and Gasteiger charges were applied using Autodock tools (ADT) (Morris et al., 2009). All the natural product ligands which were identified and retrieved from NCBI-PubChem chemical database (https://pubchem.ncbi.nlm.nih.gov/) (Kim et al., 2019) were saved in sdf format. All these files were converted to mol2 format using the OpenBabel software (O'Boyle et al., 2011).

Molecular docking

Molecular docking studies were performed using the Autodock 4.2.6 and ADT tools. The selected protein was refined by deleting the crystal ligands, chain B and crystal water molecules. The downloaded ligands were saved in .pdbqt file format, and grid maps were setup using a grid box with coordinates of X=-11.708, Y=-42.266 Z=21.559 having a number of points of 60 for all the x,y,z dimensions. Finally, the Lamarckian genetic algorithm was incorporated for docking ligands into the binding pocket region (Morris et al., 2009).

ADME properties

ADME properties play a crucial role in predicting the druggable properties of small molecules (Katsila, Spyroulias, Patrinos, & Matsoukas, 2016). The 24 best active natural compounds were selected based on results from the molecular docking studies. All the selected molecules were analyzed for ADME analysis using a SwissADME server (Daina, Michielin, & Zoete, 2017).

Statistical analysis

All the experiments were done in triplicate, and all the data were shown as mean ± SD. The data were analyzed using the Graphpad Prism 5 program trial version. Statistical differences between the experimental groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's Multiple Comparison test. Mean values were considered statistically significant when $p<0.001$.

RESULTS

Toxicity studies

All the NHF1, NHF2, NHF3, NHF4, NHF5, and NHF6 formulations were found to be safe and no mortality was seen in both acute and sub chronic toxicity studies, even at the high dose escalation of 2000 mg/kg. All the animals were observed to be normal in the consumption of food, behavior and physical activity during and at the end of the observation period of 14 and 28 days for acute and sub chronic toxicities, respectively. After the observation period, blood was collected from the tail for hematological analysis. Hematological results showed (Table 3) no significant variation in hemoglobin, platelet count and total WBC.

Pharmacological screening

Locomotor activity test

The test groups which were treated with NHF2 and NHF4 nootropic herbal formulations revealed a statically significant reduction in escape latency compared to the control and standard, indicating greater activity. The results were shown in a bar diagram with a statistical significance value in Figure 1.

Pole climbing apparatus

The test groups which were treated with NHF2 and NHF6 nootropic herbal formulations revealed a statically significant reduction in escape latency compared to the control, indicating greater activity. However, NHF5 shows significant compared with the standard. The results were shown in a bar diagram with statistical significance value in Figure 2.

Table 3. Group mean – hematology reports of animals treated with nootropic herbal formulations.

Formulations	Dose	Mean ± SD	Hb (g %)	Platelets ($x10^5$/c)	Total WBC ($x10^3$/cmm)
Control		Mean	11.33%	1.9	7.5
		± SD	0.00412	0.126	0.54
NHF1	Medium	Mean	11%	2.5	8.9
		± SD	0.00358	0.113	0.34
	High	Mean	11.14%	2.93	10.37
		± SD	0.00431	0.103	0.489
NHF2	Medium	Mean	11.19%	1.8	6.5
		± SD	0.00398	0.25	0.34
	High	Mean	11.24%	1.96	7.06
		± SD	0.00427	0.103	0.15
NHF3	Medium	Mean	11.22%	0.9	1.2
		± SD	0.00336	0.0816	0.26
	High	Mean	11.00%	0.358	1.95
		± SD	0.00701	0.0917	0.08
NHF4	Medium	Mean	11%	1.5	8.4
		± SD	0.00228	0.2	0.98
	High	Mean	10.95%	1.9	9.333
		± SD	0.00055	0.126	0.816
NHF5	Medium	Mean	11.26%	1.6	8
		± SD	0.00521	0.34	0.86
	High	Mean	11%	1.9	8.83
		± SD	0.00854	0.126	1.16
NHF6	Medium	Mean	11.20%	1.6	6.8
		± SD	0.002	0.14	0.87
	High	Mean	11.05%	1.4	6.56
		± SD	0.00089	0.12679	0.69

Values are mean of triplicate determination (n=3) ± standard deviation

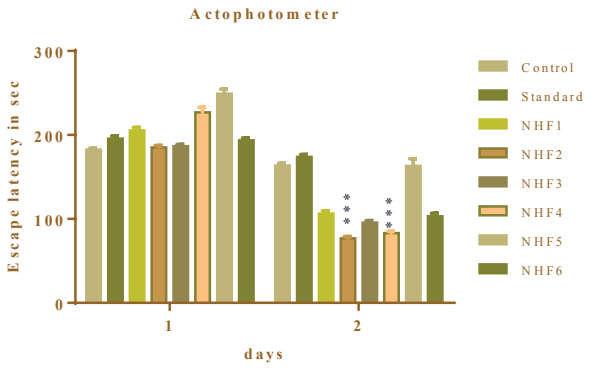

Figure 1. Bar graph of escape latency of rat in sec using actophotometer ***=p<0.001 standard (donepezil) vs. NHF2 and NHF4.

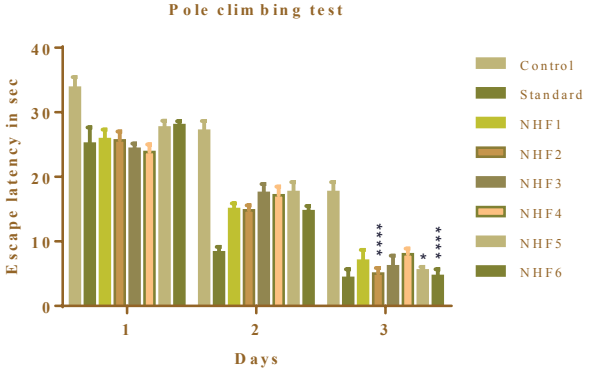

Figure 2. Bar graph of escape latency of rat in sec using cook's pole climbing apparatus ***=p<0.001 Standard (donopezil) vs. NHF5.

Rod walking test

The test groups which were treated with nootropic herbal formulations revealed a statically significant reduction in escape latency compared to the control, indicating greater activity. The results were shown in a bar diagram with statistical significance value in Figure 3.

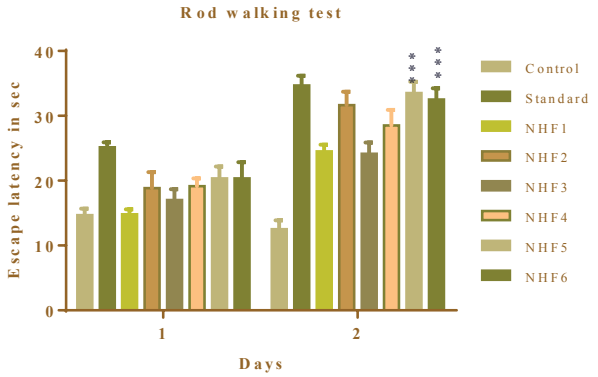

Figure 3. Bar graph of escape latency of rat in seconds using cook's pole climbing apparatus ***=p<0.001 Standard (donepezil) vs. NHF5.

In vivo acetylcholinterase estimation

In vivo acetylcholinesterase activity was measured using a modified Ellman's method. The yellow color observed was due to the reduction of DTNB by certain substances in the brain homogenate and due to non-enzymatic hydrolysis of the substrate. After the instrument was calibrated, the absorbance change per minute of the sample was read at 420 nm. Acetylcholine is converted to thiocholine and acetate by the action of the acetylcholinesterase enzyme. The thiocholine, which was broken from acetylcholine, reacts with dithiobisnitrobenzoate and produces a yellow color. More yellow color represents less inhibition, whereas, less yellow color represents more inhibition of the acetylcholinesterase activity. The absorbance of NHF5 and NHF1 was found to be lower than that of the standard drug donepezil, which directly indicates that the NHF5 and NHF1 herbal formulations were more active than the standard drug. The values were represented in a bar diagram represented in Figure 4.

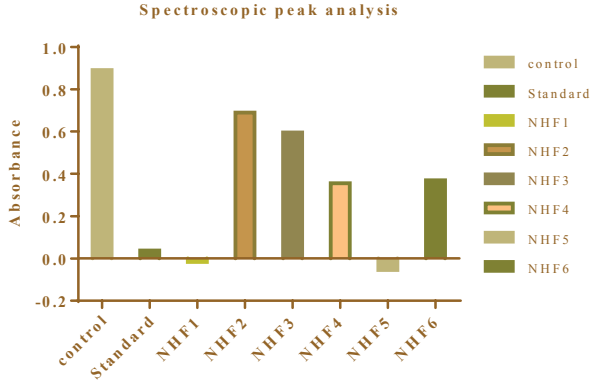

Figure 4. Graph shows spectroscopic absorbance peak analysis of control, standard and nootropic herbal formulations at 420 nm.

Computational results

Molecular docking analysis

In this molecular docking study, about 39 natural products were selected from the plant parts which belong to the NHF1 and NHF5 formulations through a thorough search of the literature reports. The target protein acetylcholinesterase was retrieved from a protein data bank (PDB) having PDB ID: 4M0E_A, which contains 542 amino acids and x-ray diffraction resolution of 2.0 Å. The most active residues in the binding area interacting with the ligands were "Tyr 341, Ser 293, Glu 292, Phe 295, Ser203, Arg 296, Glu 202, Ser 125, Tyr 124, Asp 74, and Trp 286". Among 39 docked phytochemicals, 3 molecules such as Sarsasapogenin (-10.75 kcal/mol), racemosol (-10.63 kcal/mol), and beta-sitosterol (-10.25 kcal/mol) showed the best binding energies. The superimposition of the 3 best active phytochemicals were inserted into the active site of the protein and represented in Figure 5.

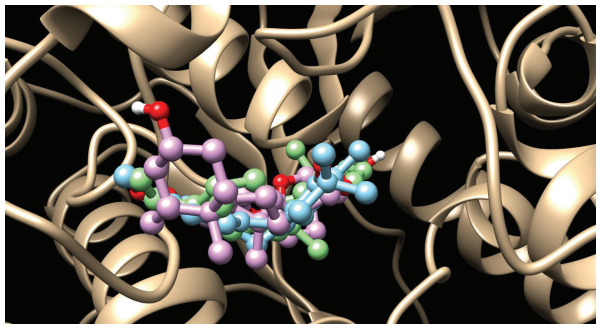

Figure 5. Superimposition of the best 3 ligands from the docking studies were superimposed in binding region of the 4M0E protein.

ADME analysis

In this study, about 24 best active molecules out of 39 compounds from molecular docking studies were analyzed for ADME parameters by the SwissADME online tool to understand the drug likeness nature of nootropic herbal chemicals. In this study, physicochemical, lipophilicity, water solubility, pharmacokinetics and drug likeness parameters were analyzed, which were shown in the Table 4.

DISCUSSION

The nootropic herbal formulations which were prepared were firstly tested for phytochemical analysis for ensuring the presence of the most important class of phyto-constituents such as alkaloids, glycosides and flavonoids, and the phytochemical tests show the presence of those phytochemicals. Secondly, all the nootropic herbal formulations were evaluated for acute and sub-chronic toxicity studies with the dose escalation of 2000 mg/kg body weight of the animal. Hematological reports suggest that all the formulations were safe for administration, and no mortality was observed during the study.

In vivo pharmacological studies such as locomotor activity, pole climbing study, and rod walking studies were performed using actophotometer, rod walking apparatus, and cook's pole

Table 4. SwissADME properties of best active phytochemicals.

S.No	Compound ID	MW	HB-A	HB-D	TPSA	iLOGP	GI-absorption	BBB -permeation	CYP2D6 inhibition	CYP3A4 inhibition	Lipinski violation	Bioavailability Score	Brenk alerts	SA
1	14632996	218.33	1	0	17.07	3.11	High	Yes	No	No	0	0.55	1	4.53
2	5281157	299.28	5	4	106.86	1.19	High	No	No	No	0	0.56	2	2.36
3	10087955	329.3	6	4	116.09	1.78	High	No	No	No	0	0.56	2	2.57
4	11723200	315.28	6	5	127.09	0.71	High	No	No	No	0	0.56	3	2.47
5	222284	414.71	1	1	20.23	5.07	Low	No	No	No	1	0.55	1	6.30
6	196216	218.33	1	0	17.07	3.14	High	Yes	No	No	0	0.55	1	4.17
7	9064	290.27	6	5	110.38	1.33	High	No	No	No	0	0.55	1	3.5
8	420422	305.37	5	0	48	3.41	High	Yes	Yes	No	0	0.55	0	4.25
9	156777	354.35	6	3	96.22	2.63	High	No	Yes	Yes	0	0.55	0	4.08
10	585939	258.27	4	3	69.92	1.7	High	Yes	Yes	No	0	0.55	0	3.05
11	442770	340.37	5	3	86.99	1.98	High	No	Yes	Yes	0	0.55	1	3.79
12	5281855	302.19	8	4	141.34	0.79	High	No	No	No	0	0.55	3	3.17
13	72276	290.27	6	5	110.38	1.47	High	No	No	No	0	0.55	1	3.5
14	5280961	270.24	5	3	90.9	1.91	High	No	Yes	Yes	0	0.55	0	2.87
15	5280520	270.24	5	3	90.9	1.36	High	No	Yes	Yes	0	0.55	0	2.89
16	5282074	286.24	6	4	111.13	1.48	High	No	Yes	Yes	0	0.55	0	2.95
17	5280863	286.24	6	4	111.13	1.7	High	No	Yes	Yes	0	0.55	0	3.14
18	119269	356.37	6	4	107.22	2.24	High	No	Yes	Yes	0	0.55	1	3.85
19	71629	306.27	7	6	130.61	1.19	High	No	No	No	1	0.55	1	3.76
20	5089889	576.5	12	9	209.76	1.8	Low	No	No	Yes	3	0.17	1	5.85
21	5280343	302.24	7	5	131.36	1.63	High	No	Yes	Yes	0	0.55	1	3.23
22	624971	340.41	4	2	58.92	3.23	High	Yes	Yes	Yes	0	0.55	0	4.04
23	92095	416.64	3	1	38.69	4.54	High	Yes	No	No	1	0.55	0	6.88
24	5282230	327.33	5	2	84.86	2.68	High	No	No	No	0	0.56	1	2.57

MW: Molecular weight, HB-A: Hydrogen Bond Acceptor, HB-D: Hydrogen Bond Donor, TPSA: Topological Polar Surface Area, S.A: Synthetic accessibility

climbing apparatus respectively. In the actophotometer test, the NHF2 and NHF4 formulations were found to be more active than the standard drug. In the cook's pole climbing test, NHF5 was found better than the standard. Similarly, NHF5 was found to be active in the rod walking test.

In vivo acetylcholinesterase activity was analyzed using a modified Ellman's method in which the color intensity was measured as absorbance against the enzyme activity. The more yellow color indicates the more enzyme activity, whereas a less yellow color indicates the less enzyme activity which is due to more inhibition of the acetylcholinesterase enzyme by the inhibitors. The NHF5 and NHF1 were found to be more active than the standard drug donepezil.

Finally, docking studies performed on the acetylcholinesterase revealed that all the docked molecules have a good binding affinity. However, about 7 molecules have a range of -9.00 kcal/mol to -10.75 kcal/mol binding energy and good interaction with the protein residues. Among which 3 molecules shows the most active. Hence, it is believed that the presence of these phytochemicals might be directly responsible for the acetylcholinesterase activity. The ADME parameters predicted from the SwissADME server also supported that all the most active 3 molecules have good drug-like properties.

CONCLUSION

The nootropic herbal formulations which were used in this study showed significant results in behavioral and physiological activities. Specifically, the nootropic herbal formulations NHF1 composed of *Cinnamon zeylanicum, Vigna mungo, Avena sativa, Asparagus racemosus, Areca catechu* and NHF5 composed of *Zingiber officinalis, Convolvulus pluricaulis, Curcuma*

longa, Phyllanthus emblica, Mentha piperita, Hibiscus rosa sinensis showed greater impact in elevation of neuronal acetylcholine in the brain via significant demotion of acetylcholinesterase activity and it is clearly evident in the *In vivo* acetylcholinesterase study. Further study conducted to evaluate the chemical constituents responsible for the activity was predicted using molecular docking studies which suggest that Sarsasapogenin (-10.75 kcal/mol), racemosol (-10.63 kcal/mol), and beta-sitosterol (-10.25 kcal/mol) have the best binding energy and greater interactions with the acetylcholinesterase enzyme. The ADME parameters predicted from the SwissADME server further support that all the best active compounds are proven to be druggable molecules and can permeate through the blood brain barrier (BBB). These shreds of evidence suggest that the neuroprotective and acetylcholinesterase inhibition nature of these formulations maybe due to the presence of the chemical constituents sarsasapogenin, racemosol, and beta-sitosterol. Therefore, these formulations might clinically help patients of dementia and Alzheimer's in recovery by symptomatic relief, and improvement of cognition.

Peer-review: Externally peer-reviewed.

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Original Article

The inhibitory effects of plant extracts, vitamins and amino acids on myeloperoxidase activity

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ABSTRACT

Background and Aims: Myeloperoxidase (MPO, EC 1.11.2.2) is a vital antimicrobial enzyme, having a crucial role in host defense. Obstructing the activity of MPO is a possible pharmacological approach for the hindrance and management of a wide array of inflammatory illnesses. Consequently, blocking the activity of MPO is a potential pharmacological strategy for prevention and treatment of a broad range of inflammatory diseases.
Methods: In our study, inhibitory effects of 6 different sulfur containing plant extracts, 16 different vitamins and amino acids were studied for MPO inhibitory activities. The MPO enzyme activity was determined spectrophotometrically according to the method of Wei and Frankel.
Results: Among the aqueous plant extracts, black cabbage extract having IC_{50}=0.92±0.07 mM showed the highest inhibition. Among the vitamins and amino acids studied, the highest MPO enzyme inhibition was exhibited by ascorbic acid with IC_{50}=0.01±0.003 and cysteine with IC_{50}=1.09±0.73 mM.
Conclusion: Based on the outcomes, it was observed that all the examined plant extracts, vitamins and amino acids inhibited MPO enzyme at certain ratios.

Keywords: Myeloperoxidase, enzyme, inhibition, plant extract, vitamins, amino acids

INTRODUCTION

Myeloperoxidase (MPO, EC 1.11.2.2) is a lysosomal hemoprotein found in the azurophilic granules in neutrophils (Unubol et al., 2015). Compared to neutrophils, the human monocytes have fewer MPO-positive granules which are lost into tissue macrophages during differentiation (Malle, Furtmüller, Sattler, & Obinger, 2007).

In the presence of H_2O_2, Cl^- is oxidized to HOCl by MPO. It also functions as classic peroxidase, thereby producing a series of free radicals and reactive oxygen species (ROS). The inhibitors of MPO have high potentials for the treatment of many inflammatory diseases (Wurtz et al., 2018; Regasini et al., 2008). While many compounds (e.g. azides, anilines, phenols, hydrazides, and hydroxamic acids) are potent inhibitors of MPO activity in vitro, (Wurtz et al., 2018; van der Veen, de Winther, & Heeringa, 2009; Forbes et al., 2013) they are essentially toxic, consequently inappropriate for use as therapeutic agents (Tian, Ding, Peng, & Lu, 2017). For these reasons, researchers are constantly in search of new natural medications.

This study was aimed at examining the inhibitory activities of 6 sulfur containing plant extracts, as well as 16 different vitamins and amino acids on MPO enzyme.

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MATERIALS AND METHODS

Chemicals

All reagents used in the inhibition of MPO enzyme activity were of analytical grade and commercially available.

Preparation of aqueous plant extracts and other drugs

Plant materials were washed with water and dried at room temperature. Dried plants (20 g) were extracted by adding 200 mL of distilled water and refluxed for 8 hours. The extracts were then filtered and the filtrates were taken to the pre-weighed glass flasks. The glass flasks were placed in a rotary evaporator and the water of the mixtures was evaporated under reduced pressure. Then, extracts were kept in Eppendorf tubes at -20°C. Before use, all extracts were dissolved in distilled water at different concentrations. Also, vitamins, amino acids and peptides were prepared by being dissolved in distilled water.

Enzyme inhibitory activity assay

Rat gastric tissues homogenates were used as the enzyme source. The gastric tissues were homogenized in 0.9% saline to make up a 10% (w/v) homogenate. The homogenate was centrifuged at 3000 rpm for 30 minutes at 4°C and the supernatant was used for enzyme inhibition experiments.

MPO enzyme inhibitory activity was determined spectrophotometrically according to Wei and Frenkel's method (Wei & Frenkel, 1991). In a test tube, 1.3 mL of 4-aminoantipyrine (25 mM in 2% phenol) and 1.5 mL hydrogen peroxide solutions (1.7 mM) was shaked for 4 min, and 0.1 mL inhibition solution were added and stirred. The reaction was started by adding 0.2 mL of homogenate. Then, the change in absorbance was measured at 510 nm for 5 min. Reference measurements were performed without inhibitors (control value). Quercetin was used as standard.

The potent inhibition of MPO activity was calculated as follows:

$$\text{MPO Inhibition (\%)} = \frac{(A-B)}{A} \times 100$$

A is the enzyme activity without inhibitor. B is the activity in presence of inhibitor. The IC_{50} was determined as the concentration of plant extract required to inhibit MPO activity by 50%. The results are given as half maximal inhibitory concentrations (IC_{50}) values calculated from the regression equations prepared from the concentrations of the samples. Low IC_{50} values indicate higher enzyme inhibitory activity.

RESULTS

MPO inhibition values of different sulfur containing plant extracts are given in Table 1. High MPO inhibitory action correlates to a low IC_{50} value. Decreased inhibition values of plant extracts according to the lowest IC_{50} values are as follows: black cabbage > quercetin > white cabbage > purple cabbage > onion > brussels sprouts > cauliflower. As observed from the results, black cabbage (IC_{50}=0.92 ± 0.07 mg/mL) and white cabbage (IC_{50}=8.64 ± 0.98 mg/mL) displayed the highest MPO inhibitory effects when compared to other species and onion extracts (Table 1).

Table 1. The inhibition of myeloperoxidase activity by various plant extracts.

Plant extracts	Concentration (mg/mL)	Inhibition (%)*	IC_{50} Value (mg/mL)*
Black cabbage	0.25	13.2±4.17	0.92±0.07
	0.5	29.2±5.45	
	1.0	54.2±4.74	
Brussels sprouts	25	16.3±3.39	95.04±1.43
	50	36.3±5.59	
	100	50.2±0.85	
Cauliflower	25	6.3±2.05	131.13±3.68
	50	11.6±2.83	
	100	37.7±0.64	
Onion	1.25	5.0±1.98	24.09±7.71
	2.5	6.1±2.47	
	5.0	12.7±5.23	
Purple cabbage	1.25	4.6±2.90	23.96±9.61
	2.5	8.2±1.13	
	5.0	13.2±2.19	
White cabbage	0.2	6.0±0.00	8.64±0.98
	0.5	14.5±4.95	
	2.0	23.3±4.88	
	5.0	31.5±4.24	
Quercetin	0.2	37.36±2.64	1.49±0.17
	0.3	43.68±2.63	
	0.6	56.32±8.67	
	1.0	70.69±3.45	

*Mean ± SD

The inhibitory effects of vitamins are shown in Table 2. All the tested compounds exhibited MPO inhibitory activity. Ascorbic acid was found to be the most effective MPO inhibitory agent among the vitamins, its IC_{50} value was 0.01 ± 0.003 mg/mL (Table 2). Decreased inhibition values of vitamins to the lowest IC_{50} values are as follows: ascorbic acid > DL-α-tocopherol > quercetin > lipoic acid > pyridoxal-5 as-phosphate > DL-methionine methyl sulfonium chloride > nicotinamide > β carotene > routine hydrate > riboflavin > anorine hydrochloride.

According to Table 3, the amino acid with the lowest IC_{50} value was L-cysteine. Cysteine was found to have the most effective MPO inhibitory activity among the amino acids, with an IC_{50} value of 1.09 ± 0.73 mg/mL (Table 3). The highest inhibition values of the peptides and amino acids are as follows: L-cysteine > reduced glutathione > quercetin > L-lysine > L-glutamic acid > L-methionine > L-alanine.

DISCUSSION

MPO is a heme enzyme which uses H_2O_2 and Cl^- to catalyse the production of the reactive and cytotoxic oxidant hypochlorous acid (HOCl) (Daugherty, Dunn, Rateri, & Heinecke, 1994). The initial product of the MPO H_2O_2-Cl^- system is the potent antimicrobial oxidant hypochlorous acid/hypochlorite (HOCl/OCl^-). However, under pathological conditions, persistent activation of the MPO-H_2O_2 system of activated phagocytes may adversely affect tissues. HOCl is able to initiate modification reactions targeting lipids, DNA and (lipo)proteins, including halogenation, nitration and oxidative cross-linking (Malle et al., 2007). Also, MPO – mediated damage is involved in the pathogenesis of several inflammatory conditions, atherosclerosis, demyelinating diseases of the central nervous system and some tumors (Unubol et al., 2015).

As a result of systematic studies, plants have been used in modern medicine, phytotherapy and pharmacy according to

Table 2. The inhibition of myeloperoxidase by vitamins.

Plant extracts	Concentration (mg/mL)	Inhibition (%)*	IC_{50} Value (mg/mL)*
Anorine hydrochlorid	2.5	5.8±2.19	40.86±5.05
	5.0	9.8±2.90	
	10.0	14.4±3.25	
Ascorbic acid	2.5	68.6±8.06	0.01±0.003
	5.0	83.8±3.82	
	10.0	91.5±1.77	
β-carotene	1.0	7.9±0.99	12.85±1.12
	2.5	12.2±1.91	
	5.0	22.2±2.47	
(±)-α-Lipoic acid	0.3	2.3±0.49	5.05±0.22
	0.5	9.9±2.62	
	1.0	10.5±0.42	
DL-methionine methylsulfonium chloride	0.5	7.0±1.13	8.31±2.18
	1.0	8.7±2.90	
	2.5	18.3±5.44	
Nicotinamide	1.0	6.5±2.05	11.54±3.07
	2.5	12.9±2.05	
	5.0	24.0±5.66	
Pyridoxal-5'-phosphate	0.5	4.2±2.89	7.20±1.17
	1.0	4.7±0.89	
	2.5	17.8±1.51	
Riboflavin	1.0	4.8±1.56	16.14±7.17
	2.5	8.7±1.13	
	5.0	30.8±0.50	
Routine hydrate	2.5	31.3±1.77	13.00±2.67
	5.0	38.9±0.78	
	10.0	43.3±2.97	
DL-α-tocopherol acetate	2.5	5.3±3.75	0.03±0.01
	5.0	8.6±4.03	
	10.0	19.5±6.93	
Quercetin	0.5	37.36±2.64	1.49±0.17
	1.0	43.68±2.63	
	2.0	56.32±8.67	
	3.0	70.69±3.45	

*Mean±SD

Table 3. The inhibition of myeloperoxidase by amino acids and peptides.

Plant extracts	Concentration (mg/mL)	Inhibition (%)*	IC_{50} Value (mg/mL)*
L-Alanine	10.0	3.0±1.77	215.99±55.54
	25.0	5.6±1.20	
	50.0	12.5±2.26	
L-Cysteine	5.0	59.4±3.25	1.09±0.73
	10.0	85.3±1.20	
	20.0	98.8±0.57	
L-Glutamic acid	10.0	14.3±5.16	57.73±2.71
	25.0	29.1±1.20	
	50.0	43.7±0.71	
L-Lysine	2.5	3.0±0.85	54.91±19.41
	5.0	6.2±0.00	
	10.0	10.4±4.03	
L-Methionine	5.0	2.3±1.41	83.80±17.19
	10.0	5.6±2.33	
	20.0	11.6±1.84	
Reduced glutathione (GSH)	5.0	33.4±0.49	7.43±0.32
	10.0	71.8±2.05	
	20.0	92.3±0.42	
Quercetin	0.5	37.36±2.64	1.49±0.17
	1.0	43.68±2.63	
	2.0	56.32±8.67	
	3.0	70.69±3.45	

*Mean±SD

the effects of secondary metabolites and their composition. The use of synthetic drugs in general provides an effective and rapid treatment. But in some cases, high dose intake causes various side effects on the organism and systems. Thus, the use of herbal medicines or their active components represents alternatives for the treatment of numerous inflammatory disease (Castro, Ocampo & Franco, 2014). Some food derived polyphenols flavonoids and phenolic antioxidants have inhibitory effects on MPO (Kato, Nagao, Terao, & Osawa, 2003). Many studies demonstrate that extracts of different parts of various plant species such as *Peganum harmala* (Bensalem et al., 2014), *Ginkgo biloba* (Tian et al., 2015) *Careya arborea* (Begum, Sharma, Pillai, Aeri, & Sheliya, 2015), *Tragopogon graminifolius* (Farzaei et al., 2015), *Costus igneus* (Krishnan, Mathew & Vijayalakshmi 2014), *Urera aurantiaca* (Riedel, Marrassini, Anesini, & Gorzalczany, 2015) *Arctium lappa* (Wu et al., 2014) *Punica granatum* (Bachoual, Talmoudi, Boussetta, Braut, & El-Benna, 2011), *Onosma armeniacum* (Cadirci, Suleyman, & Aksoy, 2007), *Vaccinium corymbosum* (Torri et al., 2007), *Mangifera indica* (Garrido, González, Lemus, Delporte, & Delgado, 2006) and *Iberis amara* L. (Schempp, Hippeli, Weiser, Kelber, & Elstner, 2004) have shown significant effects on MPO enzyme inhibition.

Studies on cabbage species have shown that they contain flavonoids, ascorbic acid, DL-α-tocopherol acetate and DL-methionine methyl sulfonium chloride (vitamin U) (Podsędek, 2007; Sokmen, Tunali, & Yanardag, 2012). Podsedek (2007) reported that the vitamin C level of Brassica vegetables considerably differ among and within their subspecies. As observed from the results of this study, black and white cabbage exhibited the highest inhibitory activities at the concentration of 1 mg/mL (54.2±4.74%) and 5 mg/mL (31.5±4.24%), respectively on MPO enzyme activity. These inhibition values are thought to be caused by significant amounts of ascorbic acid, carotenoids, DL-α-tocopherol acetate and phenolic compounds in black cabbage and white cabbage. Many studies reported that flavonoids and polyphenols in natural extracts inhibit MPO at micromolar concentrations (Regasini et al., 2008; Kostálová, Mísíková, & Gáborová, 2001), and most of them are competitive substrates for MPO, through the production of HOCl and other hypohalides (Sokmen, Tunali, & Yanardag, 2012).

Ascorbic acid is a hydrophilic compound and acts directly by scavenging lipid hydroperoxide, superoxide and hydroxyl radicals, or indirectly by playing an important role in recycling tocopherol, a process that results in the conversion of ascorbic acid into a semiascorbyl radical (Bursać-Mitrović et al., 2016). In this study, ascorbic acid was found as having the most effective MPO inhibitory activity among the vitamins, its IC_{50} value was 0.01±0.003 mg/mL. Black cabbage contains considerably high levels of ascorbic acid.

L-Cysteine is an amino acid containing the sulfhydryl group that has a critical role in preventing oxidative stress in cells. In our study, cysteine was found to have the most effective MPO inhibitory activity among the amino acids, with an IC_{50} value of 1.09±0.73 mg/mL . Sagone et al. (1989) demonstrated that a sulfur centered compound - dimethylthiourea inhibited the

MPO enzyme by blocking the formation of HOCl, and can also react with H_2O_2 in certain experimental systems (Sagone Jr., Husney, Wewers, Herzyk, & Davis, 1989). On the other hand, sulfides were found to exhibit protective effects against several pathological diseases. Pálinkás et al., (2015) and Garai et al., (2017) suggested that this effect is possibly due to the obstruction of MPO-mediated oxidant production and/or synthesis of sulfane sulfur (via MPO catalysed sulfide oxidation by neutrofil-produced H_2O_2) (Garai et al., 2017).

CONCLUSION

The results of this study indicate that sulfur containing plant extracts, vitamins and amino acids are effective inhibitors of MPO activity. It may be suggested that these compounds and the plant extracts serve as alternative and complementary treatments in regulating immune responses in inflammatory regions when appropriate concentrations are added to a controlled diet.

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Original Article

An ethnobotanical study in Pöhrenk village (Çiçekdağı-Kırşehir province / Turkey)

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ABSTRACT

Background and Aims: This article presents important ethnobotanical information obtained in Pöhrenk village (Çiçekdağı-Kırşehir) which has the experience of severe migration. The aim of this study is to gather traditional ethnobotanical knowledge of wild plants used in this village which is located in the Central Anatolia Region of Turkey, and to identify the uses and local names of these wild plants.
Methods: The ethnobotanical study was carried out in Pöhrenk village between July 2018 and June 2019. The information, including the traditional uses of wild plants, was obtained from local people through face to face interviews, and during this study, 36 people (25 female and 11 male) were interviewed. During this period, demographic characteristics of participants, names of the local plants, their utilized parts and preparation methods were investigated and recorded.
Results: A total of 51 wild taxa belonging to 23 families were collected. According to the obtained data, the plants are mostly used as food (32 taxa), traditional folk medicine (9 taxa), making goods (6 taxa) and fodder (4 taxa). Also, the most represented families are Rosaceae (21.56%), Asteraceae (15.68%), Lamiaceae (5.88%) and Fabaceae (5.88%). Furthermore, the study was compared with three ethnobotanical studies conducted in nearby regions.
Conclusion: The data obtained in this study provided clues to ethnobotanists (or botanists), pharmacologists, and perhaps future local development projects.

Keywords: Çiçekdağı, Ethnobotany, Kırşehir, Pöhrenk, Traditional knowledge

INTRODUCTION

Since ancient times, the importance of plants in human life has been a known fact (Bulut, 2015). Traditional plant knowledge has always been verbally transmitted from generation to generation. This important information, compiled with ethnobotanical studies, is valuable for conservation, and the establishment of the local and indigenous plant usages has significant benefits (Sõukand & Pieroni, 2016).

Detailed ethnobotanical studies in Turkey were started since the beginning of the 19th Century (Ertuğ, 2014). Turkey, with the number of taxa of around 12000, has a rich flora, and about 3,800 of these taxa are endemic. In addition to this, many different cultures also live together in Turkey (Güner, Aslan, Ekim, Vural, & Babaç, 2012). Therefore, it has a great wealth both in terms of traditional use of plants and local names of plants (Erik & Tarikahya, 2004). However, the traditional use of plants has been adversely affected due to migration from rural to urban areas and factors such as people's orientation to synthetic drugs.

Ethnobotanical studies and studies on folk medicinal plants were carried out in Kırşehir province and nearby regions (Ayandın, 2010; Han & Bulut, 2012; Şenkardeş, 2014; Vural, Karavelioğulları, & Polat, 1997). In addition, a previous ethnobotanical study on

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Çiçekdağı and the surroundings of Kırşehir was published (Vural et al., 1997). Although Pöhrenk village, which is our area of study, is located within the borders of Çiçekdağı, it does not cover this study due to its distance to the district.

The aim of this study is to conduct a detailed ethnobotanical study in Pöhrenk Village (Çiçekdağı / Kırşehir) to avoid the disappearance of ethnobotanical knowledge, to relay this knowledge to new generations and to provide resources for future scientific studies.

MATERIALS AND METHODS

Study area

This study was conducted in Pöhrenk village, in the Çiçekdağı district (Kırşehir Province), which is located in the Central Anatolia region of Turkey. Pöhrenk village is one of 44 villages in the Çiçekdağı district (Figure 1). This region belongs to the Irano-Turanian Plant Geography Region and falls within the B-5 grid square according to the Grid classification system, developed by Henderson (1961). The geographical location of the study area is 39°25'56.8" North and 34°27'14.18" East. Its altitude is approximately 1150 meters. The average annual temperature in the province is 10.2°C, and the annual rainfall is 420 mm (Climate Data, 2019).

Pöhrenk village is 60 kilometers away from the center of Kırşehir, and 22 kilometers from the center of Çiçekdağı (Çiçekdağı Governor, 2019). The village residents immigrated from Adıyaman (East of Turkey), by the Ottoman Empire settlement laws in 1865 (Yıldırım, Ceyhan Suvari, İşoğlu, & Bozkurt, 2006).

Socio-economic structures

The economy of the region is based on agriculture and animal husbandry. Wheat, barley and sunflower are the most usual cultivated crops in the region (Kırşehir Governor, 2019). Additionally, sheep breeding is common due to the fact that the study area is a natural vegetation steppe (Çiçekdağı Governor, 2019). However, the unemployment rate has increased due to the decline in agriculture in recent years, and there has been a high volume of migration from the village to big cities in Turkey, such as Istanbul and Ankara, and to European countries, such as Germany and Austria.

Interviews with native people

A total of 69.44% female and 30.56% male informants were interviewed. The informants had varying levels of education, with 29.87% having no education, 46.77% having a primary level, 15.53% having a secondary level and only 7.83% having a tertiary level of education.

The interviews were conducted with local people without much difficulty because one author (B.Ç.) is local to the area and has relations there. A questionnaire was administered to the local people through face-to-face interviews. Interviews were conducted in the fields and houses. We visited the fields during all seasons.

The International Society of Ethnobiology Code of Ethics was taken into account in the interviews (ISE, 2006).

Plant materials

The field studies were carried out between May 2018 and August 2019. During this period, the collected plants were pressed

Figure 1. The location of Pöhrenk village in Turkey and Kırşehir.

in the field and prepared for identification. These specimens were initially identified with the help of the Flora of Turkey (Davis, 1965-1985; 1988; Güner, Özhatay, Ekim, & Başer, 2000), "A Checklist of the Flora of Turkey (Vascular Plants)" (Güner et al., 2012), "Illustrated Flora of Turkey Vol 1" (Güner et al., 2014) and "Illustrated Flora of Turkey Vol 2" (Güner et al., 2018) and "Türkiye'nin Doğal-Egzotik Ağaçları ve Çalıları" (Akkemik, 2018), and then they were compared with specimens in the Herbarium of the Faculty of Pharmacy of Istanbul University (ISTE). The scientific names of the plant taxa were identified according to "A Checklist of the Flora of Turkey (Vascular Plants)" (Güner et al., 2012). The plants were kept in ISTE.

RESULTS

The ethnobotanical knowledge about 51 taxa belonging 23 families was recorded. The local names of three taxa (*Verbascum cheiranthifolium* var. *cheiranthifolium*, *Salvia dichroantha, Lotus corniculatus* var. *corniculatus*) are unknown. The detailed knowledge including scientific name, voucher number, family name, life form, local name, used part(s), use, utilization method and preparation are summarized in Table 1. The most common families are Asteraceae (15.68%), Rosaceae (21.56%), Lamiaceae (5.88%) and Fabaceae (5.88%). The plants are used for food (32 taxa), traditional folk-medicine (9 taxa), making goods (6 taxa), fodder (4 taxa), firewood (4 taxa), ornament (2 taxa), cosmetic (1 taxon), fragrance (1 taxon) and evil eye (1 taxon). The percentages of plants' use are shown in Figure 2.

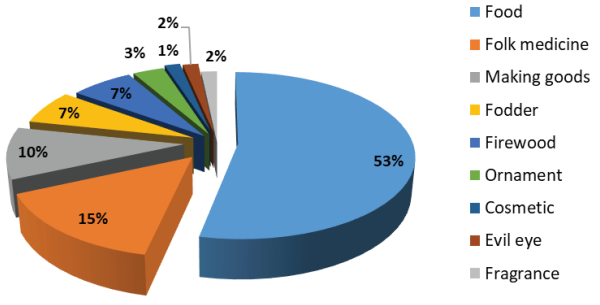

Figure 2. The percentages of plants' use in Pöhrenk village.

The edible plants are consumed eaten raw, prepared spice, soap, 'pilav' with bulgur, 'sarma', compote, marmalade, jam, 'şerbet' and tea, fried with onion or prepared as pancakes. Sarma is a cooked leaf rolled around a filling made from rice and/or minced meat (Doğan, Nedelcheva, & Pieroni, 2017). The use of the spice prepared with the leaves of *Mentha longifolia* is very common (Figure 3).

The most commonly used parts of plants are the aerial parts (19 taxa), fruits (11 taxa), leaves (8 taxa), flowers (4 taxa) and capitulums (2 taxa) (Figure 4). The aerial parts and leaves of raw consumed plants as food are usually collected in early April. Most of the plants whose fruits are consumed are in the Rosaceae family, and they are usually consumed raw or consumed as compote (Figure 5 and 6). The parts of all plants used as fodder are the aerial parts.

Additionally, the life forms of the used plants are herbs (68.62%), trees (19.60%) and shrubs (11.76%), in descending order. It was reported that the most important plants were *Polygonum cognatum, Teucrium polium, Malva neglecta, Mentha longifolia, Prunus cocomilia, P. divaricata* and *P. spinosa*.

Three of the collected taxa are endemic. These taxa are *Salvia dichroantha, Anchusa leptophylla* subsp. *incana* and *Crocus ancyrensis* (Figure 7).

DISCUSSION

Ethnobotanical studies became widespread in Turkey at the beginning of the 90s, and more folk-medicinal uses were recorded in the studies conducted at that time. It is possible to observe the same feature in a previous study which was conducted in Çiçekdağı's center and its surroundings (Vural et al., 1997). When we compare our data with this study, 10 taxa (*Chenopodium album, Crataegus orientalis, Gundelia tournefortii, Peganum harmala, Polygonaum cognatum, Potentilla reptans, Pyrus elaeagnifolia, Rosa canina, R. hemisphaerica, Teucrium polium*) are common, and six of them (*Polygonaum cognatum, Chenopodium album, Peganum harmala, Rosa canina, R. hemisphaerica, Teucrium polium*) have the same use. Also, *Gundelia tournefortii, Peganum harmala, Polygonaum cognatum* and *Rosa canina* have the same local name. However, *Rosa canina*

Figure 3. The spice of *Mentha longifolia*, A) dried leaves of *M. longifolia*, B) Yoghurt soup with its spice.

Table 1. The ethnobotanical uses of plants in Pöhrenk village (Çiçekdağı-Kırşehir).

Scientific name, Voucher number	Family name	Life form	Local name	Use	Plant part used	Utilization method and preparation
Amaranthus albus L. ISTE 116220	Amaranthaceae	Herb	-	Fodder	Aerial parts	Directly
Amygdalus orientalis Miller. ISTE 116079	Rosaceae	Shrub	Acı badem	Food	Seeds	Eaten raw
**Anchusa leptophylla* Roem. & Schult. subsp. *incana* (Ledeb.) D.F. Chamb. ISTE 116035	Boraginaceae	Herb	Emzik, Sormuk, Tımıt	Food	Flowers	Its nectar sucked
Anthemis cretica L. subsp. *anatolica* (Boiss.) Grierson ISTE 116108	Asteraceae	Herb	Papatya	Medicinal	Aerial parts, Capitulums	Externally; decoction, for common cold. For infertility in women, boiled as a mixture with Arpa *(Hordeum* sp.) and Tolik (*Malva neglecta*) and then the woman sitting over steaming water. Internally; decoction as sedative, curing shortness of breath
Artemisia absinthium L. BÇ23	Asteraceae	Shrub	Hawşan	Fragrance Making goods	Aerial parts	Hung on the wall, Broom
Capsella bursa-pastoris (L.) Medik. ISTE 116029	Brassicaceae	Herb	Noncic	Food	Leaves	Eaten raw
Chenopodium sp. ISTE 116875	Chenopodiaceae	Herb	Sılmastık	Food	Aerial parts	Fried with onion or prepared pancake with cheese
Chenopodium album L. subsp. *album* var. *album* ISTE 116072	Chenopodiaceae	Herb	Sılmastık, Sılmastıke toke	Food	Leaves	Fried with onion or prepared pancake with cheese
Chenopodium botrys L. ISTE 116073	Chenopodiaceae	Herb	Bostan güzeli, Yabani semiz	Food	Aerial parts	Preparing 'cacık' (with yogurt), pancake
Cichorium intybus L. ISTE 116089	Asteraceae	Herb	Çıtlık, İstriye çavi zer	Medicinal, food, Making goods	Capitulums Leaves Aerial parts	Eaten directly for curing diabetes and for curing fatty liver Eaten raw, Broom (Şışın)
Convolvulus arvensis L. ISTE 116 099	Convolvulaceae	Herb	Sırmaşığ, Sarmaşık	Fodder	Aerial parts	Fresh

Crataegus monogyna var. *monogyna* Jacq. ISTE 116023	Rosaceae	Tree	Suruk	Food	Fruits	Eaten raw or as compote.
Crataegus orientalis subsp. *orientalis*Pallas ex M. Beib. ISTE 116075	Rosaceae	Tree	Gaheşik, Alıç, Şilan	Medicinal food	Fruits	Internally; eaten as cardiotonic, decoction as diuretic Eaten raw or as compote
**Crocus ancyrensis* (Herb.) Maw ISTE 116015	Iridaceae	Herb	Pivonk	Food	Corms flowers	Eaten raw
Cyanus depressus (M.Bieb.) Soják ISTE 116 036	Asteraceae	Herb	Gökbaşı	Fodder	Aerial parts	Fresh
Daucus carota L. ISTE 116018	Apiaceae	Herb	Yabani havuç	Food	Roots	Eaten raw
Echinops spinosissimus Turra subsp. *bithynicus*(Boiss.) Greuter ISTE 116070	Asteraceae	Herb	Yabani ceviz, güz, sriye güz	Food	Receptacles	Eaten raw
Eryngium campestre L. var. *virens* Link ISTE 116038	Apiaceae	Herb	Noncüz,	Food	Stem	Young stems are eaten after peeling off the outer part
*Glaucium grandiflorum*Boiss. & A. Huet subsp. *refractum* var. *refractum* (Nab.) Mory ISTE 116112	Papaveraceae	Herb	Gül, Gulasur	Food	Flowers	Preparing 'sherbet'
Gundelia tournefortii L. BÇ30	Asteraceae	Herb	Kenger	Medicinal	Latex	Chewing gum as digestive
Juncus inflexus L. ISTE 116031	Juncaceae	Herb	Kındırga	Making goods	Aerial parts	Baskets
Lotus corniculatus var. *corniculatus* L. ISTE 116110	Fabaceae	Herb	-	food	Seeds	Eaten raw
Malva neglecta Wallr. BÇ21	Malvaceae	Herb	Tolık, Ebegümeci	Medicinal Food	Aerial parts	Externally: for infertility in women, boiled as mixture with Arpa *(Hordeum* sp.) and Papatya (*Anthemis cretica* subsp. *anatolica)* then the woman sitting over steaming water. Externally: slopes prepared with cracked wheat is used for swelling of the skin. Internally: decoction, as an anti-inflammatory, intestinal cleanser. Eaten raw
Medicago sativa subsp. *sativa* L. ISTE 116105	Fabaceae	Herb	Fige baji	Food	Seeds	Eaten raw

Mentha longifolia (L.) L. subsp. *typhoides* (Briq.) Harley ISTE 116039	Lamiaceae	Herb	Punk, Narpuz	Food	Leaves	As spice
Onopordum turcicum Danin. ISTE 116019	Asteraceae	Herb	Kangal	Food	Stems	Eaten raw as a snack after peeling off the outer part
Peganum harmala L. ISTE 116042	Zygophyllaceae	Herb	Üzerlik	Ornament evil eye	Dried aerial parts fruits seeds	Hanging on the wall as amulets, roasting on the fire
Polygonum cognatum Meissn. ISTE 116033	Polygonaceae	Herb	Madımalak, Mardımalak, Madımak	Food	Aerial parts	Fried with onion Prepared pancake Prepared 'pilav' with bulgur
Potentilla reptans L. ISTE 116 098	Rosaceae	Herb	Henna çuçıkan	Cosmetic	Aerial parts	as henna, crushing on stones (children)
Prunus cocomilia Ten. ISTE 116 028	Rosaceae	Tree	Heruge baji, Erik	Food	Fruits	Eaten raw or as compote
Prunus divaricata Ledeb.var. *pis-sardii* Ledeb. ISTE 116 027	Rosaceae	Tree	Erige baji, Sarı erik	Food	Fruits	Eaten raw or as compote
Prunus spinose L. ISTE 116024	Rosaceae	Tree	Erige baji, Yaban erik	Food	Fruits	Eaten raw
Pyrus elaeagnifolia subsp. *elaeag-nifolia* Pall. ISTE 116021	Rosaceae	Tree	Çortuk	Food	Fruits	Eaten raw
Pyrus syriaca subsp. *syriaca* Boiss. ISTE 116115	Rosaceae	Tree	Yabani çortuk	Food	Fruits	Eaten raw
Rhamnus lycioides L. subsp. *oleoides* (L.) Jahandiez & Maire ISTE 116030	Rhamnaceae	Shrub	Çalıyeraş	Food	Fruits	Eaten raw
Rosa canina L. ISTE 116025	Rosaceae	Shrub	Kuşburnu, Şilan	Medicinal food	Fruits	Internally; decoction in the flu Jam, marma-lade and tea
Rosa hemisphaerica J. Herrm. ISTE 116085	Rosaceae	Shrub	Kunpank	Food	Fruits	Jam, marmalade and tea
Rumex crispus L. ISTE 116097	Polygonaceae	Herb	Tırşigo, Efelik	Food	Leaves	Eaten raw or as a wrapping material for 'sarma'
Salix alba L. ISTE 116016	Salicaceae	Tree	Sogut, Söğüt	Firewood	Branches Stems	Walking stick Heating
Salix excelsa S.G. Gmelin ISTE 116017	Salicaceae	Tree	Sogut, Salkım söğüt	Firewood	Branches Stems	Walking stick Heating

**Salvia dichroantha* Stapf. ISTE 116092	Lamiaceae	Herb	-	Food	Flowers	Its nectar sucked
Scabiosa argentea L.	Caprifoliaceae	Herb	Süpürge	Making goods	Aerial parts	Broom
Sinapis arvensis L. ISTE 116080	Brassicaceae	Herb	Xardal, Xardale zar	Food	Leaves	Eaten raw
Tamarix parviflora DC. ISTE 116 109	Tamaricaceae	Shrub	Hawşan	Making goods Firewood	Aerial parts	Broom
Taraxacum sp. ISTE 116877	Asteraceae	Herb	Nancamus	Food	Aerial parts	Eaten raw
Teucrium polium L. ISTE 116041	Lamiaceae	Herb	Mırada	Medicinal	Aerial parts	Internally; decoction, treatment of allergy, the treatment of jaundice as incense for shortness of breath, appetizing
Trifolium physodes var. *physodes* Steven &M.Bieb. ISTE 116091	Fabaceae	Herb	Yonca	Fodder	Aerial parts	Fresh
Typha domingensis Pers. ISTE 116032	Typhaceae	Herb	Kamuş, Kamış	Ornament	Aerial parts	Branches
Ulmus minor Miller ISTE 116 100	Ulmaceae	Tree	Karaağaç	Making goods, firewood	Branches Stems	Walking stick Heating
Verbascum cheiranthifolium Boiss. var. *asperulum* (Boiss.) Murb. ISTE 116063	Scrophulariaceae	Herb	-	Medicinal	Leaves	Externally; for hemorrhoids, boiled then directly put on the wounds or the patient sitting over steaming water
Verbascum cheiranthiflium var. *cheiranthifolium* Boiss. ISTE 116064	Scrophulariaceae	Herb	-	Medicinal	Leaves	Externally; for hemorrhoids, boiled then directly put on the wounds or the patient sitting over steaming water

*Endemic taxa.

is used for the treatment of flu in Pöhrenk, while it is used only as food and tea in the Çiçekdağı study. The use of *Teucrium polium* is, in general, the same in both regions, but differently, the plant is used for the treatment of jaundice in Pöhrenk. *Peganum harmala, Potentilla reptans* and *Pyrus elaeagnifolia* subsp. *elaeagnifolia* are used for medicinal purposes in Çiçekdağı, but these taxa are used for different purposes in Pöhrenk (Table 2).

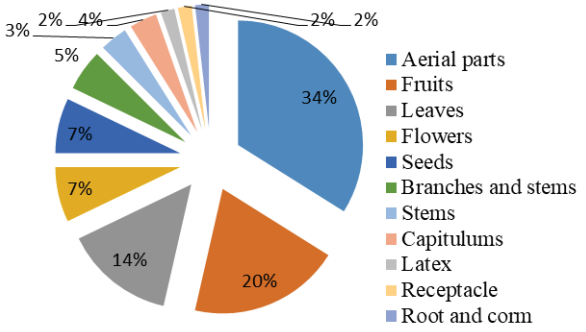

Figure 4. The percentages of plants used parts in Pöhrenk.

The comparison of all the plants used in the present study with previous ethnobotanical studies (Ayandın, 2010; Han & Bulut, 2012; Şenkardeş, 2014; Vural et al., 1997) in the nearby regions is given in Table 3. *Anthemis cretica* subsp. *anatolica*, *Chenopodium botrys* and *Verbascum cheiranthifolium* are only used for medicinal purposes in Pöhrenk. Also, *Chenopodium botrys, Crocus ancyrensis, Glaucium grandiflorum* subsp. *refractum* var. *refractum, Lotus corniculatus* var. *corniculatus, Mentha longifolia* subsp. *typhoides, Prunus cocomilia, Prunus spinosa, Pyrus syriaca, Rhamnus lycioides* subsp. *oleoides* and *Salvia dichroantha* are only consumed as food in Pöhrenk. Additionally, the preparation of sherbet from *Glaucium grandiflorum* flowers is recorded only in Pöhrenk. However, *Teucrium polium* and *Rosa canina* are also used for medicinal purposes in nearby studies (Ayandın, 2010; Şenkardeş, 2014). *Chenopodium album, Polygonum cognatum* and *Rosa canina* are also consumed as food in tree nearby studies (Ayandın, 2010; Şenkardeş, 2014; Vural et al., 1997). Additionally, *Peganum harmala* is used for evil eye in both studies (Figure 8).

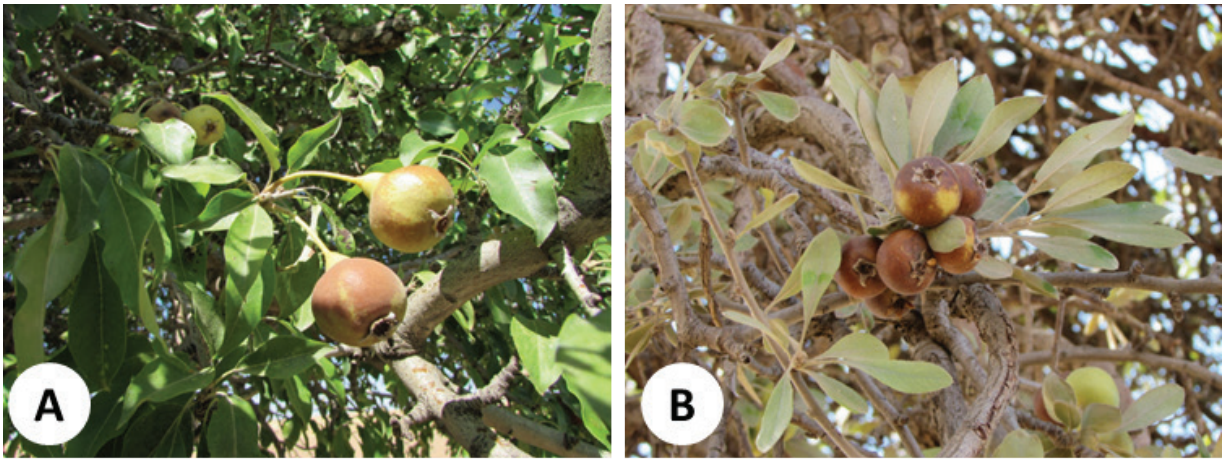

Figure 5. The fresh consumed fruits; A) *Pyrus syriaca* var. *syriaca*, B) *Pyrus elaeagnifolia* subsp. *elaeagnifolia*.

Figure 6. The compote of *Prunus cocomilia* and *P. divaricata* var. *divaricata*.

Figure 7. *Crocus ancyrensis*.

Table 2. The comparison of the intended use of common plants in Pöhrenk Village and Çiçekdağı (Vural et al., 1997).

Scienific name	Intended use in Pöhrenk	Intended use in Çiçekdağı
Chenopodium album	Food	Food
Crataegus orientalis* subsp. *orientalis	Medicinal (Cardiotonic and diuretic)	Food, medicinal (Antihypertensive)
Peganum harmala	Incense, evil eye, ornament	Medicinal (skin diseases, hemorrhoids), evil eye
Polygonum cognatum	Food	Food
Potentilla reptans	Cosmetic	Medicinal (antidiarrheal)
Pyrus elaeagnifolia* subsp. *elaeagnifolia	Food	Medicinal (blood purifier)
Rosa canina	Food, Medicinal (flu)	Food
Rosa hemisphaerica	Food	Food
Teucrium polium	Medicinal (allergy, appetizing, shortness of breath, jaundice)	Medicinal (appetizing, shortness of breath)

Table 3. The comparison of the intended use of common plants in Pöhrenk Village and in Çiçekdağı (Kırşehir) (Vural et al., 1997), Kadışehri (Yozgat) (Han & Bulut, 2012), Nevşehir (Acıgöl, Derinkuyu, Gülşehir, Nevşehir-Merkez, Ürgüp) (Şenkardeş, 2014) and Polatlı (Ankara) (Ayandın, 2010).

Scientific name	Pöhrenk (Kırşehir)	Çiçekdağı (Kırşehir)	Kadışehri (Yozgat)	Nevşehir	Polatlı (Ankara)
Amaranthus albus	Fodder	-	-	-	-
Amygdalus orientalis	Food	-	-	Food, medicinal	Food
**Anchusa leptophylla* subsp. *incana*	Food	-	-	Food	Food
Anthemis cretica subsp. *anatolica*	Medicinal	-	-	-	-
Artemisia absinthium	Fragrance, making good	-	-	-	-
Capsella bursa-pastoris	Food	-	-	Food	-
Chenopodium album	Food	Food	-	Food, medicinl	Food, fodder
Chenopodium botrys	Food, medicinal, making good	-	-	-	-
Cichorium intybus	Medicinal	-	-	Food, medicinal	Making good
Convolvulus arvensis	Fodder	-	Medicinal	Fodder	Medicinal
Crataegus monogyna	Food	Food, medicinal	-	Food, medicinal, making good	-
Crataegus orientalis	Food, Medicinal	-	-	Food, medicinal, making good	Food, making good
**Crocus ancyrensis*	Food	-	-	-	-
Cyanus depressus	Fodder	-	-	-	-
Daucus carota	Food	-	-	-	Food, fodder
Echinops spinosissimus subsp. *bithynicus*	Food	-	-	-	Fodder
Eryngium campestre var. *virens*	Food	-	Medicinal	Food, medicinal	Making good

Glaucium grandiflorum subsp. *refractum*	Food	-	-	-	-
Gundelia tournefortii	Medicinal	-	-	Food, medicinal	Food
Juncus inflexus	Making good	-	-	-	-
Lotus corniculatus var. *corniculatus*	Food	-	-	-	-
Malva neglecta	Food, medicinal	-	Medicinal	Food, medicinal, making good	Food
Medicago sativa subsp. *sativa*	Food	-	-	-	Fodder
Mentha longifolia subsp. *typhoides*	Food	-	-	Food, medicinal	-
Onopordum turcicum	Food	-	-	-	Food, fodder
Peganum harmala	Evil eye, ornament	Medicinal, evil eye	Medicinal	Medicinal, making good, evil eye	Evil eye
Polygonum cognatum	Food	Food	Medicinal	Food	Food
Potentilla reptans	Making good	Medicinal	-	-	-
Prunus cocomilia	Food	-	-	-	-
Prunus divaricata subsp. *divaricata*	Food	-	Medicinal	Food	-
Prunus spinosa	Food	-	Medicinal	-	-
Pyrus elaeagnifolia	Food	Medicinal	-	Food, making good	Food, medicinal
Pyrus syriaca	Food	-	-	-	-
Rhamnus lycioides subsp. *oleoides*	Food	-	-	-	-
Rosa canina	Food, medicinal	Food	Medicinal	Food, medicinal	Food, medicinal
Rosa hemisphaerica	Food	Food	-	Food, medicinal	Ornament
Rumex crispus	Food	-	Medicinal	Food, medicinal	-
Salix alba	Firewood	-	Medicinal	Medicinal, making good, fodder	Making good
Salix excelsa	Firewood	-	-	-	-
**Salvia dichroantha*	Food	-	-	-	-
Scabiosa argentea	Making good	-	-	-	-
Sinapis arvensis	Food	-	-	Food, medicinal	Food, medicinal
Tamarix parviflora	Firewood, making good	-	-	Firewood	-
Teucrium polium	Medicinal	Medicinal	Medicinal	Medicinal	Making good
Trifolium physodes var. *physodes*	Fodder	-	-	-	-
Typha domingensis	Ornament	-	-	-	-
Ulmus minor	Making good	-	-	Medicinal	Evil eye, firewood
Verbascum cheiranthifolium	Medicinal	-	-	-	-

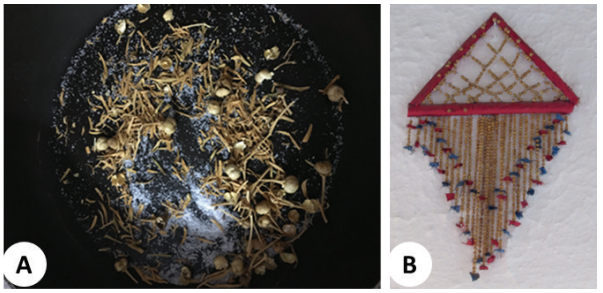

Figure 8. The use of *Peganum harmala*; A) Burning of *P. harmala* with salt to protect from evil eye, B) the ornament made of fruits of *P. harmala*.

Since the center of Çiçekdağı is close to the study area, it is expected that there will be more common plants compared to other regions, whereas fewer common plants are observed. The most common plants are observed in Nevşehir (24 taxa) and Polatlı (22 taxa).

CONCLUSION

The traditional knowledge is no longer being passed down from older to younger generations in Pöhrenk, because most of the residents (generally only the middle-aged and elderly) of the Pöhrenk village spend only the summer months in the village. The compiling of traditional ethnobotanical knowledge in this area is critical. This reveals the importance of this study, and this study will close the gap about traditional ethnobotanical knowledge.

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Original Article

The role of community pharmacists in public health and public health related problems which they encounter

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ABSTRACT

Background and Aims: This study was conducted in order to identify the role of community pharmacists in public health and the public health related problems which are encountered.
Methods: In this cross-sectional study, 87 community pharmacists answered a questionnaire form about sociodemographic characteristics, public health counseling roles and barriers. The data was summarized as mean ± standard deviation and percentage. Chi-square test was used to compare the categorical data.
Results: 93.1% of the community pharmacists considered that they had an active role in the protection of public health, while the percentage of those who thought they were able to realized it was 78.2%. Limited authority (63.2%), insufficient time and workload (52.6%) were the leading causes of ineffectivity of the pharmacists in public health. The causes that prevented receiving consultancy services were shame (64.4%), the education level of the client (51.7%) and gender difference between the pharmacist and client (48.3%). The pharmacists thought that their professional reputation should be improved in order to improve their consulting role (35%).
Conclusion: Although there are many occupational problems of pharmacists in Turkey, pharmacists voluntarily provide a consultacy service about many subjects for the protection of public health. Legal arrangements are needed to make pharmacies public health counseling centers.

Keywords: Pharmacists, Public health, Turkey, Pharmacy, Health promotion

INTRODUCTION

Pharmacists are the most accessible healthcare professionals in the society (World Health Organization, 1998; Eades, Ferguson, & O'Carroll, 2011; Beshir & Bt Hamzah, 2014). Community pharmacies are continuosly in communication with the society, and there is no need to make an appointment for receiving consultancy. Healthy individuals meet with health professionals as well as patients in pharmacies, so pharmacies have an important potential for public health (Anderson, Blenkinsopp, & Armstrong, 2003; Erku et al., 2017). Due to these characteristics of them, they can provide solutions to the problems related to health protection and health improvement in public health services (Erku et al., 2017).

In addition to their pharmacological services, pharmacists also have consultancy and health education roles in health promotion programs. From this point of view, pharmacists play an active role in the provision of public health services (World Health Organization, 1998; Eades et al., 2011). Pharmaceutical public health has been defined as: "The application of pharmaceutical knowledge, skills and resources to the science and art of preventing disease, prolonging life, promoting, protecting and improving health for all through the organised efforts of society" (Walker, 2000). In many countries, such as England, America, Canada

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and Sweden, community pharmacists are active in public health issues, such as smoking cessation, diabetes monitoring, following up on cholesterol and blood pressure, folic acid and pregnancy, asthma, immunization, oral health, emergency hormonal contraception (Anderson et al., 2003; Saramunee et al., 2015; Agomo, Udoh, Kpokiri, & Osuku-Opio, 2018).

Turkey ranks first and France second in terms of the number of pharmacies. According to the number of registered pharmacies in the country, the top five countries are Italy, France, Spain, Germany and Turkey. There were 34,870 pharmacists in Turkey and 25,896 of them were community pharmacists according to the data of 2018 (Turkish Pharmacists Association, 2018). Turkish Pharmacists' Association defined pharmaceutical public health competencies of pharmacists. These competences include providing information and advice on medications and other health products, health promotion and improvement, as well as identifying basic health needs, prevention and control of diseases, advising on promoting a healthy lifestyle (Turkish Pharmacists Association, 2015).

Emergency contraception was provided in community pharmacies in Turkey while diabetes and asthma management services were provided within the scope of My Counsellor Pharmacy Project. The public health services, such as blood pressure measurement, cholesterol measurement, glucose measurement, weight measurement, pregnancy testing, treatment of smoking cessation, hypertension management and vaccination services are not officially provided in pharmacies. The My Counsellor Pharmacy Project was launched in December 2014 by the Turkish Pharmacists Association. This project supports the continuous professional development of pharmacists. This project has been developed for pharmacists to receive health training regularly and train their counselees. At the present time the project is carried out as a pilot application in Turkey and it is aimed to spread the project across the nation in the future. The project is a step taken for the continuous training of pharmacists. Continuing education and professional development are not compulsory in Turkey while they are compulsory in many European countries. Therefore, this project is important for the professional development of pharmacists (Turkish Pharmacists Association, 2018).

This study was carried out to determine the current roles of community pharmacists in public health and their problems.

MATERIALS AND METHODS

This is a cross-sectional study. In Turkey there are 81 provinces and the city Niğde was chosen for the researcher to access easily. The study consisted of community pharmacists (n=100) in Niğde province of Turkey. The sample was not selected; it was aimed to reach the whole universe. 87 pharmacists were included in the study. Firstly, all pharmacies were informed about the study via the Chamber of Pharmacists in the Niğde. The pharmacies where pharmacists were not present, were continuously visited by the researcher until the pharmacists were met. A 17-question, questionnaire form was prepared by the researcher according to the literature, for the purpose of determining the characteristics of the pharmacists and pharmacies (age, sex, educational background, duration of pharmacy business, location of pharmacy, position of pharmacy, number of staff except the pharmacist, daily number of patients), ways of accessing information, characteristics of counselees, service provided by pharmacy, consultancy subjects and problems encountered. Questionnaire questions varied according to question types and they included questions that could be answered as yes/no/I have no idea, multiple-option questions for pharmacists and open-ended questions. The data was collected by using this questionnaire form applied by face to face interviews with the pharmacists who accepted to participate in the study.

The data were analyzed with SPSS 21.0 (version 21.0, SPSS, Inc, Chicago, IL, 2012). The data were transferred to the computer and summarized as mean±standard deviation and percentage. Chi-square test was used to compare categorical data (used to compare qualitative variables such as pharmacists' active role in public health, providing counselling and having adequate information about counselling with qualitative variables such as age, sex, occupational year, expertise, location of pharmacy, number of staff and number of patients). $p<0.05$ was accepted as statistically significant.

Ethical approval: The study was carried out with the approval of Niğde Ömer Halisdemir University Ethics Committee (No:2018/11-05). Permission was also obtained from the Niğde Chamber of Pharmacists.

RESULTS

In this study, 87 of 100 pharmacies in the Niğde province of Turkey were included. 42.5% of the pharmacists were female. According to the Turkish Statistics for the year 2018, the ratio of female pharmacists in Niğde was lower than the ratio of female pharmacists in Turkey (Turkish Pharmacists Association 2018). The mean age was 44.3±16.0 years. 20.7% of the pharmacists were 65 years old or older. 13.8% of them were senior pharmacists. Their mean working year as a pharmacist was 19.0±14.4 years. 55.2% of the pharmacies were located in the city center. 32.2% of the pharmacies were close to a hospital; 33.3% were close to a family health center; 34.5% of them were in the center of province. 56.3% of the pharmacies had 2 or more personnel except pharmacists. 52.9% of the pharmacies had 51-100 patients a day (Table 1).

More than half of the pharmacists (55.2%) thought that the public health course in the University education was not sufficient. The pharmacists reached up-to-date information on health through the Internet (87.4%), journals-books (58.6%) and social media (54%). Nearly half of the pharmacists (49.4%) stated that they obtained information from their colleagues. 75.9% of the pharmacists stated that they could use the internet effectively. While 93.1% of the pharmacists believed that they played an active role in protecting public health, one fifth of them (21.8%) thought that they were not active in protecting public health, considering their present condition. It was determined that there was no significant difference between the pharmacists in the city center and in the countryside in terms of being active in public health ($\chi^2=0.104$, $p=0.748$). The pharmacists who were near the hospital thought that they were more active ($\chi^2=7.741$, $p=0.021$).

Table 1. Sociodemographic characteristics of pharmacists and pharmacy locations.

Sociodemographic Characteristics	N	%
Gender		
Female	37	42.5
Male	50	57.5
Age		
25-40	47	54.0
41-55	14	16.1
56-75	26	29.9
Education Level		
Undergraduate	75	86.2
Master	12	13.8
PhD	-	-
Working experience as a pharmacist		
1-10 years	33	37.9
11-20 years	21	24.1
21-30 years	10	11.5
31 years or longer	23	26.4
Location of the Pharmacy		
City Center	48	55.2
District	34	39.1
Town	5	5.7
Location of the Pharmacy		
Near a Hospital	28	32.2
Near a Family Health Center	29	33.3
On a Central Street	30	34.5
Number of Personnel in Pharmacy except pharmacists	49	56.3
0-2	38	43.7
3 or more		
Daily Patient Number		
50 or below	33	37.9
51-100	46	52.9
101 or above	8	9.2

N: Number of respondents

A question was addressed to the pharmacists who thought that they were not active in public health, containing their reasons for not being active, as well as multiple options and open-ended expression. Accordingly the health system, the limitation of authority (63.2%), insufficent time and workload (52.6%) were among the main reasons for the ineffective role of the pharmacists in public health (Table 2).

Table 2. Reasons of "Pharmacists' Inactive in Public Health"*

Reason	N	%
Health system	12	63.2
Authority limitation	12	63.2
Insufficient time	10	52.6
High workload	10	52.6
Unsuitable place	6	31.6
Personnel inadequacy	5	26.3
Other	5	26.3

*Multiple choices were marked, N: Number of respondents

77% of the beneficiaries of pharmacy services were the individuals with low educational and socioeconomic status. The question of "In your opinion, what are the first three occupational groups in which the community receives counseling on health-related issues?" was answered as pharmacists (50.6%), physicians (40.2%), and health personnel (55.2%). In pharmacies, prescription medicine (98.9%), counseling (34.5%) and non-prescription medicine (21.8%) services were provided, respectively.13.8% of the pharmacists stated that consultancy service increased; 54% of them stated that consultancy service had not changed; %14.9 of them stated that it decreased in the last 6 months.

The obstacles for receiving counseling services from pharmacists are presented in Table 3. Feeling ashamed by the clients (64.4%), their educational status (51.7%), gender difference between a pharmacist and a client (48.3%) were the obstracles determined, respectively (Table 3). Nowadays, the internet has an important place in our lives. The rate of pharmacists who thought that the widespread use of the internet affected the pharmacists' counseling roles was 69.4%. The pharmacists thought that most of the clients came to the pharmacy with the wrong information because of increased internet usage (71.2%). While, for the pharmacists, the widespread use of the internet increased their access to health information (35.6%) (Table 4). 47.1% of the pharmacists made open-ended suggestions for the purpose of increasing counselling services. Most of them answered as increasing the professional reputation

Table 3. Obstacles for "Receiving Consultancy Services"*

Obstacle	N	%
Client's educational status	45	51.7
Gender difference	42	48.3
Shame	56	64.4
Being crowded of pharmacy	21	24.1
Inability to communicate appropriately	17	19.5
Busying of the pharmacist	19	21.8
Other	7	8.0

*Multiple choices were marked, N: Number of respondents

Table 4. Effect of internet usage on pharmacists' counseling roles*

Variable	N	%
My counseling role decreased	20	33.9
My counseling role increased	10	16.9
My Access to information increased	21	35.6
Clients came with wrong information	42	71.2
Clients are informed	9	15.3
Other	2	3.4

*Multiple choices were marked, N: Number of respondents

Table 5. Pharmacists' counselling rate and their sufficiency in counselling.

			Knowledge Sufficient	
Consultancy Issues	**N**	**%**	**N**	**%**
Smoking cessation			70	80.5
Yes	75	86.2		
No	12	13.8		
Alcohol cessation			66	75.9
Yes	33	37.9		
No	54	62.1		
Substance Addiction			62	71.3
Yes	20	23.0		
No	67	77.0		
Healthy Nutrition			69	79.3
Yes	64	73.6		
No	23	26.4		
Weight Control			69	79.3
Yes	57	65.5		
No	30	34.5		
Physical Activity			67	77.0
Yes	50	57.5		
No	37	42.5		
Communicable Diseases			61	70.1
Yes	49	56.3		
No	38	43.7		
Vacines			64	73.6
Yes	55	63.2		
No	32	36.8		
Hypertension			75	86.2
Yes	77	88.5		
No	10	11.5		
Diabetes			73	83.9
Yes	76	87.4		
No	11	12.6		
Hyperlipidemia			75	86.2
Yes	72	82.8		
No	15	17.2		
Asthma-COPD*			75	86.2
Yes	74	85.1		
No	13	14.9		
Rational Drug Use			68	78.2
Yes	71	81.6		
No	16	18.4		
Emergency Contraception			63	72.4
Yes	36	41.4		
No	51	58.6		
Family Planning Methods			68	78.2
Yes	42	48.3		
No	45	51.7		
Sexually Transmissible Diseases			69	79.3
Yes	34	39.1		
No	53	60.9		
Suicide Risk			54	62.1
Yes	11	12.6		
No	76	87.4		
Osteoporosis			68	78.2
Yes	65	74.7		
No	22	25.3		
Total	**87**	**100**		

*Chronic Obstructive Pulmonary Disease, N: Number of respondents

Table 6. Issues in which pharmacists provide counselling according to their sex.

	Female Pharmacists				Male Pharmacists					
	Yes		No		Yes		No			
Issues	N	%	N	%	N	%	N	%	X^2	P
Smoking cessation	32	86.5	5	13.5	43	86.0	7	14.0	0.004	0.948
Alcohol cessation	7	19.0	37	81.0	26	52.0	24	48.0	9.884	**0.002**
Substance Addiction	6	16.2	31	83.8	14	28.0	36	72.0	1.668	0.197
Healthy Nutrition	32	86.5	5	13.5	32	64.0	18	32.0	5.529	**0.019**
Weight Control	28	75.7	9	24.3	29	58.0	21	42.0	2.941	0.086
Physical Activity	22	59.5	15	40.5	28	56.0	22	44.0	0.104	0.747
Communicable Diseases	19	51.3	18	48.7	30	60.0	20	40.0	0.647	0.421
Vaccines	21	56.8	16	43.2	34	68.0	16	32.0	1.156	0.282
Hypertension	34	91.9	3	7.1	43	86.0	7	14.0	0.726	0.394
Diabetes	33	89.2	4	10.8	43	86.0	7	14.0	0.196	0.658
Hyperlipidemia	32	86.5	5	13.5	40	80.0	10	20.0	0.627	0.428
Asthma-COPD*	33	89.2	4	10.8	41	82.0	9	18.0	0.865	0.352
Rational Drug Use Emergency	31	83.8	6	16.2	40	80.0	10	20.0	0.203	0.652
Contraception	21	56.8	16	43.2	15	30.0	35	70.0	6.276	**0.012**
Family Planning Methods	21	56.8	16	43.2	21	42.0	29	58.0	1.854	0.173
Sexually Transmissible Diseases	12	32.4	25	67.6	22	44.0	28	56.0	1.195	0.274
Suicide Risk	4	10.8	33	89.2	7	14.0	43	86.0	0.196	0.658
Osteoporosis	33	89.2	4	10.8	32	64.0	18	32.0	7.141	**0.008**

*Chronic Obstructive Pulmonary Disease, N: Number of respondents

(35%) and providing informative regular meetings and in-service training (33.3%). In addition, the following recommendations were given: not seeing pharmacies as a commercial establishment, paying hospital admissions in hospitals, reducing commercial concerns of pharmacists, reducing pharmaceutical procedures, introducing pharmacy, increasing professional unity, paying wages for counseling services, availability of a pharmacist in a pharmacy, communication of pharmacists with patients, compliance of pharmacists with the ethical rules, the inspection of internet products, improving pharmacists by themselves, increasing the authorities of pharmacists, establishing an official web site where pharmacists can benefit about counselling issues.

Several consultancy services were given about many public health issues in the pharmacies (Table 5). The main issues were related to hypertension (88.5%), diabetes (87.4%) and smoking cessation (86.2%). The issues in which the pharmacist thought that they had insufficient information, were suicide risk (37.9%), infectious diseases (29.9%) and substance addiction (28.7%).

The state of receiving counseling was compared with variables such as age, gender, occupational year, speciality, location of pharmacy, number of personnel and number of patients. According to the location of pharmacy; the pharmacists in the city center provided more counseling for physical activity (χ^2*=10.457, p=0.005*) and emergency contraception (χ^2*=10.009, p=0.007*), compared to the pharmacists in the countryside. Also, significant differences were found in some issues in which counseling was provided according to the pharmacist's gender. Accordingly, the female pharmacists provide more counseling about healthy nutrition, emergency contraception and osteoporosis than the male pharmacists, whereas, the male pharmacists provide more counseling about alcohol cessation compared to the female pharmacists (Table 6).

The male pharmacists thought that they had more adequate information about smoking cessation (χ^2 *=6.806, p=0.009*), alcohol cessation (χ^2 *=4.252, p=0.039*), drug addiction (χ^2 *=4.381, p=0.036*) and rational drug use (χ^2 *=4.232, p=0.040*) than the female pharmacists.

DISCUSSION

When mean numbers of personnel working in a pharmacy are compared between European countries, the pharmacies in Turkey have the lowest mean number of employees as 3 while the pharmacies in Denmark have the highest mean number of employees as 14 (Turkish Pharmacists Association, 2018). Similarly, more than half of the pharmacists enrolled in our study had 2 or more personnel.

More than three quarters of the pharmacists use the Internet effectively (75.9%). In contrast to our study, a study performed in the United Kingdom in 2017 identified the reluctancy of the pharmacists to use technology and social media as one of the obstacles in public health (Agomo, Portlock, & Ogunleye, 2017).

More than one-fifth of the pharmacists thought that they did not have an active role in the protection of public health due to the health system, authority limitation, insufficient time and high workload. In a study conducted on pharmacists, similar results to our findings were obtained, such as the inability to allocate adequate time to patients/inability to give satisfactory answers to patients, providing a consultancy service for the Social Security Institution, frustration, lack of time for self-improvement (Gülpınar, Uzun, & Yalım, 2015). In another study

conducted in the United Kingdom, the health system and time limitation were defined as an obstacle for pharmacists to carry out public health roles (Agomo et al., 2017). More than three quarters of the beneficiaries of pharmacy services have low education and income levels. The level of education and health literacy are related concepts (Balcık, Taskaya, & Sahin, 2014). This result shows that the counseling service is necessary on its own and pharmacies are a good opportunity for counseling.

Although the occupational group was asked, the third occupation group which was consulted on health issues was determined as neighbour, friend, television, internet and herbalist by 30% of the pharmacists. In a study, the rate of using traditional medical methods was found to be 65.8% (Oral, Ozturk, Balcı, & Sevinc, 2016). This result shows that traditional practices still play an important role in health. The most important factors affecting the counseling situation were shame, education, and gender. This situation can be explained by the cultural characteristics of Turkish society except the lack of education.

As is the case in different areas, especially in the field of health, the internet which is used effectively and widely can cause undesired results when not being used carefully (Gorkemli, 2017). Unhelpful or wrong advices found on the Internet and social media may lead to important health problems (Prasad, 2013). The fact that the clients came to the pharmacy with wrong information showed that they did not use the internet resources rationally.

The pharmacists thought that their professional reputation should be increased. Similarly, the pharmacists thought that their professional reputation decreased in another study conducted with the pharmacists (Gülpınar et al., 2015).

The role of community pharmacists as an active contributor to public health in addition to their traditional role in medication is recognized around the World (Eades et al., 2011). Although it is officially limited, pharmacists consult on numerous public health issues such as hypertension, diabetes, smoking cessation, hyperlipidemia, asthma- Chronic Obstructive Pulmonary Disease (COPD) and weight control. While, the counseling for alcohol abuse, substance abuse and suicide risk were least provided. In contrast to our study, it was found that a small number of pharmacies provided counseling services on issues, such as smoking cessation, weight management, hypertension screening, diabetes, dyslipidemia in a study conducted in Ethiopia. The counseling for screening of infectious diseases, sexually transmitted diseases, emergency contraception methods and alcohol dependence were most provided (Erku & Mersha, 2017). This result shows that each country has different public health problems and needs for counseling.

The subjects in which the pharmacists thought that they had insufficient knowledge were suicide risk, communicable diseases and substance abuse. Professional development is not compulsory for pharmacists in Turkey. In order to meet the educational needs systematically, professional development should be made compulsory.

The fact that the pharmacists in the city center provided more counseling for physical activity than the pharmacists in the countryside might be associated with the fact that people in cities lead a more sedentary life and thus, need physical activities more. On the other hand, the fact that counseling for emergency contraceptive methods was provided more in the city center might be associated with the fact that urban women want fewer children due to reasons, such as being more aware of the presence of emergency contraceptive methods, greater participation in the labor force and higher educational level. The female pharmacists mainly provided counseling for issues concerning women and affecting their health, such as healthy nutrition, emergency contraception and osteoporosis. On the other hand, the male pharmacists mainly provided counseling for alcohol cessation, which is encountered in men. According to these results, it is possible to conclude that the counselees consult with fellow pharmacists for some issues.

Keeping pharmacists away from bureaucratic obstacles in Turkey, improving their professional reputation, preventing pharmacies from being perceived as trading houses, providing financial support to pharmacies' public health services as in the United States and fulfilling their training needs systematically, are important. According to these findings, it can be suggested for the Turkish Union of Pharmacists to conduct studies in order to determine problems faced by pharmacies across Turkey and to lead in establishing a system for continuous training of pharmacists. Public health services which are provided voluntarily in pharmacies in Turkey should be made formal via laws.

CONCLUSION

In conclusion, there were no studies within this scope in Turkey before this study. In this study, the current situation of the pharmacists in Turkey and their problems in terms of public health were identified. Although pharmacists in Turkey face professional problems (such as bureaucratic obstacles, work load, limitation of authority), it is possible to state that they usually play a voluntarily active role in protecting and developing public health. There is a need for more scientific studies in order to make the active participation of pharmacists in public health seem visible and to enhance it.

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Short Paper

Determination of chlorpheniramine enantiomers in pharmaceutical formulations by HPLC on chiral column with PDA detection

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ABSTRACT

Background and Aims: An HPLC method with photodiode array detector on a chiral column was proposed for enantioselective determination of chlorpheniramine (CLP) enantiomers in dosage forms.
Methods: The enantioselective determination was achieved on amylose tris(3,5-dimethylphenylcarbamate) column, using n-hexane-(propan-2-ol)-diethylamine (97.5:2.5:0.025, v/v/v) mobile phase. The peaks were detected at 258 nm. Diphenhydramine was used as an internal standard (IS). A new sample preparation procedure was developed to avoid the interference of the other ingredients present in the formulations.
Results: Limit of quantification of the proposed method was 0.88 and 1.31 µg/mL for S-(+)-CLP and R-(-)-CLP, respectively.
Conclusion: The method is linear, sensitive, specific and can be used for the enantioselective assay of CLP enantiomers in pharmaceutical formulations.

Keywords: Chlorpheniramine, amylose tris(3,5-dimethyl phenylcarbamate), enantioselective determination, HPLC-PDA, chirality

INTRODUCTION

Stereochemistry of drugs is an important topic for the pharmaceutical industry and the regulatory authorities because enantiomers of chiral drugs may exhibit different biological activities with only one of the enantiomers exhibiting therapeutic value while the others are less effective or toxic. There has been increasing interest in the stereospecific analysis of chiral drug molecules (Calcaterra & D'Acquarica 2018).

Chlorpheniramine (CLP), 3-(4-chlorophenyl)-*N,N*-dimethyl-3-(pyridin-2-yl)propan-1-amine, is a first generation histamine H1 receptor antagonist (Figure 1).

CLP is mostly marketed as a racemate, only a few dosage forms are available as a single S-(+)-CLP. In tissues, the S-(+) enantiomer of chlorpheniramine (dexchlorpheniramine) has a 13-fold greater affinity than its R-(-) enantiomer to H1 receptors (Tanda, Kopajtic,

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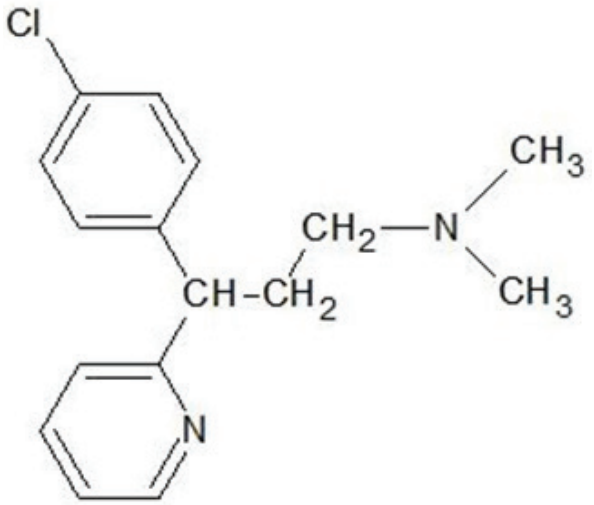

Figure 1. Chemical structure of chlorpheniramine.

& Katz, 2008). S-(+)-CLP has antihistaminic activity and R-(-)-CLP exhibits sedative side effects (Tagawa et al., 2002).

Studies on the enantioselective determination of rac-CLP have been limited. Studies for stereoselective determination have used β-cyclodextrin chiral stationary phase with mass spectrometric detection (Fried, Young, Yasuda, & Wainer, 2001), coupled achiral (cyanopropyl)–chiral (Amylose) stationary phase with UV detection (Hiep, Khanh, Hung, Thuillier, & Gimenez, 1998), or ODS column with β-cyclodextrin as a mobile phase additive (Chen, Jeong, Hwang, Kim, & Kang, 2008).

Here, we describe an enantioselective determination of CLP enantiomers in syrup containing a high dose of paracetamol by HPLC on an amylose column with PDA detection. The proposed novel method was validated according to ICH guidelines in terms of precision, linearity and accuracy.

MATERIALS AND METHODS

Instrumentation

The HPLC system consisted of LC-20AT liquid system (Shimadzu Corporation Analytical System) equipped with a degasser (DGU-20A5R), photodiode array detector (SPD-M20A PDA) and a rheodyne syringe sample injector (50 µL). The enantioselective separation of S-(+) and R-(-) enantiomers was performed on a Chiralpak AD-H column (250 mm x 4.6 mm i.d.) with 5 µm particle size (Daicel).

The mobile phase consisted of a mixture of n-hexane-(propan-2-ol, IPA)-diethylamine (DEA) (97.5:2.5:0.025, v/v/v). Flow rate was 1.2 mL/min at isocratic mode. The eluents were monitored at 258 nm. All analyses were performed at 25°C.

Chemicals

Racemic chlorpheniramine maleate (rac-CLP-M) and diphenhydramine hydrochloride (DPH) were kindly supplied by Bilim İlaç A.Ş. (Istanbul, Turkey). S-(+)CLP-M was obtained from Kiwidrug (New Zealand). n-Hexane (HPLC grade), propan-2-ol (HPLC grade), were purchased from Merck (Darmstadt, Germany). Diethylamine (DEA) was obtained from Fluka (Switzerland).

Preparation of stock solutions

The stock solutions of rac-CLP-M (400 µg/mL, calculated as free base) and DPH (IS) (1 mg/mL) were prepared in distilled water. Quality control samples containing 2, 4, 6, 8, and 10 µg/mL of corresponding enantiomers of CLP were prepared by diluting the rac-CLP-M stock solution with distilled water. All solutions were stored at 4°C.

Sample preparation procedure

1 mL aliquots of quality control samples, 25 µL of IS and 100 µL of 0.1 M NaOH solutions were placed in a 15 mL conical glass centrifuge tube. The samples were extracted with 1.5 mL of n-hexane-dichloromethane (2:1 v/v) by vortex-mixing for 2 min and centrifuged at 2500 rpm for 10 minutes. 1 mL of the organic phase was separated and evaporated to dryness under a gentle stream of nitrogen and reconstituted in 300 µL of mobile phase. A 50 µL aliquot of the solution was injected into the chromatographic system.

Assay of pharmaceutical dosage forms

1 mL aliquot of commercial syrup (160 mg paracetamol and 1 mg rac-CLP-M in 5 mL of syrup) was diluted to 10 mL with distilled water, sonicated and 1 mL aliquot of the solution was used for analysis according to the sample preparation procedure. The concentration of CLP enantiomers in syrup was calculated using the regression equation (n=6).

Method validation

The developed method was validated according to ICH guidelines (ICH Guideline, Q2(R1), 2005). Calibration lines were constructed by plotting the peak area ratio (PAR) against the corresponding concentration of enantiomers. Limit of quantification (LOQ) and limit of detection (LOD) were determined using 10 σ/s and 3.3 σ/s, respectively. Intra- and interday precision was determined by performing four consecutive injections at three concentration levels (4, 6, 8 µg/mL) of S-(+)- and R-(-)-CLP enantiomers. Accuracy of the method was determined by adding standard CLP solutions at 8.5 µg/mL and 5.5 µg/mL levels to syrup samples.

RESULTS AND DISCUSSION

Method development

Effects of modifier and solvent composition on retention and separation of enantiomers were investigated. n-Hexane-IPA-DEA (97.5:2.5:0.025, v/v/v) provided the best results for the separation of IS and CLP enantiomers.

A representative chromatogram of CLP enantiomers under optimum conditions is shown in Figure 2. Baseline separation was achieved for enantiomers with α value of 1.24. Peak resolution value was 3.80. S-(+)-CLP was used to identify the peaks of the CLP enantiomers.

The total run time of the analysis was 15 min. The average retention time and standard deviation of ten replicates were 9.63±0.05 min and 11.36±0.08 min for S-(+) and R-(-) enantiomers, respectively**.** No interfering peaks were observed at the same retention times of IS, S-(+)-CLP and R-(-)-CLP, and confirmed the specificity of the developed method.

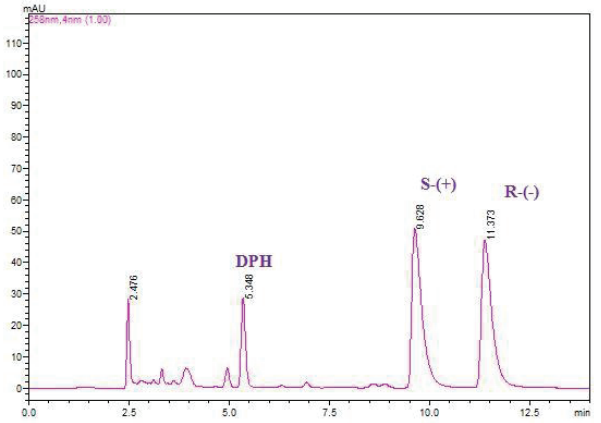

Figure 2. Representative chromatogram of CLP enantiomers and DPH (IS) on amylose tris(3,5-dimethyl phenylcarbamate) column. Conditions: mobile phase: n-Hexane-IPA-DEA (97.5:2.5:0.025 v/v/v) at 1.2 mL/min flow rate; detection: 258 nm.

Assay validation

The method was found linear within the range 2-10 µg/mL with a correlation coefficient (r) of 0.999 for both S-(+)- and R-(-)-CLP enantiomers. The regression equations were found to be y=0.4175(±0.17)x-0.017(±0.007) and y=0.4195(±0.17) x-0.011(±0.005) for S-(+)- and R-(-)-CLP, respectively.

The limit of detection (LOD) and limit of quantification (LOQ) of the CLP enantiomers were determined using calibration standards. LOD of the proposed method was 0.29 and 0.44 µg/mL for S-(+) and R-(-)-CLP, respectively. LOQ of the proposed method was 0.88 and 1.31 µg/mL for S-(+) and R-(-)-CLP, respectively.

Intraday and interday precision values of the method were determined by analysing the samples on the same day and on three different days at three different concentrations for each analyte (n=4). Precision of the method was expressed by relative standard deviation (RSD %). Interday precision values were found in the range of (RSD %) 0.24-0.61 and 1.28-1.40 for S(+) and R(-) enantiomers, respectively. Intraday precision values were found in the range of (RSD %) 0.25-1.40 and 1.34-1.50 for S-(+) and R-(-) enantiomers, respectively.

The accuracy of the method was evaluated by spiking the syrup formulation with standard rac-CLP –M solution. The mean percent recovery (RSD %) values were found as 99.41 (0.04) and 99.64 (0.04) at 8.5 µg/mL level for S-(+) and R-(-) enantiomers, respectively. RSD % values were found as 99.64 (0.06) and 101.82 (0.02) at 5.5 µg/mL level for S-(+) and R-(-) enantiomers, respectively.

Analysis of commercial syrup

CLP enantiomers in two dosage forms were analysed according to the validated method. Analyzed commercial syrups contain high doses of paracetamol (160 mg/5 mL) compared to rac-CLP-M (1 mg/5 mL). Paracetamol is insoluble in nonpolar- and chlorohydrocarbons (Granberg & Rasmuson, 1999). Different extraction solvents were tested. Using the n-Hexane-dichloromethane (2:1) mixture allowed selective extraction of CLP enantiomers from syrup without any interference of ingredients in the formulation.

The content of S-(+)-CLP and R-(-)-CLP enantiomers in the syrup were found as (mean % ±SD) 99.2±0.09 and 97.8±0.07 for batch 1; 98.2±0.08 and 98.0±0.09 for batch 2 respectively.

CONCLUSION

In this study, we propose a novel, simple, and rapid chiral HPLC method for the determination of CLP enantiomers in formulations containing high concentration of paracetamol. In this novel extraction procedure, the use of the non-polar solvent system n-hexane-dichloromethane (2:1) provides selectivity, and the use of diphenhydramine as an internal standard allows sensitive, precise and linear enantiomer determination.

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