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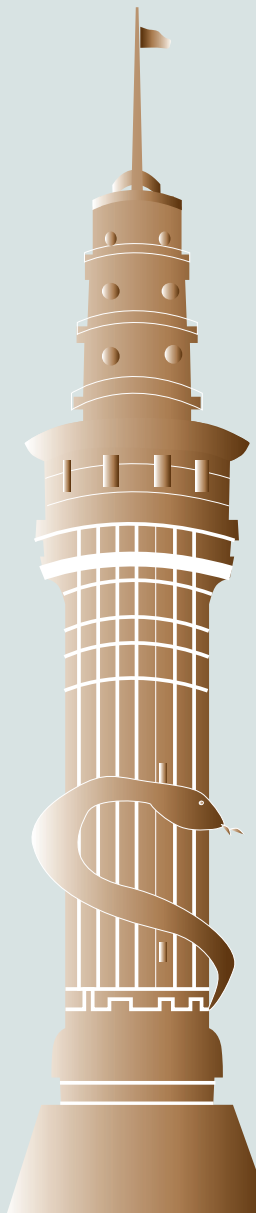
İstanbul Journal of Pharmacy



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İstanbul Journal of Pharmacy

INDEXING AND ABSTRACTING

Web of Science - Emerging Sources Citation Index (ESCI)

TÜBİTAK-ULAKBİM TR Dizin

CAS Source Index

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İstanbul Journal of Pharmacy

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PUBLISHER

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Publication Type: Periodical

İstanbul Journal of Pharmacy

EDITORIAL MANAGEMENT BOARD

İstanbul Journal of Pharmacy

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İstanbul Journal of Pharmacy

INSTRUCTIONS TO AUTHORS

AIMS AND SCOPE

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

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

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

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 (https://publicationethics.org/resources/guidelines-new/principles-transparency-and-best-practice-scholarly-publishing).

İstanbul Journal of Pharmacy is currently indexed in Web of Science-Emerging Sources Citation Index, TUBITAK ULAKBIM TR Index and CAS database.

Processing and publication are free of charge with the journal. No fees are requested from the authors at any point throughout the evaluation and publication process. All manuscripts must be submitted via the online submission system, which is available at http://dergipark.gov.tr/iujp. The journal guidelines, technical information, and the required forms are available on the journal's web page.

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

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İstanbul Journal of Pharmacy

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

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Editor-in-Chief is responsible for the contents and overall quality of the publication. He/She must publish errata pages or make corrections when needed.

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

Reviewers must have no conflict of interest with respect to the research, the authors and/or the research funders. Their judgments must be objective.

Reviewers must ensure that all the information related to submitted manuscripts is kept as confidential and must report to the editor if they are aware of copyright infringement and plagiarism on the author's side.

A reviewer who feels unqualified to review the topic of a manuscript or knows that its prompt review will be impossible should notify the editor and excuse himself from the review process.

The editor informs the reviewers that the manuscripts are confidential information and that this is a privileged interaction. The reviewers and editorial board cannot discuss the manuscripts with other persons. The anonymity of the referees must be ensured. In particular situations, the editor may share the review of one reviewer with other reviewers to clarify a particular point.

PEER REVIEW PROCESS

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Editor-in-Chief does not allow any conflicts of interest between the authors, editors and reviewers and is responsible for final decision for publication of the manuscripts in the Journal.

Reviewers' judgments must be objective. Reviewers' comments on the following aspects are expected while conducting the review.

- Does the manuscript contain new and significant information?
- Does the abstract clearly and accurately describe the content of the manuscript?
- Is the problem significant and concisely stated?
- Are the methods described comprehensively?
- Are the interpretations and consclusions justified by the results?
- Is adequate references made to other Works in the field?

- Is the language acceptable?

Reviewers must ensure that all the information related to submitted manuscripts is kept as confidential and must report to the editor if they are aware of copyright infringement and plagiarism on the author's side.

A reviewer who feels unqualified to review the topic of a manuscript or knows that its prompt review will be impossible should notify the editor and excuse himself from the review process.

The editor informs the reviewers that the manuscripts are confidential information and that this is a privileged interaction. The reviewers and editorial board cannot discuss the manuscripts with other persons. The anonymity of the referees is important.

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

All submissions are screened by a similarity detection software (iThenticate by CrossCheck) at any point during the peer-review or production process. Even if you are the author of the phrases or sentences, the text should not have unacceptable similarity with the previously published data.

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- Research participants should participate in the research voluntarily, not under any coercion.
- Any possible harm to participants must be avoided. The research should be planned in such a way that the participants are not at risk.
- The independence of research must be clear; and any conflict of interest or must be disclosed.
- In experimental studies with human subjects, written informed consent of the participants who decide to participate in the research must be obtained. In the case of children and those under wardship or with confirmed insanity, legal custodian's assent must be obtained.
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The manuscripts should be prepared in accordance with ICMJE-Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals (updated in December 2015 - http://www.icmje.org/icmje-recommendations.pdf). Authors are required to prepare manuscripts in accordance with the CONSORT guidelines for randomized research studies, STROBE guidelines for observational original research studies, STARD guidelines for studies on diagnostic accuracy, PRISMA guidelines for systematic reviews and meta-analysis, ARRIVE guidelines for experimental animal studies, and TREND guidelines for non-randomized public behavior.

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- Copyright Agreement Form
- Author Form
- Title Page

during the initial submission.

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- The full title of the manuscript as well as a short title (running head) of no more than 50 characters,
- Name(s), affiliations, and highest academic degree(s) and ORCID ID(s) of the author(s),
- Grant information and detailed information on the other sources of support,
- Name, address, telephone (including the mobile phone number) and fax numbers, and email address of the corresponding author,
- Acknowledgment of the individuals who contributed to the preparation of the manuscript but who do not fulfill the authorship criteria.

Abstract: An structured abstract should be submitted with Original Articles (Background and Aims, Methods, Results, Conclusion). Please check Table 1 below for word count specifications.

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

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

Statistical analysis to support conclusions is usually necessary. Statistical analyses must be conducted in accordance with international statistical reporting standards (Altman DG, Gore SM, Gardner MJ, Pocock SJ. Statistical guidelines for contributors to medical journals. Br Med J 1983: 7; 1489-93). Information on statistical analyses with specified statistical software and descriptive details of the chemical used should be provided with a separate subheading under the Materials and Methods section.

Units should be prepared in accordance with the International System of Units (SI).

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

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

Tables

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

Figures and Figure Legends

Figures, graphics, and photographs should be submitted as separate files (in TIFF or JPEG format) through the submission system. The files should not be embedded in a Word document or the main document. When there are figure subunits, the subunits should not be merged to form a single image. Each subunit should be submitted separately through the submission system. Images should not be labeled (a, b, c, etc.) to indicate figure subunits. Thick and thin arrows, arrowheads, stars, asterisks, and similar marks can be used on the images to support figure legends. Like the rest of the submission, the figures too should be blind. Any information within the images that may indicate an individual or institution should be blinded. The minimum resolution of each submitted figure should be 300 DPI. To prevent delays in the evaluation process, all submitted figures should be clear in resolution and large in size (minimum dimensions: 100 × 100 mm). Figure legends should be listed at the end of the main document.

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

Table 1. Limitations for each manuscript type

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

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

For plant materials, herbarium name (or acronym), number, name and surname of the person who identified the plant materials should be indicated in the Materials and Methods section of the manuscript.

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

Reference Style and Format

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)

Citations with more than six authors;

(Çavdar et al., 2003)

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]. *İstanbul 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

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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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Evaluation of disease and medication knowledge levels of diabetic patients

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ABSTRACT

Background and Aims: Diabetes mellitus is a public health problem with a significant economic burden to society. Patient attitude is a significant determinant of adherence and clinical outcomes. We aimed to evaluate the disease and medication knowledge level of diabetic patients and possible patient-related predictive factors.

Methods: Adult diabetic patients were included in the study. Patients' demographic and health-related data were collected using a pre-prepared form. Two questionnaires, KAP knowledge tool and Medication Assessment tool were used to assess the disease and medication knowledge levels of patients.

Results: The mean age of 159 patients was 54.44±12.24 years. The disease and medication knowledge scores were high with an average of 13.9±1.74 and 5.26±0.53, respectively. Higher scores were linked to being male ($p=0.042$; $p=0.007$) and higher educational status ($p<0.001$; $p=0.006$). Lower scores were recorded in patients with comorbidities ($p=0.002$; $p<0.001$), older patients ($p<0.001$), longer disease duration ($p<0.001$; $p=0.009$), longer antidiabetic drug use ($p<0.001$; $p=0.009$) and using more drugs ($p=0.002$; $p=0.006$). None of the patients could mention any possible side effects of their medications.

Conclusion: It can be deduced that patient-related factors are significant predictors of patients' disease and medication knowledge. Patient-specific education in addition to rational pharmacological intervention is necessary to achieve better clinical outcomes.

Keywords: Diabetes mellitus, disease knowledge, medication knowledge, patient factors

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INTRODUCTION

Diabetes mellitus is a chronic disease with a high increase in prevalence worldwide. The population of diabetes patients is estimated to reach 578 and 700 million by 2030 and 2045, respectively (Saeedi et al., 2019). The Turkish Diabetes Epidemiology Project (TURDEP) recorded a rise in diabetes prevalence from 7.2% in 1998 to 13.7% in 2010 (Satman et al., 2013). In 2019, Turkey had the highest age-adjusted comparative prevalence (11.1%) of diabetes in the European region and diabetes-related expenditure accounts for 23.8% of the total health expenditure (IDF, 2019). The social and economic burden of the disease cannot be undermined as Turkey is projected to have 10.4 million patients by 2045 (IDF, 2019; Saeedi et al., 2019).

The increase in prevalence is related to the ageing society, urbanization and sedentary lifestyle (Satman et al., 2013). Disease risk can be reduced by taking appropriate precautions and modifying causative factors. Patient education and compliance to treatment are very crucial in providing metabolic control, preventing complications, and improving quality of life.

Several chronic diseases increase the risk of diabetes and vice versa. Hypertension is one of the most common comorbidities present in diabetic patients. Accompanying comorbidities in diabetic patients increase the risk and progression of both macrovascular and microvascular complications of diabetes (American Diabetes Association, 2022). Therefore, educating patients, on the risk of new onset of certain diseases, preventive measures, present comorbidities, and compliance with better management measures, is essential.

One of the main patient factors affecting therapeutic outcomes is adherence. The World Health Organisation defines adherence as "the extent to which a person's behaviour, taking medication, following a diet, and/or executing lifestyle changes, corresponds with agreed recommendations from a health care provider", and it is influenced by multiple factors (Brown and Bussell 2011; Gast and Mathes 2019; WHO, 2003). One of the factors that increases patient adherence, perhaps the most important, is the patient's level of knowledge about the disease and treatment (Albright, Parchman, and Burge 2001). Also, disease knowledge has been shown to affect patient quality of life, and depressive risk has been associated with a low knowledge level (Akin, 2013).

Detailed education on diabetes including probable risks and necessary lifestyle changes is indispensable. Self-care activities such as healthy eating, physical activity, glycemic index calculation, blood glucose monitoring and many more are essential. Patient monitoring by health care providers to assess whether the given information is well understood and applied appropriately is mandatory. Furthermore, updating the knowledge of the diabetic patient at regular intervals and promoting self-care skills are of vital importance in terms of adaptation to the disease (Shrivastava, Shrivastava, and Ramasamy, 2013). Community pharmacists may have an important role to play in this situation as they dispense medications to patients and are the most easily accessible healthcare provider. Their involvement in the care process has been shown to lead to better clinical outcomes and be of economic benefit (Abdulrhim, Sankaralingam, Ibrahim & Awaisu, 2020; Milosavljevic, Aspden, and Harrison 2018; Newman et al., 2020).

Predictive factors can give a better insight into patients' needs from which patient-specific education can be established. This study aimed to evaluate the disease and medication knowledge level of diabetic patients and determine possible predictive factors of patients' knowledge levels.

MATERIALS AND METHODS

This single-centre prospective study was carried out between May-June 2022 in a community pharmacy. Adult diabetic patients using at least one prescribed antidiabetic medication who gave consent were included in the study. Patient demographic and health-related data were collected using a pre-prepared form by a 5th-year pharmacy student. Two questionnaires, 'KAP knowledge questionnaire' (Akin 2013) and 'Medication knowledge evaluation tool' (Okuyan, Sancar, & Izzettin 2013) were used to measure patients' disease and medication knowledge respectively.

The KAP knowledge questionnaire consists of 18 disease-related questions with multiple answer options of which only one is correct. Patients were asked to give the most suitable answer for each question. All correctly answered questions are scored '1', and wrong ones '0', and a total score was obtained for each patient. The Medication knowledge evaluation tool consists of seven open-ended questions. The questions were answered for all dosage forms used by each patient and an average was obtained for each patient. All patients' responses were recorded, and a point was given for each correct response to each question. An additional point was granted to the patients who could state the exact mechanism of action of their medication. The average score of each patient was recorded as their medication knowledge score.

SPSS Version 25.0 was used for statistical analysis. The Kolmogorov-Smirnov test was performed to determine whether the data were parametric. Continuous variables are expressed as mean ± standard deviation; ordinal and nominal data are expressed as n (%). Pearson's correlation analysis was used to analyse the relationship between continuous variables. Mann Whitney U Test was used to determine whether there was a difference between two parametric parameter variables, and Kruskal Wallis-H test was used to determine whether there was a difference between non-parametric variables with multiple subgroups. A p-value < 0.05 within a confidence interval of 95% was considered significant.

This study was approved by Istanbul Medipol University Ethics Committee with decision No:387 (E-10840098-772.02-2721).

RESULTS

A total of 159 patients with an average age of 54,44 participated in the study. The gender distribution of participants was equal (50.1 % female and 49.9% male). The average duration of diabetes disease and duration of treatment were 11.43 years and 11.2 years, respectively. Patients' health and demographic details are given in Table 1. All patients were overweight or

Table 1. Patients' health and demographic data.

Parameters	Mean±SD (range)	n	%
Age (years)	54,44±12,24 (26-83)		
Sex			
Female		81	50,9
Male		78	49,1
Body mass index	29,04±1,30 (26.29-33.59)		
<30		124	78
>30		35	22
Duration of diabetes disease	11,43±8,14 (1-34)		
Duration of antidiabetic drug use	11,2±8,11 (1-34)		
Frequency of daily blood glucose measurement	4,32±1,42 (1-6)		
Patients with comorbidities		91	57,2
Number of comorbidities	0.86±0.9 (0-4)		
Types of comorbid diseases			
Hypertension		64	54.2
Hyperlipidemia		37	31.4
Hypothyroidizm		16	13.6
Heart failure		1	0.8
Educational status			
Primary		24	15,1
Middle school		67	42,1
High school		45	28,3
University/ Post-graduate		23	14,5
Routine doctor visits - Once every			
3 months		19	11,9
6 months		79	49,7
12 months		61	38,4
Dosage forms used	2.59±1.09 (1-5)		
Active agents used	3.09±1.19 (1-6)		
Antidiabetic dosage forms used	1,76±0.76 (1-4)		
Antidiabetic active agents used	2.24±0.96 (1-4)		

obese, and comorbidities were present in 57.2% of the patients. The most common comorbidity was hypertension reported by 64 patients and all patients reported using at least one medication for their comorbidity. A total of 412 drug preparations containing 491 active agents were used by patients. The maximum number of drug preparations used was five, and the maximum number of active agents was six reported by eight and three patients, respectively. The majority (n=116, 73%) of patients were using combination therapy for their diabetes. All patients reported measuring their blood glucose at least once a day with an average of 4.3 measurements/day. Only 19 patients reported visiting their physician at 3-month intervals. The educational status of patients was averagely low with only 14.5% of the patients having a university degree.

Age positively correlates to disease duration ($p<0.001$) and the number of drugs used ($p<0.001$). The frequency of daily glucose management negatively correlates to age ($p=0.021$) and disease duration ($p=0.001$), but positively correlates to the number of antidiabetic medications used ($p=0.025$). There was a positive correlation between the number of drugs used and disease duration ($p=0.001$) and the number of antidiabetic medications used ($p<0.001$).

Disease knowledge scores

The average disease knowledge score was 13.9 ± 1.74. The minimum score was 7 obtained by one patient, and the maximum score was 17 obtained by 4 patients (Table 2). All patients were able to correctly answer four questions which include "The most accurate method of monitoring diabetes is..."; "The important factors that help in controlling blood sugar are…."; "Treatment of diabetes comprises....."; and "For proper foot care, a diabetic patient.... ". On the contrary, the questions "In a diabetic patient, high blood pressure can increase or worsen...." and " A diabetic patient should measure his or her blood pressure...." were only answered correctly by five and 32 patients, respectively.

Medication knowledge scores

A total of 412 questionnaires were completed. The distribution of patients' responses to the questions is given in Table 3. The general scores of patients were high. All patients expressed knowing how to use their drugs and when to take them, but none of them knew the possible side effects of their medications. The average score was 5.26 ± 0.53. The minimum score was 4 obtained by 5 patients, and the maximum score was 6 obtained by 47 patients.

Predictors of patients' disease and medication knowledge scores

There was a positive correlation between the disease and medication knowledge scores ($r=0.268$; $p=0.001$). The disease and medication knowledge scores were negatively correlated to age ($p<0.001$), disease duration ($p<0.001$; $p=0.009$); and the number of dosage forms used ($p=0.002$; $p=0.006$). While medication knowledge score was positively correlated to the frequency of blood glucose monitoring ($p<0.001$), there was no relevant correlation between knowledge scores and other patient parameters. Details are given in Table 4.

Table 2. Response to disease knowledge questions.

Questions	Patients who answered correctly n (%)	
Diabetes is a condition in which the body contains...	87	(54.7)
The major cause of diabetes is.....	95	(59.7)
The symptom(s) of diabetes is/are.....	76	(47.8)
Diabetes, if not treated.....	148	(93.1)
The most accurate method of monitoring diabetes is...	159	(100.0)
In a diabetic patient, high blood pressure can increase or worsen....	5	(3.1)
A diabetic patient should measure his or her blood pressure.....	32	(20.1)
The lifestyle modification(s) required for diabetic patients is/are.....	146	(91.8)
A diabetic patient should have his or her eyes checked....	101	(63.5)
The important factors that help in controlling blood sugar are	159	(100.0)
Treatment of diabetes comprises.....	159	(100.0)
Diabetes cannot be treated with.....	153	(96.2)
Upon control of diabetes, the medicines.....	159	(100.0)
How do you manage hypoglycemic symptoms.....?	149	(93.7)
Regular urine tests will help in knowing.....	136	(85.5)
A regular exercise regimen will help in.....	133	(83.6)
The well-balanced diet includes.....	154	(96.9)
For proper foot care, a diabetic patient.....	159	(100.0)

Table 3. Response to the Medication knowledge evaluation tool.

Questions	n	%
Can you list the names of all medications you are currently taking?	155	97.5
Can you tell me why you are taking this medication?	158	99.4
Do you know how to take your medicine?	159	100.0
Do you know when to take your medicine?	159	100.0
Do you know the possible side effects of your medicine?	0	0
Do you know what to do if your medication's side effects occur?	142	89.3
Do you know what to do if you miss a dose of your medicine?	108	67.9

Table 4. Correlation between knowledge scores and patient parameters.

	Disease knowledge score		Medication knowledge score	
Patient parameter	**r**	**p**	**r**	**p**
Age	-0.591	<0.001**	-0.314	<0.001**
Body mass index	0.146	0.066	0.02	0.803
Duration of diabetes disease	-0.509	<0.001**	-0.207	0.009**
Duration of antidiabetic drug use	-0.513	<0.001**	-0.206	0.009**
Daily glucose monitoring	0.146	0.066	0.293	<0.001**
Number of drugs used	-0.240	0.002**	-0.215	0.006**
Number of antidiabetic drug used	-0.01	0.898	0.054	0.498

Pearson's correlation, p<0.05 indicates statistical significance

Higher disease knowledge scores were recorded for male patients (p=0.042) and in more educated patients (p<0.001). Medication knowledge scores were positively correlated to being male (p=0.007), having higher education (p=0.006) and having fewer doctor visitations (p=0.036). The presence of co-morbidity was associated with lower disease (p=0.006) and medication knowledge (p<0.001). As seen in Table 5, the general knowledge of patients with only primary education was significantly low compared to the other groups, while only the medication knowledge scores of patients that visited the doctor every 3 months were significantly lower than the other two groups. There was a significant difference between these patient groups. The frequency of doctors' visits was associated with having more comorbidities (p<0.001) and medications (p<0.01).

DISCUSSION

Complete patient compliance is required in chronic diseases like diabetes to achieve disease control. Disease and medication knowledge of patients affect their adherence and attitude towards medications and self-care practices. Better glycaemic control is achieved in more knowledgeable patients (Bukhsh

Table 5. Association between knowledge scores and patient parameters.

Patient parameter		N	Disease knowledge score		Medication knowledge score	
			Median (IQR)	p	Median (IQR)	p
Sex[a]	Female	81	14 (2)	0.042*	5 (0.4)	0.007*
	Male	78	15 (2)		5 (1)	
Presence of comorbidities[a]	Yes	91	14 (3)	0.002*	5 (0)	<0.001*
	No	68	15 (2)		5 (1)	
Educational status[b]	Primary	24	11 (3)	<0.001*	5 (0)	0.006*
	Middle school	67	14 (2)		5 (0.6)	
	High school	45	15 (2)		5 (1)	
	University graduate	23	15 (1)		5 (1)	
Routine doctor visits - Once every[b]	3 months	19	14 (2)	0.078	5 (0.2)	0.036*
	6 months	79	14 (2)		5 (1)	
	12 months	61	15 (2)		5 (1)	

[a]Mann-Whitney Test; [b]Kruskal-Wallis Test; p<0.05 *indicates statistical significance

et al. 2019). In this study, the disease and medication awareness of patients and patient-related predictive factors were assessed. The participants were averagely young, which implies a high incidence of early-onset diabetes. Also, age was significantly correlated to the duration of disease, which implies even older patients were diagnosed at an early age. A similar age average was reported in previous studies (Bukhsh et al., 2019; Okuyan, et al., 2013; Quinton, Lewis, Ali, Morgan, & Bertelli, 2013). A significant increase in the incidence of diabetes and impaired glucose tolerance in the 20-40 age group was previously reported in the TURDEP-II Study (Satman et al., 2013), and this increase is attributed to the increase in the incidence of obesity which is a known risk factor for diabetes (American Diabetes Association, 2022). The body mass index of all the patients was above normal with an average of 29.04 kg/m^2, and 35 patients were obese. The presence of comorbid diseases was also high in the participants. Hypertension and hyperlipidaemia were the most recorded comorbidities. These two diseases are common among diabetic patients (American Diabetes Association 2022). The number of medications used increases with comorbidities. Although the average number of drugs used was relatively low, 66 patients were using at least three antidiabetic medications. The gender distribution of the study population was relatively equal as there were only three more female patients than males. Epidemiology studies in Turkey have shown a higher prevalence of diabetes among female patients (Satman et al., 2013).

The disease knowledge of our patients was relatively high as the scores of 105 patients were above average. This was reflected in patients' attitudes towards daily blood glucose monitoring. Similar results were reported in a recent study (Muhammad Haskani et al., 2022). Self-care activities have been associated with higher knowledge (Bukhsh et al., 2019). However, more frequent doctor visits were reported by less knowledgeable patients. These patients had more comorbid diseases and more medications. Unfortunately, most patients lacked knowledge of the risks of the comorbidities on their prognosis, which is an important issue. A multidisciplinary approach to patients especially when there are comorbidities is essential in the effective rational management of all diseases. Education must be personalised to encompass all aspects of a patient's particular needs.

Male patients had more disease knowledge than their female counterparts. Although some studies have found gender-related differences in disease knowledge (Bukhsh et al., 2019), Sweileh et al. reported that sex did not affect disease knowledge in their study (Sweileh et al., 2014). The knowledge level increased with educational status, as reported in other studies (Akin 2013; Al-Adsani, Moussa, Al-Jasem, Abdella, & Al-Hamad, 2009; Bukhsh et al., 2019; Guler and Oguz 2011). Correspondingly, educational status was shown to be a significant predictor of self-care practices (Bukhsh et al., 2018). The disease knowledge of patients significantly dropped with age. The decrease in knowledge level in older patients has been reported in other studies (Al-Adsani et al., 2009; Guler and Oguz 2011; He & Wharrad 2007). Correspondingly patients with longer disease duration had lower disease knowledge scores.

The average medication knowledge level of patients was relatively high in contrast to a recent study which used the same measurement tool as ours (Muhammad Haskani et al., 2022). Higher scores were associated with being male, absence of comorbidities, higher educational status, and more frequent doctor visits. We recorded lower scores in older patients, who have longer disease duration and use more medications. Okuyan et al. reported higher scores in female patients and younger patients (Okuyan et al., 2013). In their study, they calculated the medication knowledge score for a randomly picked drug for

each patient while we calculated the average score of medication knowledge of all drugs for each patient.

Although average medication knowledge was high, no patient could mention any possible side effects of their medications as similarly reported in another study (Muhammad Haskani et al., 2022). Medication-related side effects like hypoglycaemia are common with antidiabetic medication. These side effects may have detrimental consequences. Good communication between healthcare professionals and patients is important to reduce medication-related mortality and morbidity risk (American Diabetes Association, 2022). Patient awareness of self-manageable side effects and the side effects that need emergency attention are necessary. Patients must be educated on necessary self-management strategies.

Community pharmacists have an important role to play as the most easily accessible healthcare providers. Community pharmacist-led interventions have been shown to improve clinical outcomes in the management of diabetic patients (Korcegez, Sancar, & Demirkan, 2017). Involvement of pharmacies in patient education especially in medication-related issues will improve patient knowledge, clinical outcome, rational drug use and thus economic outcomes.

Study Limitations

The study was carried out in one-centre, which limited the number of patients and the socioeconomic diversity of patients. We also could not measure the effect of knowledge level on patient adherence and prognosis as the duration of the study was limited.

CONCLUSION

From this study, it can be deduced that patient-related factors are significant predictors of patients' disease and medication knowledge. Less knowledge was linked with older age, presence of comorbidities, number of drugs used, longer disease duration and antidiabetic drug use. Patient-specific education and training in addition to rational pharmacological intervention are necessary to achieve better clinical outcomes.

Informed Consent: Written consent was obtained from the participants.

Peer-review: Externally peer-reviewed.

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Original Article

Evaluation of vitamin D prescribing and consumption in Turkiye

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ABSTRACT

Background and Aims: Vitamin D plays several roles in keeping the body's cells healthy and functioning the way they should. Most people do not get enough Vitamin D, so supplements are common. However, it is also possible for this vitamin to build up and reach toxic levels in the body. This study investigated the prescription of Vitamin D and Analogues in primary care and outpatient consumption in Türkiye between the years 2015- 2018. Also, the number of protocols created with Hypervitaminosis D was evaluated.

Methods: In this study, drug consumption data were evaluated by Prescription Information System (PIS) and ATC/DDD (Anatomical, Therapeutic and Chem- Defined Daily Dose) Methodology. Descriptive statistics were used for evaluation of the data, and numerical values were presented via tables and graphics. Statistical analysis of the data was performed using the SPSS 23 (Statistical Package for the Social Sciences) package program. The Chi-square test was used to evaluate the relationship between the variables, and the P value below 0.05 was accepted as a statistical significance.

Results: Between 2015 and 2018, a total of 11.874.898 prescriptions containing drugs with the A11CC (Vitamin D and Analogues) ATC code were created by family physicians. The total number and the percentage of prescriptions containing Vitamin D and Analogues increased over these years. There was a statistically significant difference between the number of prescriptions. While the percentage of prescriptions containing Vitamin D and Analogues was 0.89 in 2015, it was 3.38 in 2018. It was determined that the percentage of prescriptions increased over these years. The consumption of the drugs with the A11CC ATC code in outpatients increased over these years according to the ATC/DDD methodology.

Conclusion: The study showed that the prescription of preparations containing Vitamin D by family physicians increased significantly over the years and there was an increase in Vitamin D consumption in outpatients. In addition, it was shown that the number of protocols established for the diagnosis of Hypervitaminosis D increased over the years in Türkiye.

Keywords: Drug, drug consumption, rational drug use, Vitamin D, prescribing

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INTRODUCTION

The World Health Organization (WHO) defined a drug as a substance used to examine or change physiological systems or pathological conditions for the benefit of the living creature (Kayaalp, 2005).

Like the consumption of drugs, the unconscious consumption and abuse of drugs are as old as civilization. Drug abuse is the use of drugs outside of medical indications or in a way that does not comply with the cultural and social structure of that society (Vural, 2005).

All drugs are open to unnecessary, inappropriate and unconscious consumption, and undesirable effects can be observed even at treatment doses. One of the drugs thought to be used unconsciously in Turkiye is Vitamin D. Vitamin D is an important vitamin for almost all systems of our body (Kayaalp, 2005).

Vitamin D regulates calcium and phosphorus metabolism through especially three target organs, namely, kidney, bone and small intestines, maintains the body's Ca and P balance, and also has important effects on our body (Kayaalp, 2005; Holick, 2007). Vitamin D is a fat-soluble vitamin and is transported to the tissue level by binding to the carrier protein in the blood (Kayaalp, 2005; Holick, 2007; Holick, 2017; Osteoporoz ve Metabolik Kemik Hastalıkları Tanı ve Tedavi *Kılavuzu,* 2022).

Awareness of using Vitamin D has increased in recent years. It's use has become popular with the influence of media such as the internet, social media, television, radio and newspaper. It should not be forgotten that Vitamin D is also a drug, it is stored in the body by dissolving in fat and its use in high doses can cause toxic effects. The awareness, knowledge and conscience level of health professionals and the public should be increased about these issues. Like all drugs, great attention should be paid to the pharmaceutical form, frequency and dose of Vitamin D.

The aim of this article is to evaluate the prescription and consumption of Vitamin D in Turkiye. The situation assessment was made by evaluating Vitamin D prescribing in primary health care institutions in Turkiye through the Prescription Information System (PIS) and examining Vitamin D consumption in outpatients according to the ATC/DDD Methodology (Anatomical Therapeutic Chemical /Defined Daily Dose). In addition, the number of protocols created with the diagnoses of "Hypervitaminosis D", "Poisoning with Vitamins" and "Side Effects Caused by Vitamins" in health institutions in Turkiye were evaluated.

In Turkiye, oral solution, injection solution, oral drops, ampoule, tablet and capsule formulations containing "Vitamin D and its Analogues" are available (TİTCK SKRS E-Reçete İlaç ve Diğer Farmasötik Ürünler Listesi, 2022).

Vitamin D levels that can be consumed daily according to the age group are stated on the official website of the Turkish Medicines and Medical Devices Agency (TMMDA).

Although there is no consensus on the optimal level of Vitamin D as a result of the studies, in most guidelines, the Vitamin D level is defined as:

• Deficiency if it is<10 ng/ml (25 nmol/L),

• Insufficiency if it is between 10 - 20 ng/ml (25-50 nmol/L),

• Sufficient if it is >20 ng/ml (50 nmol/L) (Holick, 2009; Manson, Brannon, & Rosen, 2016; Pilz et al., 2019; Altieri et al.; 2009; Sempos et al., 2018 Boullion & Carmet, 2018; Giustina et al., 2019; Lips et al., 2019; Dawson-Hughes, 2022).

According to the Turkish Society of Endocrinology and Metabolism Osteoporosis and Metabolic Bone Diseases Working Group, the Vitamin D level is:

• Insufficient if it is between 10-20 ng/ml (25-50 nmol/L),

• Deficient if it is <10 ng/ml (25 nmol/L),

• Sufficient for bone health if it is >20 ng/ml (50 nmol/L),

• Sufficient for its extra-bone effects if it is between 30-50 ng/ml (75-125 nmol/L) (TİTCK KÜB/KT Listesi 2022).

Vitamin D intoxication and hypervitaminosis D are different cases.

According to the related sources:

Serum 25-OH Vitamin D level:

• >100 ng/ml (250 nmol/L) is considered D hypervitaminosis• >150 ng/ml (375 nmol/L) is considered as Vitamin D intoxication (Giustina et al., 2019; Lips et al., 2019; Dawson-Hughes, 2022; Galior, Grebe & Singh, 2018; Osteoporoz ve Metabolik Kemik Hastalıkları Tanı ve Tedavi *Kılavuzu,* 2022).

According to the Endocrine Society serum 25-OH Vitamin D level >150 ng/L and calcium level >10.5 mg/dl are defined as Vitamin D intoxication (Dawson-Hughes, 2022).

With the increase in awareness about Vitamin D all over the world, the use of exogenous Vitamin D has increased, and there has been an increase in cases of hypervitaminosis D/Vitamin D intoxication (19-44). When the PIS data obtained from TMMDA are examined, it has been determined that there has been an increase in the cases of hypervitaminosis D in Turkiye over the years.

According to the the cases examined; The most common causes of irrational use of Vitamin D have been found to be:

• Vitamin D is not seen as a drug by the society,

• The perception that Vitamin D will not harm no matter what dose is used,

• Inappropriate doses of Vitamin D are usually given to the baby during infancy,

• Health professionals such as physicians, pharmacists, and nurses recommend to the parents that the baby should take

a high dose of Vitamin D, even though the baby is not diagnosed with Vitamin D deficiency and/or rickets,

• The parents want their babies to teethe early, accelerate bone development, walk early, and use inappropriate doses of Vitamin D on their babies, voluntarily, based on hearsay information,

• The desire of the elderly to consume high doses of Vitamin D without any recommendation,

• Consuming the ampoule form of Vitamin D although it is not needed,

• Consuming more ampoule forms than necessary,

• Oral consumption of the ampoule form,

• Long-term use of Vitamin D above the daily dose (Özkan, Hatun, & Bereket, 2012; Hossein-Nezhad & Holick, 2013; Urbańska, Łukaszkiewicz, Płudowski, & Jones, 2018; Ketha, Wadams, Lteif, & Singh, 2015; Belaidi et al., 2016; Garbim et al., 2017; Ertl at al., 2017; Scheelings, Slocombe, & Bayley, 2017; Sharma, Dutta, Sharma, & Gadpayle, 2017; Vincentis et al., 2021 De Paula et al., 2020; Bozacı, Avcı, Nalbant, Vasi, Soydan M., 2018; Balkan and Ünal, 2013; Yılmaz, Gül, Çakan, & Aydın, 2014; Uytun, Ertural, Baş, & Torun, 2014; Ünal et al., 2007; Taşkesen Gümüş & Katar, 2009; Yasar, Fırat, Özer, Özer, & Erdemir, 2011; Kara, Yıldırım Baş, & Öngel, 2012; Sarı, Bülbül, Benzer, Akçay, Özkul Sağlam, 2014; Türkmenoğlu et al., 2014; Uğur et al., 2016; Buluş, İnan, Demet, & Andıran, 2016; Aydın & Dokuzlar, 2018).

MATERIALS AND METHODS

Materials

In this study, the data was evaluated by both Prescription Information System (PIS) and ATC/DDD Methodology.

PIS is an electronic system managed by the Turkish Medicines and Medical Devices Agency of the Ministry of Health, which analyzes and evaluates the drugs prescribed by physicians, and enables them to follow up and inform the physicians about their own prescriptions

ATC/DDD Methodology is a drug classification system developed, managed and supported by WHO. The ATC/DDD is a unique methodology which allows the presentation and comparison of drug consumption statistics on national and international platforms. The differences such as amount, dose, duration, and population are eliminated with this methodology. The use of the ATC/DDD methodology in drug use research continues to become increasingly common around the world.

The ATC/DDD methodology is also an important comparison method used in drug consumption studies and is accepted worldwide. With this method, a defined daily dose is examined in proportion to the population, and thus, drug consumption comparisons can be made more concretely (Aksoy, Alkan, İşli, 2015; TİTCK Akılcı İlaç Kullanımı Web Sitesi 2022).

In this article, the consumption of Vitamin D and Analogues in outpatients in Turkiye was examined using the ATC/DDD methodology, which is a method recommended by WHO and where comparisons can be made between data in national and international fields. A comparison of the consumption of Vitamin D and Analogues between 2015 and 2018 was made by calculating the "defined daily dose (DID) per 1.000 people in a day".

A drug in the Vitamin D and Analogues group in the ATC classification system is in the main group A (Level 1) and has the code A11CC (WHO ATC/DD Index 2022/).

Defined daily dose (DDD) expresses the average daily maintenance dose assumed to be used in adults for the main indication of a drug in the ATC system. DDD is a statistical measure of drug consumption determined by WHO and is used to standardize the comparison of drug use in different drugs or in different health care settings TİTCK Akılcı İlaç Kullanımı 2022).

DDD is based on main indication, maintenance dose and route of administration. DDD can only be assigned to drugs with an ATC code. DDD is a unit of measurement and does not need to reflect the daily dose recommended or prescribed by the doctor (TİTCK Akılcı İlaç Kullanımı Web Sitesi 2022).

Doses required for patients or patient groups are often different from DDD and vary according to individual characteristics such as age, body weight, and pharmacokinetic characteristics. When calculating DDD, it is assumed that the average weight of an adult is 70 kg. Except for some cases, DDD calculation is not made for pediatric drugs. DDD is not given for immune serums, vaccines, general and local anesthetics, topical preparations, antineoplastic drugs, allergen extracts, and contrast agents. Medication consumption, given as DDD units, gives a rough estimate of the amount of medication actually consumed and does not reflect the actual amount of medication consumed. DDD is a fixed unit of measurement independent of price and formulation. It offers researchers the opportunity to identify and compare drug consumption trends. DDD amount = ["Number of boxes" x "Number of tablets in box" x "Tablet weight in grams"] / "DDD value of drug in grams". "Defined daily dose per package (DPP)" can be calculated by "Package Content" / DDD formula (TİTCK Akılcı İlaç Kullanımı Web Sitesi 2022).

Method

Data usage permission was obtained from TMMDA for this article. In Turkiye, the prescribing of Vitamin D in primary health care institutions through the Prescription Information System and the consumption of Vitamin D in outpatients according to the ATC/DDD Methodology were examined and a situation assessment was made. In addition, the number of protocols created with the diagnosis of Hypervitaminosis D, Poisoning with vitamins and side effects caused by vitamins in Turkiye were evaluated. All prescriptions created electronically by family physicians and registered in PIS for the years 2015-2018 were examined retrospectively. Prescriptions containing the A11CC (Vitamin D and Analogues) ATC code were also evaluated. In this context, the data were obtained separately for each year. This manuscript does not contain information about dietary supplements.

Statistical evaluation of the data was made and the data was presented with tables and figures.

Descriptive statistics were used for evaluation of the data, and numerical values were presented via tables and graphics. Statistical analysis of the data was performed using the SPSS 23 (Statistical Package for the Social Sciences) package program. The Chi-square test was used to evaluate the relationship between the variables and the P value below 0.05 was accepted as statistical significance.

RESULTS

Between 2015 and 2018, a total of 561.269.736 prescriptions were created by family physicians. It was determined that the total number of prescriptions created by family physicians increased over the years. There was a statistically significant difference between the number of prescriptions over the years ($p<0.05$).

Table 1. Number of prescriptions created by family physicians by years.

Year	Number of Prescriptions
2015	130.098.241
2016	134.235.120
2017	141.625.433
2018	155.310.942
Total	561.269.736
P	p<0.05
x^2	2.622.215.8

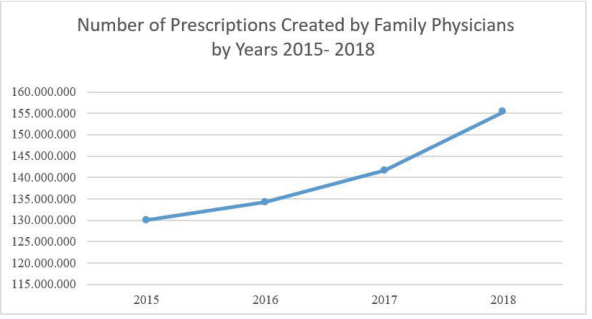

Figure 1. Number of Prescriptions Created by Family Physicians by Years.

Between 2015 and 2018, a total of 11.874.898 prescriptions containing drugs with the A11CC (Vitamin D and Analogues) ATC code were created by family physicians. It was determined that the total number of prescriptions containing A11CC (Vitamin D and Analogues) ATC-coded drugs created by family physicians increased over the years. It was seen that there was a statistically significant difference between the number of prescriptions over the years ($p<0.05$).

While the percentage of prescriptions containing A11CC (Vitamin D and Analogues) ATC-coded drugs created by family physicians was 0.89 in 2015, it was 3.38 in 2018. It was determined that the percentage of prescriptions increased over the years.

Table 2. Number of prescriptions containing the drugs with A11CC (vitamin D and analogues) ATC code generated by family physicians by years.

Year	Number of Prescriptions
2015	1.163.954
2016	2.309.867
2017	3.149.651
2018	5.251.426
Total	11.874.898
P	p<0.05
x^2	3.009.626

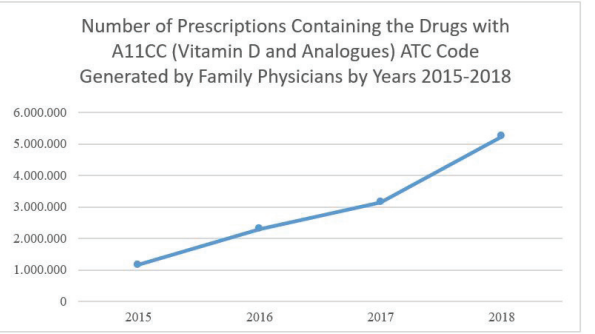

Figure 2. Number of Prescriptions Containing the Drugs with A11CC (Vitamin D and Analogues) ATC Code Generated by Family Physicians by Years.

The number of prescriptions containing A11CC ATC-coded medicines created by family physicians for the 0-2 age group between 2015 and 2018 constitutes only 5% of the total prescriptions.

Table 3. Percentage of prescriptions containing drugs with A11CC ATC code generated by family physicians by years.

Year	Percentage of Prescription
2015	0.89
2016	1.72
2017	2.22
2018	3.38

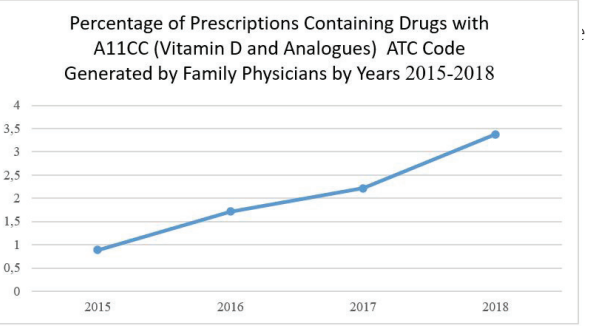

Figure 3. Percentage of Prescriptions Containing Drugs with A11CC ATC Code Generated by Family Physicians by Years.

A11CC (Vitamin D Analogues) ATC code in outpatients increased over the years according to the ATC/DDD methodology.

Table 4. Consumption of the drugs with A11CC ATC code in outpatients according to ATC/DDD methodology by years.

Year	DID
2015	120.74
2016	163.65
2017	169.88
2018	201.7

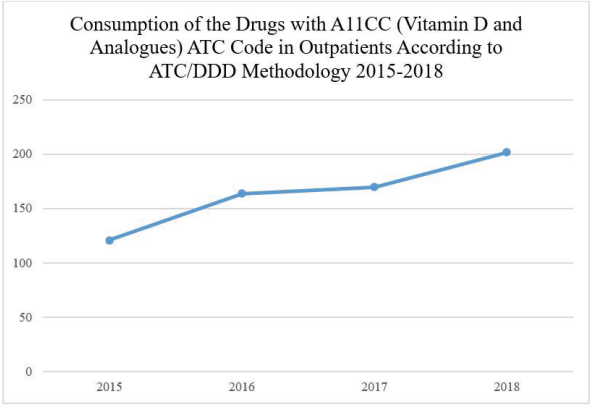

Figure 4. Percentage of Prescriptions Containing Drugs with A11CC ATC Code Generated by Family Physicians by Years.

Family physicians prescribed the drugs with the A11CC (Vitamin D and Analogues) ATC code for a total of 8.610.282 patients between 2015 and 2018. It was determined that there was an increase in the number of patients whose prescriptions contained the drugs with the A11CC ATC code over the years. It was found that the total number of patients increased over the years. It was seen that there was a statistically significant difference between the number of patients over the years ($p<0.05$).

Table 5. Number of patients prescribed containing A11CC ATC-coded drugs by family physicians by years.

Year	Number of patients
2015	816.854
2016	1.655.771
2017	2.303.903
2018	3.833.754
Total	8.610.282
P	p<0.05
x^2	2.267.162.8

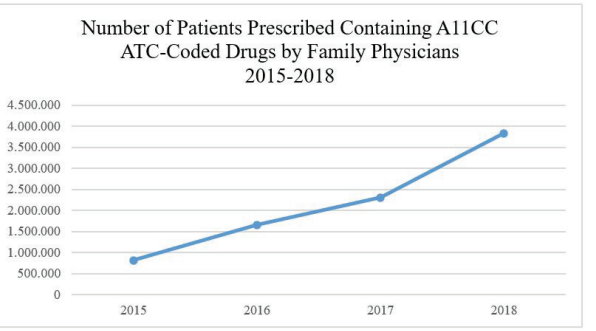

Figure 5. Number of Patients Prescribed Containing A11CC ATC-Coded Drugs by Family Physicians by Years.

A total of 4.209 protocols were created with the E67.3 ICD-10 diagnosis code (Hypervitaminosis D) between 2015 and 2018. It was seen that there was a statistically significant difference between the number of protocols over the years ($p<0.05$).

Table 6. Number of protocols created with E67.3 ICD-10 diagnosis code by years.

Year	Number of Protocols
2015	580
2016	524
2017	1.260
2018	1.665
Total	4.029
P	p<0.05
x^2	906.0221

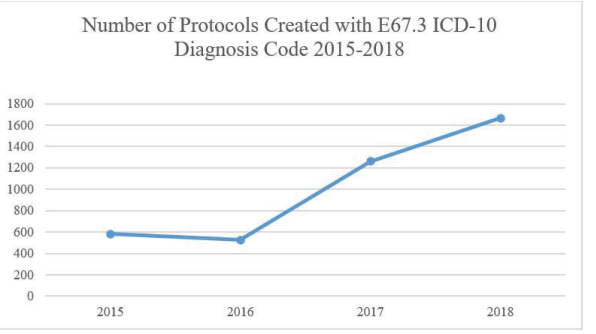

Figure 6. Number of Protocols Created with E67.3 ICD-10 Diagnosis Code by Years.

DISCUSSION

As a result of the data evaluation for the years 2015-2018 taken from TMMDA, it was determined that Vitamin D prescribing, consumption and cases of hypervitaminosis D increased significantly over the years in Turkiye. It can be concluded that hypervitaminosis D/Vitamin D intoxication develops as a result of false information and beliefs about Vitamin D insufficiency and its prevention.

According to the data evaluation of the years 2015-2018, it was determined that the total number of prescriptions and the number of prescriptions containing drugs with the A11CC ATC code created by family physicians increased significantly over the years. In addition, the percentage of prescriptions containing the drugs with the A11CC (Vitamin D and Analogues) ATC code has also displayed an increasing trend over the years. A11CC (Vitamin D and Its Analogues) ATC-coded Drugs were analyzed according to the ATC/DDD methodology, and it was determined that the consumption of Vitamin D in outpatients increased over the years.

In our study, it was determined that there was a significant increase in the number of patients who were prescribed A11CC (Vitamin D and Analogues) ATC-coded drugs by family physicians over the years.

One of the limitations of this study was that the prescription of the drugs containing Vitamin D and its Analogues was evaluated only among primary care physicians in this study. In addition, no evaluation was made about the usage of the prescribed drugs by the patients.

In order to prevent unconscious consumption of Vitamin D, there are some studies of TMMDA. "Dear Pharmacist's Letter", which is one of the documents sent directly to healthcare professionals by TMMDA and aims to ensure the safe and effective use of drugs, was published in 2016 and shared with relevant stakeholders. Information was given about the high number of cases of hypercalcemia and Vitamin D intoxication, especially in children, as a result of the selling without prescription and unconscious use of drugs containing high doses of Vitamin D in pharmacies. It was emphasized that maximum attention should be paid to the fact that drugs containing high doses of Vitamin D are sold by prescription in pharmacies (TİTCK Eczacı Bilgilendirme Mektubu, 2016).

In 2020, an announcement about the use of preparations containing Vitamin D was shared with the public by TMMDA. In this announcement, it was stated that the indications, posology, warnings and precautions sections in the Summary of Product Characteristics (SPC) and Instructions for Use (IU) of drugs containing Vitamin D should be revised. The term "oral" cannot be used in the names of parenteral products containing Vitamin D and in SPC-IU information. It was denoted that these products should be regulated as indicated only in patients with gastrointestinal malabsorption in the case of Vitamin D deficiency in the "indications" section of these parenteral products. For orally taken products containing Vitamin D, the indication information should be arranged to show that the product is indicated for the treatment, maintenance and prophylaxis of Vitamin D deficiency. It was indicated that the doctor has to decide how to use the drug and that the drug must be used according to the doctor's recommendation in the SPC-IU information of all oral and parenterally administered products containing Vitamin D (TITCK, D Vitamini Içeren Ilaçlar Hakkında Yazı, 2020).

CONCLUSION

In this study, a situation assessment was made by examining the prescribing of Vitamin D in primary health care institutions through the Prescription Information System (PIS) and the consumption of Vitamin D in outpatients using the ATC/DDD Methodology in Turkiye. The study showed that the prescription of preparations containing Vitamin D by family physicians increased significantly over the years and there was an increase in Vitamin D consumption in outpatients. In addition, it was shown that the number of protocols established for the diagnosis of Hypervitaminosis D, Vitamin Poisoning increased over the years in Turkiye. It is obvious that Vitamin D is a very important vitamin for our body, and its deficiency causes serious problems. The effect of Vitamin D on many systems such as the skeletal, muscular, cardiovascular and immune systems has become more known in recent years. Awareness of the benefits of Vitamin D in healthcare professionals and the general public is increasing. It is thought that the popularization of Vitamin D in social media, TV, radio and newspaper leads to an increase in the irrational use of irrational Vitamin D and its unconscious consumption among the public.

"Dear Pharmacist's Letter" published by TMMDA in 2016 and shared with the relevant stakeholders, plus the writing shared with the public in 2020 regarding the use of preparations containing Vitamin D reveal the importance given to the rational use of Vitamin D.

According to the prescribing and consumption data, it was seen that the number of patients receiving Vitamin D supplementation was increasing.

It is thought that providing training to health professionals and the public about the use of Vitamin D will reduce the irrational and unnecessary use of Vitamin D and the risk of intoxication. Training and awareness activities can be organized in order to increase awareness among health professionals and the public. Health professionals and the public should be aware that unconscious and high-dose Vitamin D consumption may cause Vitamin D intoxication, and Vitamin D should be used in case its deficiency is detected by laboratory methods.

In our society, vitamins are not generally accepted as medicine. Therefore, it may be difficult to obtain a history of high-dose Vitamin D consumption in some patients. Anamnesis should be taken carefully in patients with hypercalcemia. There is a perception that vitamins are not drugs, and therefore excessive consumption is harmless. For this reason, when taking the patient's anamnesis, it should also be questioned whether there is a use of Vitamin D in addition to drug use.

The most important step in preventing Vitamin D intoxication is not starting the treatment with high-dose Vitamin D without proving Vitamin D deficiency with laboratory results. It is thought that using the drop form instead of the ampoule form in patients with Vitamin D deficiency will reduce the risk of toxicity. Appropriate dose treatment should be planned for patients and patients taking Vitamin D supplements should be followed closely.

In case of any adverse reaction to the drug, it is necessary for health professionals and patients to inform the health authority about it. Persons in the risk group in terms of Vitamin

D deficiency/insufficiency should be tested and, if necessary, replacement should be made in line according to the test results. The person should be evaluated according to age, body weight, and additional diseases, and a treatment plan should be created accordingly. The physician should decide how to use the preparations containing Vitamin D. It should be used according to the doctor's recommendation and under the advice of a pharmacist. It is recommended that patients receiving long-term treatment with high-dose Vitamin D be informed of the symptoms of a possible overdose. People who use Vitamin D should be told to what they should pay attention.

It is thought that raising awareness among health professionals and the public will make a great contribution to the rational use of Vitamin D-containing preparations.

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Original Article

Comparing the effects of aromatherapy and mindfulness meditation on university students' stress levels

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ABSTRACT

Background and Aims: Aromatherapy is the combination of aroma and therapy and literally means treatment with fragrance. Massage, inhalation, compresses, and baths are the most common applications of aromatherapy. According to several studies, inhalation of essential oils greatly reduces anxiety and tension. On the other hand, the last 30 years has seen an increase in the popularity of mindfulness meditation for reducing the risk of depressive relapse while also lowering stress and anxiety levels. The aim of this study is to compare the effects of aromatherapy and mindfulness practices on university students' stress levels.

Methods: A total of 78 Altınbaş University students participated in this study, which uses the Beck Depression Inventory-II and Perceived Stress Scale to collect data. Five experimental groups were created in which aromatherapy and mindfulness meditation studies were applied, with a control group also used in an experimental investigation that includes pretest and posttest assessments. *Cedrus atlantica* (Endl.) G.Manetti ex Carrière (CA) and *Cananga odorata* (Lam.) Hook.f. & Thomson (CO) essential oils were used in the aromatherapy applications.

Results: As a result, this study has found the participants' anxiety levels to decrease, with all intervention groups having lower posttest scores than their pretest levels.

Conclusion: The results of this study show a decrease in post-intervention test scores for all experimental groups compared to the control group, particularly for the groups where aromatherapy and mindfulness meditation were administered together. Aromatherapy and mindfulness emerge as viable therapeutic options for anxiety, with CA and CO essential oils being particularly useful.

Keywords: Aromatherapy, Cananga odorata, Cedrus atlantica, essential oils, mindfulness meditation

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INTRODUCTION

Aromatherapy is a term that combines the phrases scent and therapy. Aroma as a term has a variety of meanings, such as sweet perfume and spice in Latin, as well as spice in Greek. Therapy comes from the Greek *therapeia*, which means cure/healing. Purists believe that the term aromatherapy should only be used to refer to the use of essential oils in ways that are consistent with its definition, such as inhalation as opposed to massage or other application techniques (Price & Price, 2011). Essential oil analysis has become increasingly popular in recent years. Essential oils are secondary metabolites generated by aromatic plants. They are volatile, natural, complex compounds with a strong odor (Lucchesi, Chemat, & Smadja, 2004; Shaw, 1979). The use of essential oils to promote relaxation has become commonplace, with the calming effects of lavender, cedarwood, and vetiver making them effective for treating anxiety and sadness. Aromatherapy is frequently used topically and during massage (Suyono et al., 2020).

Cedrus atlantica (cedarwood) essential oil includes hydrocarbons (sesquiterpenes such as β-himachalene at 30.8–40.4%, α-himachalene at 10.3–16.4%, and γ-himachalene at 6.7–9.7%), alcohols (sesquiterpenols such as epi-β-cubenol at 1.1–2.5%), and ketones (sesquiterpenones such as α-atlantone at 5.2–13.4% and γ-atlantone at 1.2–3.9%; Price & Price, 2011; Tisserand & Young, 2013). The antispasmodic, astringent, decongestant, insecticide, sedative, and soothing properties of cedarwood essential oil have all been utilized. According to Suyono et al (2020), rats exposed to stress from extended swimming had their plasma cortisol levels decreased using *Cedrus* essential oil balms. The amount of cortisol is reduced more effectively when a higher quantity of essential oils is applied. *Cedrus* essential oil's 30% concentration produces a diazepam-like action (Suyono, Jong, & Wijaya, 2020). One study investigated the effect of textile products produced with essential oils on stress . The 20 female participants reported feeling less stressed after using the aroma-therapeutic textiles created for their study. Their findings suggested that inhaling the lavender and cedarwood aroma-therapeutic textiles to have significantly reduced stress in the individuals compared to the control textile. A statistically significant reduction in skin conductance, respiration rate, and heart rate (PNS activity) was also observed when using the aroma-therapeutic textiles compared to the aroma-less control textile (Mehta & MacGillivray, 2016).

Cananga odorata (CO; ylang-ylang) essential oil includes hydrocarbons (sesquiterpenes such as β-caryophyllene at 38.2%, α-caryophyllene at 9.2%, germacrene D at 8.3%, and δ-cadinene at 6.0%) and alcohols (monoterpenols such as linalool at 5.6%). CO essential oil was also recorded to have antidiabetic, antiseptic, antispasmodic, balancing, calming, hypotensor, tonic, reproductive stimulant, and sedative properties (Price & Price, 2011; Tisserand & Young, 2013). According to Hongratanaworakit and Buchbauer , CO as an essential oil has calming effects and offers some support when used together with medicines to lower blood pressure or treat depression and stress in people(Hongratanaworakit & Buchbauer, 2006). Exposure to the smell of CO may be able to counteract the anxiety-inducing effects of piperazine (m-CPP). The extracellular signal-regulated kinase 1/2 (ERK1/2) and cAMP response element-binding protein (CREB) pathway in the hippocampus and the serotonin system were found to both be involved in the anxiolytic effects of CO essential oil (Zhang, Zhang, Feng, & Yao, 2018).

Mindfulness meditation refers to a subset of meditation techniques known as mindfulness meditation. Mindfulness meditations such as Zen/Chan and Vipassana are common (Jalali-Heravi, Parastar, & Sereshti, 2010). Dr. Kabat-Zinn introduced mindfulness-based stress reduction into psychotherapy in the late 1970s to assist patients in managing stress, pain, mood, and comfort in life (Kabat-Zinn, 1982). Individuals who are experiencing unpleasant emotions or difficult life circumstances such as stress, depression, anxiety, or fear can learn to stop so-called action mode (i.e., automatic, unconscious, habitual reaction mode) by using mindfulness treatment. Mindfulness-based stress reduction (MBSR) is well acknowledged to have a positive effect on young people's ability to regulate their emotions and has been attracting ever greater attention. Medical students and young children who participate in MBSR interventions can successfully lower their self-reported trait and status anxiety and improve their mental experience score measured at the end of an intervention (Shapiro, Schwartz, & Bonner, 1998). Mindfulness meditation entails establishing a unique mental characteristic known as mindfulness. When adopting an attitude of curiosity, openness, and acceptance, awareness then entails self-regulation of attention to conscious awareness of one's current experiences (Shapiro, Astin, Bishop, & Cordova, 2005). Zhou et al. (2020) chose 1,489 people from 14 studies for comparison with the controls, and their meta-analysis concluded MBSR to considerably reduced anxiety symptoms (Zhou et al., 2020).

Long-term stress is harmful to one's health. The hypothalamic-pituitary-adrenal axis is activated by stress, which raises the level of the corticosteroid hormone. Cortisol overproduction impairs metabolism, disturbs sleep, and suppresses the immune system. Exams, too many courses, and challenging living situations increase the stress levels of university students during their student years. A person experiences stress when they encounter a situation that is hard to adapt to. Stress is a state of physiological, mental, and physical stress and is an unusual physiological response that occurs in the body in response to imaginary or real harm and a variety of stimuli (Schneiderman, Ironson, & Siegel, 2005). Job stress has also been shown to emerge when an imbalance occurs between the demands of the workplace and the resources, abilities, or needs of employees (Chen et al., 2017).

MATERIALS AND METHODS

Essential oil material

CO and *Cedrus atlantica* (CA) essential oils have been widely recommended by pharmacists and used through inhalation to reduce stress and anxiety problems in Türkiye, which is why this study has chosen to apply CO and CA oils. The study only used essential oils sold in pharmacies in Türkiye.

Determining the composition of the essential oils

Gas chromatography with flame ionization detection (GC-FID) and with mass spectrometry (GC-MS) were used to test 10% (v/v) solutions of essential oil in n-hexane. The identification and quantification of the constituents of essential oils were made possible by an Agilent 7890B GC-FID (Santa Clara, CA, USA) connected to an Agilent 5977E electron impact mass spectrometer (Santa Clara, CA, USA) via a two-way capillary splitter. 1 mL of the sample solutions was injected using an Agilent G4513A auto injector (Santa Clara, CA, USA). The DB WAX column (60 m, 0.25 mm, 0.25 µm) was run on the following temperature program: 70 ^{0}C for 15 minutes, then 180 ^{0}C at an increase rate of 2^{0}C/min. After maintaining an isothermal temperature of 180 °C for 5 minutes, the column temperature was raised to 230 °C at a rate of 5°C/min. The final isothermal column temperature was set at 230°C for 15 minutes. The analysis was allowed 100 minutes. Helium was used as a carrier gas at a constant flow rate of 1.5 mL/min. The split ratio was set at 1:50. The mass detector scan range was 45-450 m/z. The chemicals were identified by matching the mass spectra of the substances included in the Wiley Registry of Mass Spectral Data (9th ed.; April 2011) with those found in the National Institute of Standards and Technology (NIST) 11 Mass Spectral Library (NIST11/2011/EPA/NIH), as well as by using real reference samples. Calculated retention indices from the co-injected saturated alkane series (C7-C40) were then used to compare the findings to earlier research and the NIST online webbook. Using calibration curves made by performing GC-FID tests on the sample chemicals, quantification was carried out using an external standard method, with three different analyses being carried out for each. This part of the analyses was conducted in the BITEM-Bezmialem University- Phytotherapy Education, Research and Application Center.

Study design and sample

A total of 78 Altınbaş University students aged 18-29 years old participated in this study. Six groups of voluntary students were selected at random. Each group had 13 participants (2 males and 11 females), and the six groups were comprised of Group 1 (G1) who used CO essential oil inhalation, Group 2 (G2) who used CA essential oil inhalation, Group 3 (G3) who used CO essential oil inhalation and practiced the mindfulness meditation program, Group 4 (G4) who used CA essential oil inhalation and practiced the mindfulness meditation program, Group 5 (G5) who practiced mindfulness meditation, and a wait-list control group (WL) who did nothing (Fig. 1).

Study procedure

Over 15 days, each student did an intervention every night between 10:00-10:30 pm according to their group. The students in the aromatherapy groups students applied 1 drop of oil to their masks and put their mask on for 30 min. The students in the meditation group practiced mindfulness meditation by listening to pre-recorded audio for 30 min. The students in the aromatherapy and mindfulness meditation groups applied 1 drop of oil to their masks and put on their masks for 30 min. while practicing the mindfulness meditation based on the audio recording. The five experimental groups and control group were included in an experimental investigation with pretest and posttest assessments. The Beck Depression Inventory-II and Perceived Stress Scale were used to collect the data. The individuals were asked to respond to the health-related questions and provide demographic data. All subjects were required to have normal body mass index (BMI) readings and to be free of respiratory difficulties (e.g., asthma, cold, flu) or any other olfactory-related illnesses. After the formation of the control and intervention groups, the Beck Depression Inventory-II (BDI-II) and Perceived Stress Scale (PSS) were administered as a pretest and filled out by all participants in the conference hall of Altınbaş University in Istanbul's Bakırköy district. This occurred again after the study as the posttest. This study was approved by the Altınbaş University's Ethics Committee on January 20, 2022 (Approval No. 97).

Instruments

The 21-question multiple-choice BDI-II was created by Aaron T. Beck and is one of the most widely used psychometric measures for determining the severity of depression (Beck, Steer, Ball, & Ranieri, 1996). When it first emerged, mental health professionals who had up until that point considered of depression as being rooted in the patient's own thoughts undertook a paradigm shift. A well-known method for measuring stress is the 14-question PSS (Cohen, Kamarck, & Mermelstein, 1983). Despite being developed in 1983, the instrument is still a well-preferred choice for helping understand how diverse situations affect feelings and how people perceive stress. This scale asks about one's thoughts and feelings from the last month. It asks how frequently one has felt or thought about each emotion or topic.

Data analysis

When the entire intervention program finished, the BDI-II and PSS were administered as a posttest and filled by all participants. The investigation program's goals were explained to all

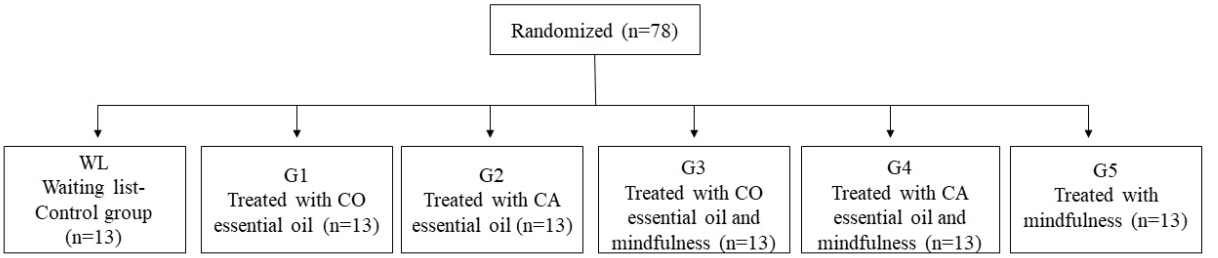

Figure 1. Study design.

participants. Prior to participating, they read and signed an informed consent form approved by Altınbaş University's Ethics Committee. The means and standard deviations were calculated. The differences according to the results were calculated and interpreted. The Wilcoxon test for paired samples was used to find significant differences between the research phases, and the Kruskal-Walls test was used to find differences between the WL group and the intervention groups. Percentages were calculated regarding the differences between the pretest and posttest results. SPSS (ver. 28 (IBM Corp., Armonk, NY, USA) was used for all statistical data analyses. Results with a 95% confidence level and $p < 0.05$ were deemed significant.

RESULTS

Essential oils composition

The findings from the GC-MS analysis of the samples are displayed in Tables 1 and 2. 93.90% of all the compounds in the *CO* essential oil were identified. The major compounds are germacrene-D (21.37%), linalool (14.45%), caryophyllene (E; 12.08%), geranyl acetate (11.10%), benzyl benzoate (7.31%), and farnesene (4.79%). 89.31% of all compounds in the *Cedrus atlantica* essential oil were identified. The major compounds are b- himachalene (49.81%), a-himachalene (18%), and longifolene (12.43%).

Comparing the effects from the aromatherapy and mindfulness meditation

As a result, the article conducted a two-week study to determine the effects of aromatherapy and mindfulness meditation on the university students' stress levels. Their stress levels were examined before and after the two-week study and compared to the globally accepted BDI-II and PSS assessments. The effects from the aromatherapy and mindfulness meditation on the students' stress levels were calculated and obtained using comparative numerical data. Table 3 lists the sociodemographic and clinical information for the study's 12 male and 66 female participants. The participants are single undergraduate university students between the ages of 18-29. The BDI-II and PSS mean scores and SDs are shown in Table 4. The BDI-II posttest scores for the five experimental groups differ significantly from those of the WL group, although G1 did not differ

Table 1. The essential oil compositions of *Cananga odorata*.

No.	Components	KI[a]	RRI[b]	Relative %	Identifaction Method
1	a-pinene	1018-1032	1027	0.23 ± 0.01	RRI, MS
2	p-methylanisole	1434-1446	1440	4.28 ± 0.03	RRI, MS
3	a-Copaene	1481-1497	1488	0.65 ± 0.04	RRI, MS
4	Linalool	1537-1553	1522	14.45 ± 0.06	RRI, MS
5	b-Elemene	1585-1600	1588	0.31 ± 0.06	RRI, MS
6	Caryophyllene (E)	1585-1612	1593	12,08 ± 0.05	RRI, MS
7	Methyl benzoate	1600-1632	1621	2.47 ± 0.02	RRI, MS
8	a-Humulene	1655-1682	1664	3.46 ± 0.02	RRI, MS
9	ɣ -Muurolene	1679-1704	1684	0.64 ± 0.02	RRI, MS
10	Bergamotene	1547-1710	1696	1.06 ± 0.03	RRI, MS
11	Germacrene-D	1699-1726	1706	21.37 ± 0.18	RRI, MS
12	a-Muurolene	1714-1740	1720	0.33 ± 0.03	RRI, MS
13	Benzyl acetate	1697-1742	1729	3.20 ± 0.09	RRI, MS
14	Farnesene -a-(E,E)	1735-1755	1749	4.79 ± 0.05	RRI, MS
15	ɣ-Cadinene	1746-1772	1753	1.65 ± 0.03	RRI, MS
16	Geranyl acetate	1743-1764	1758	11.10 ± 0.03	RRI, MS
17	Geraniol	1830-1857	1846	2.26 ± 0.02	RRI, MS
18	3-methyl-2-butenyl benzoate	N/A	2062	0.74 ± 0.02	RRI, MS
19	Cinnamyl acetate	2132-2167	2146	1.04 ± 0.01	RRI, MS
20	Tau-Muurolol	2169-2189	2179	0.34 ± 0.00	RRI, MS
21	a-Cadinol	2218-2255	2230	0.68 ± 0.03	RRI, MS
22	Farnesyl acetate	2222-2267	2264	1.06 ± 0.03	RRI, MS
23	Farnesol	2350-2367	2355	1.61 ± 0.04	RRI, MS
24	Benzyl benzoate	2577-2648	2633	7.31 ± 0.06	RRI, MS
25	Benzyl salicylate	2267-2796	2791	1.80 ±0.02	RRI, MS
	Total identified			98.92	

[a] These are found in the literature with 50% confidence intervals for the RI data ranges for each compound.
[b] Relative retention indices calculated against n-alkanes; % calculated from FID data. Identification method based on the relative retention indices (RRI) of compounds on the HP Innowax column; Mass spectrometer identification was performed on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and compared with literature data.

Table 2. The essential oil compositions of *Cedrus atlantica*.

No	Components	KI[a]	RRI[b]	Relative %	Identifaction Method
1	Limona ketone	1550-1570	1552	0.35 ± 0.01	RRI, MS
2	a-Himachalene	1652-1670	1647	18.00 ± 0.15	RRI, MS
3	Longifolene	1694-1704	1697	12.43 ± 0.11	RRI, MS
4	b- Himachalene	1700-1721	1708	49.81 ± 0.20	RRI, MS
5	ɣ-Cadinene	1752–1776	1753	1.60 ± 0.02	RRI, MS
6	a-Bisabolene	1761-1784	1771	0.60 ± 0.04	RRI, MS
7	Neoisolongifolene	1808-1820	1817	1.02 ± 0.03	RRI, MS
8	Isolongifolene	1850-1913	1858	1.47 ± 0.02	RRI, MS
9	1-Acetonaphthone, 8-methoxy	N/A	1894	1.38 ± 0.01	RRI, MS
10	a-Calarone	1912–1941	1916	0.77 ± 0.01	RRI, MS
11	b- Himachaleneoxide	2004-2045	2011	1.61 ± 0.02	RRI, MS
12	Calarene epoxide	N/A	2045	0.61 ± 0.01	RRI, MS
13	4-epi-Cubedol	2050-2088	2060	0.54 ± 0.02	RRI, MS
14	Spathulenol	2074–2150	2079	0.46 ± 0.03	RRI, MS
15	Longiborneol	2135-2154	2141	0.52 ± 0.02	RRI, MS
16	b-Cedrene	N/A	2203	0.50 ± 0.02	RRI, MS
17	b-Atlantone	2210-2245	2217	0.60 ± 0.07	RRI, MS
18	Deodarone	2226-2231	2230	1.09 ± 0.02	RRI, MS
19	ɣ-(E) Atlantone	2231-2316	2235	1.06 ± 0.01	RRI, MS
20	a-(Z) Atlantone	2231-2316	2243	1.02 ± 0.03	RRI, MS
21	a-(E) Atlantone	2231-2316	2324	2.20 ± 0.03	RRI, MS
	Total identified			97.64	

[b] Relative retention indices calculated against n-alkanes; % calculated from FID data. Identification method based on the relative retention indices (RRI) of compounds on the HP Innowax column; Mass spectrometer identification was performed on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and compared with literature data.
[a] These are found in the literature with 50% confidence intervals for the RI data ranges for each compound.

Table 3. The study participants' sociodemographic and clinical data.

Sociodemographic data	n
Gender	
Male	12
Female	66
Age (years)	
18-29	78
Education Level	
Undergraduate	78
Marital Status	
Single	78

significantly ($p_{G1} > 0.05$; p_{G2}, p_{G3}, p_{G4}, and $p_{G5} < 0.05$). When compared to the control group (WL), the perceived stress scores for the experimental groups G1, G2, G3, and G4 show significant variations or their posttest scores; however, G5 was not significant (p_{G1}, p_{G2}, p_{G3}, and $p_{G4} < 0.05$; $p_{G5} > 0.05$). When mindfulness and aromatherapy are combined, the difference in percentages regarding the pretest and posttest scores for BDI-II are lower than when these therapies are utilized separately (G1= 32.25; G2 = 34.7; G3 = 42.3; G4 = 42.4; G5 = 37.3). The mean scores for the PSS posttest show all interventions to have had an impact on perceived stress levels, with the difference percentages regarding scores being highest for the combination of mindfulness and aromatherapy (G1= 9.4; G2 = 9; G3 = 12.3; G4 = 12.4; G5 = 8.5). As a result of all these findings, both the BDI-II and PSS scores are seen to have significantly decreased (Table 5).

DISCUSSION

Stress is a phenomenon that individuals are exposed to in daily life. Changes brought about by university life, living away from family, situations encountered during education, and the anxiety of finding a job after graduation can cause students stress. The aim of this study has been to determine the perceived stress levels of university students and to monitor the effects of new complementary therapy systems as an approach to coping with stress. Coping with stress is basically seen as a response to emotions. Different approaches are found that define how an individual copes with stress. Stress and coping theory postulates two types of coping to exist. The first is the problem-focused coping strategy, which involves planned actions to address the issue causing the problem using strategies such as information gathering and decision making. The second is an emotion-focused coping strategy that exists to regulate nega-

Table 4. Group differences regarding anxiety based on the BDI-II and PSS.

Groups	Pretest	Posttest	p[b]
	Mean ± SD	Mean ± SD	
WL			
BDI-II	9.92±3.84	10.50±4.503	0.765
PSS	20±2.449	19.83±4.324	0.837
G1			
BDI-II	14.77±6.34	10±4.933	0.006
p-Value[a]		0.076	
PSS	23.77±4.952	21.54±3.573	0.258
p[a]		0.011	
G2			
BDI-II	17.31±8.25	11.31±4.75	0.006
p[a]		0.009	
PSS	23.77±4.003	21.62±5.316	0.248
p[a]		0.003	
G3			
BDI-II	16.92	9.77	0.017
p[a]		0.013	
PSS	22	19.31	0.034
p[a]		0.072	
G4			
BDI-II	20.85	12	0.008
p[a]		< .001	
PSS	23.54	20.62	0.025
p[a]		0.001	
G5			
BDI-II	18.77	11.77	0.005
p[a]		0.002	
PSS	21.92	20.08	0.017
p[a]		0.106	

[a]p-Value is calculated by Kruskal-Wallis Test between groups, [b]p-Value is calculated by Wilcoxon test between study phases

Table 5. Difference in the pretest and posttest percentages in the intervention groups.

Groups	Difference between the Pretest & Posttest Scores (%)
G1	
BDI-II	-32.25
PSS	-9.40
G2	
BDI-II	-34.7
PSS	-9
G3	
BDI-II	-42.3
PSS	-12.3
G4	
BDI-II	-42.4
PSS	-12.4
G5	
BDI-II	-37.3
PSS	-8.5

tive emotions by using strategies such as leaving behind, seeking emotional support, escaping, and avoiding (Folkman, 2020). According to a study conducted on 100 university students, the top five sources of stress involve changes in sleep patterns, holidays/breaks, changes in eating habits, increased workload, and new responsibilities. These stressors cause high levels of stress among students. This situation can be eliminated by creating stress management programs (Ross, Niebling, & Heckert, 1999). A significant relationship was found for the number of close friends an individual has and the type of school that individual attends with that individual's stress level (Durna, 2006). A study conducted with university students sought to answer how students define stress, the most important causes of stress in their lives, and how they cope with stress . Most of the students were seen to define stress as a mental state, with the causes of stress among students being lack of sleep and financial and family problems. The students were seen to have identified certain strategies such as counseling services, meditation, walking around with friends, sharing, and getting adequate sleep for coping with stress (Redhwan, Sami, Karim, Chan, & Zaleha, 2009). Following the session in their study, participants' anxiety levels were seen to have decreased, with each group having lower trait scores than state scores. In addition, the results from the current study demonstrate a reduction in anxiety scores in all experimental groups following their interventions compared to the WL group, particularly in groups G3 and G4 who received concurrent aromatherapy and mindfulness meditation. This suggests a synergy effect that had also been noted in a prior study that also discovered using aromatherapy to treat anxiety to also make mindfulness meditation more effective(Soto-Vásquez & Alvarado-García, 2017). The application of the *Cedrus atlantica* essential oil in G2 exposed them to a substantially higher reduction in anxiety levels after treatment compared to G1, who were just treated with the *Cananga odorata* essential oil. The results regarding G4, who combined the use of CA essential oil with mindfulness meditation, are also consistent with this compared to G3, who used the CO oil with mindfulness meditation.

According to data studies, the sedative effects of CO essential oils are attributed to linalool (14.45%), caryophyllene (12.08%), and benzyl benzoate (7.31%). Linalool exposure has been demonstrated to have sedative effects on a number of mouse behaviors, including locomotion, barbiturate-induced slumber, motor coordination, and body temperature regulation. Previous research has not revealed any anxiolytic action for benzyl benzoate or benzyl alcohol. According to one study, benzyl benzoate could reduce angiotensin II-induced hypertension. A bicyclic sesquiterpene known as caryophyllene works as the

active ingredient in many essential oils from spices and food plants. Studies have shown this to have a wide range of pharmacological effects as a selective cannabinoid receptor 2 (CB2) agonist, including antibacterial (against *Helicobacter pylori*), antioxidant, anti-inflammatory, analgesic (against neuropathic pain), anti-neurodegenerative, and anticancer characteristics. According to published research, the phytocannabinoid caryophyllene, which is found in food, has the ability to protect neurons by reducing oxidative stress and stabilizing mitochondria. This makes it a possible lead molecule for the development of medications for treating neurodegenerative diseases. In addition to CB2 receptor agonism, caryophyllene has been discovered to favorably regulate PPAR-γ, TLRs, and neuroimmune pathways, which are potential targets connected to the defense against neuronal death. Following their reduction of oxidative stress and/or mitochondrial dysfunction, essential oils containing caryophyllene obtained from various vegetable sources have also demonstrated promising neuroprotective effects (Machado et al., 2018; Ullah, Minno, Santarcangelo, Khan, & Daglia, 2021; Zhang, Zhang, Feng, & Yao, 2016).

The anxiolytic effects of CA essential oils are attributed to b- himachalene (49.81%) and a-himachalene (18%). According to Suyono et al., CA essential oil balms decrease the cortisol levels in rats due to α-himachalene, β-himachalene, and γ-himachalene (Suyono, Jong, & Wijaya, 2020). This shows that variations in anxiety ratings might result from a variety of chemical compositions of essential oils, although additional research is required to support this theory.

In comparison to the aromatherapy-only groups (G1 and G2), the scores for the mindfulness meditation group (G5) present a bit more reduction in the BDI-II posttest. This is similar to the findings made by other studies that observed a decrease in anxiety state, trait, or overall anxiety following mindfulness meditation therapies. This is because those who practice mindfulness may understand how to maintain a relaxed state of mind and concentrate on the present moment and to establish an attitude of acceptance and patience toward any unfavorable feelings or thoughts that may surface. However, when compared to the mindfulness meditation group in this study, the degree of change regarding the PSS scores of the aromatherapy-only groups (G1 and G2) shows a little bit more reduction regarding the post-treatment scores (G5). The biggest difference percentage regarding the variable of anxiety is shown by G4 followed by G3. This demonstrates the viability of combining mindfulness and aromatherapy to treat anxiety.

CONCLUSION

Few studies so far are found to have combined the two variables of aromatherapy and mindful meditation in this way. Through the essential oils of *Cedrus atlantica* and *Cananga odorata*, aromatherapy has developed as a successful therapeutic option for anxiety. The use of essential oils alongside mindfulness meditation is a new method in aromatherapy applications for anxiety. Further studies are nonetheless required to assist in comprehending the synergistic effects of aromatherapy and mindfulness meditation.

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Original Article

Cyto- and genotoxicity of copper (II) oxide (CuO) nanoparticles in HeLa cells

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ABSTRACT

Background and Aims: Cancer is a widespread disease responsible for the death of millions every year. Different approaches and drugs are in use to treat cancer, however, there is a need for new drugs with low cost, high activity, and low side effect risks. Nanotechnology and nanomaterials are important to develop those drugs. Copper-based nanoparticles (NPs) are shown to have biological activity as the antibacterial, and cytotoxic potential. Copper (II) oxide (CuO) NPs are widely used among Cu-based NPs. Different studies evaluated its anticancer and cytotoxic activity; however, the results are still controversial.

Methods: It was planned to characterize the NPs using Transmission Electron Microscopy (TEM) in cell culture medium and distilled water and then to evaluate their cytotoxicity in human cervical cancer cells (HeLa) using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay and neutral red uptake (NRU) assays. As one of the cytotoxicity mechanisms, the DNA damage induction potential was evaluated by Comet assay.

Results: The CuO NPs have an average diameter of about 35 nm in distilled water and 39 nm in cell culture medium. The IC50 levels of NPs were 10.7 µg/mL and 6.73 µg/mL by MTT and NRU assays, respectively. The results reveal the NPs dose-dependently increased in the DNA damage. The tail moment was 1.3-fold at 3.125 µg/mL, 2.5-fold at 6.25 µg/mL, and 3.8-fold at 12.5 µg/mL.

Conclusion: CuO NPs have high cytotoxic activity in HeLa cancerous cells. The induction of DNA damage could be an important step in the induction of cell death. Further in vivo and *in vitro* studies in need to improve the safety/low toxicity and understand the molecular mechanism of CuO-induced activity.

Keywords: Copper (II) oxide, nanoparticles, HeLa, genotoxicity, cytotoxicity

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INTRODUCTION

Cancer disease was responsible for the death of about 10 million in 2020, and it is predicted to cause the death of about 30 million in 2040 (Andleeb et al., 2021; Sung et al., 2021). Today, different approaches like hormone therapy, immunotherapy, chemotherapy, and surgeries are in use to treat/control cancer. However, research is still related to discovering and developing new chemotherapeutical agents with low side effects, low budget, and high activity (Andleeb et al., 2021). Nanotechnology and nanomaterials constitute a fertile ground for this type of research. NPs, materials with at least one dimension less than 100 nm, have superior properties, leading to wide applications in different areas (Aitken, Chaudhry, Boxall, & Hull, 2006; Sekhon 2010). Nowadays, Cu-based NPs widely used in cosmetics and medicine production and could be found in different biomedical, industrial, and commercial products such as conductors, sensors, and solar energy converters. Cu-based NPs have thermal, electrical, and catalytic properties in addition to their biological properties. (Chang, Zhang, Xia, Zhang, & Xing, 2012; Cioffi et al., 2005; Schrand et al., 2010).

The previous studies reported the accumulation of NPs in different organs (Chen et al., 2006; Kadammattil, Sajankila, Prabhu, Rao, & Rao, 2018; Lei et al., 2008; Liu et al., 2009; Meng et al., 2007) and mentioned the cellular uptake of NPs in general. And Cu-based NPs particularly lead to morphological changes in the organs and damages at the cellular level (Abudayyak, Guzel, Özhan, 2016a; 2016b; 2020; Khalid et al., 2018; Gosens et al., 2016; Thit, Selck, & Bjerregaard, 2013; 2015; Xu, Li, Xu, Xiao, & Yang, 2013). While this accumulation and cellular uptake could be evaluated negatively as a starting point for different toxic effects like hepatic damage (Chen et al., 2006; Khalid et al., 2018), nephrotic damage (Chen et al., 2006; Khalid et al., 2018; Meng et al., 2007), apoptosis in hepatocytes (Siddiqui et al., 2013; Wang et al., 2011) and inflammation in the cardiac cells (Sun et al., 2011), in the healthy organs, it also could be evaluated positively as the start of therapy for cancer and the key to cancer cells' death. Previous data enclosed the studies showed the anticancer activity of CuO NPs in different cell lines (Dadure, Mahapatra, Haldar, Potbhare, Chaudhary, 2022; Maksoudian et al., 2020; Rani & Saini, 2022). The studies vary, some of them show high activity in the cancerous cells (Abudayyak et al., 2020; Rehana, Mahendiran, Kumar, & Rahiman, 2017) the others show their safety or low toxicity (Nagajyothi et al., 2017; Oza et al., 2020). Since different factors like the cellular type play a role in the degree of toxicity in the cell, a well-known widely used HeLa was chosen to evaluate CuO NPs- induced toxicity. This will give the ability to compare the results with previous data, especially those related to biosynthesized NPs. For that, the cytotoxicity of CuO NPs was evaluated using MTT and NRU assays, and DNA damage induction using Comet assay.

MATERIALS AND METHODS

Materials: CuO NPs, MTT pigment, neutral red (NR) dye, dimethyl sulfoxide (DMSO), acetic acid, and hydrogen peroxide (H_2O_2) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Ethylene diamine tetraacetic acid (EDTA) and Triton X-100 were obtained from Biomatik (Ontario, Canada). Eagle's Minimum Essential Medium (EMEM), fetal bovine serum (FBS), antibiotic solution for cell culture, and trypsin-EDTA solution for cell culture were obtained from Multicell Wisent (Quebec, Canada).

NPs characterization: CuO NPs were suspended in a cell culture medium or distilled water, then NPs were dropped on a special carbon-coated mesh. NPs were analyzed by Transmission Electron Microscopy (TEM) (Jem-2100 HR, Jeol, USA). At least 100 NP were used in the calculation of the average size for each sample. Results were expressed as the mean±SD. Scanning Electron Microscopy (SEM) (JEOL JSM 5600, Jeol, USA) was used to evaluate the NPs' outer size and shape, for that powder was used directly.

Cell culture and exposure conditions: HeLa cell line (CCL-2™) was obtained from American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were continued in an EMEM cell culture medium supplemented with 10% FBS and 1% antibiotic. The cells, incubated at 37 °C, 95% humidity, and 5% CO_2 conditions, were collected by trypsinization. The cells were seeded at a concentration of 10^4 cells/well and $2x10^5$ cells/well for cytotoxicity and Comet assays, respectively. Before exposure, CuO NPs were prepared freshly as 1 mg/mL suspension in a complete cell culture medium. The suspension was sonicated for 15 min to prevent their aggregation/ agglomeration, then the exposure mediums with the planned concentrations were prepared by dilution. The exposure concentrations were in the range of 1.5-60 µg/mL in cytotoxicity assays. The previous data showed that CuO NPs tested for genotoxicity at concentrations arranged 5 – 50 µg/mL for 24 hours (Abudayyak et al., 2016a; 2016b; 2020; Ahamed et al., 2010; Akhtar, Ahamed, Fareed, Alrokayan, & Kumar 2012; Wang et al., 2012). In these studies, some of the tested concentrations were higher than the IC_{50} values. For that, to be able to compare the results, in the present study where the IC_{50} was about 10.7± 0.7 µg/mL, a concentration that was previously used in data and also close to our IC_{50} value (12.5 µg/mL), the half, and quarter of it (3.125, 6.25, and 12.5 µg/mL concentrations) were used in the Comet assay. For all assays, the cells were incubated for 24 hr. The unexposed cells were accepted as growth and negative control for both cytotoxicity and genotoxicity assays. DMSO (10%), for 24 hours, was used as a positive control for cytotoxicity. Cells exposed to H_2O_2 (100) µM for 2 hours were used as a positive control in Comet assays. Cytotoxicity assays were done in triplicates and repeated in different 3 days (n = 9), Comet assay was done in duplicates and repeated in different 3 days (n = 6).

Cytotoxic effect evaluation: At the end of the exposure period, MTT assay or NRU assay was applied. MTT assay is based on the evaluation of mitochondrial enzyme activity, which is accepted as a sign of cell viability. In this case, the mitochondrial enzyme of the viable cells metabolized the yellowish and water-soluble MTT to water-insoluble violet formazan crystals, while the dead cells will be unable to metabolize and so to give the violet color (Van Meerloo, Kaspers, & Cloos, 2011). NRU assay is based on the ability of viable cells to uptake by passive diffusion and then accumulate the cationic NR dye in the anionic regions in the lysosomes. Since cell death means dam-

age in lysosomes, the dead cell will be unable to accumulate the NR dyes and so to give the red color (Repetto, Del Peso, & Zurita, 2008). In the MTT assay, 25 µL of MTT solution (0.5 mg/mL) was added to each well and the plates were incubated for a further 3 hr. Then, the supernatants were thrown and 100 µL/well of DMSO was added to solve the formed violet crystals. The absorbance was measured at 590 nm using a microplate spectrophotometer system (Epoch, Germany). In the NRU assay, the exposure solutions were discharged, and 100 µL/well NR solution (50 µg/mL) was added to the plates. After incubation for 3 hours, the NR solution was discharged, and the plates were washed with warm phosphate-buffered saline (PBS) x1 twice. Then 100 µL/well of dye-solving solution (1% acetic acid, 1:1 Ethanol: Water). The absorbance was measured at 540 nm. For both assays, the cell viability and the cell death ratios were calculated compared to the negative controls. The results were expressed as the mean of concentrations that caused cell death in 50% of the cells (IC_{50}).

Genotoxic effect evaluation: The genotoxicity was evaluated by Comet assay according to the method mentioned by Collins (2004) and Speit, & Hartmann (1999). Briefly, the exposed cells were collected, washed, and adjusted to 10^6 cells/ mL. Then 100 µL of the cell suspension was mixed with 100 µL of low melting agarose and the cells spread out over slides that were previously coated with normal melting agarose. After that, coverslips were removed, and the cells in the lams were treated with a lysis solution for 12 hours and electrophoresed. Finally, the cells were treated with a normalizing solution, colored with ethidium bromide, and evaluated under the fluorescent microscope (Olympus BX53, Olympus, Tokyo, Japan) using an automated image analysis system (Comet Assay IV, Perceptive Instruments, Suffolk, UK). The tail moment was accepted as the endpoint to evaluate the DNA damage. For each sample, at least 100 cells were evaluated. The means of the tail moments for each exposure group were calculated, then the results were calculated compared to the control group and expressed as the folds of the negative control.

Statistical analysis: The significance of results calculated compared to the control groups using one-way ANOVA, post hoc, and Dunnett test (SPSS version 28.0; SPSS Inc., Chicago, IL). $p \leq 0.05$ was considered significant.

RESULTS

NPs characterization: The average diameter of NPs in water was 35 nm, and about 98% of the particles have a diameter smaller than 60 nm. The size was higher in the complete cell culture medium, and the average diameter was 39 nm with more than 79% of the particles with a diameter smaller than 60 nm (Figure 1). Additionally, the NPs were analyzed by SEM microscopy. The SEM pictures also reveal the nano size of the particles; however, it is not enough to make calculations (Figure 2).

Cytotoxicity: The exposure to CuO NPs for 24 hours induced significant cell death that increased with the increase in concentration (Figure 3). The IC_{50} value was calculated by MTT assay to 10.7±0.7 µg/mL. While the IC_{50} calculated by NRU assay was 6.73±0.1 µg/mL.

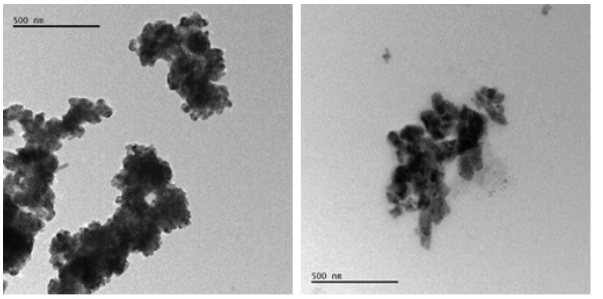

Figure 1. The TEM analysis of CuO NPs in (a) water and (b) complete cell culture medium.

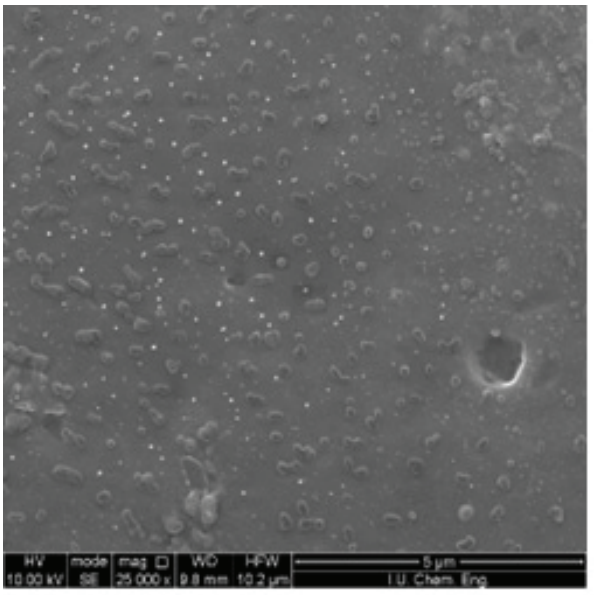

Figure 2. The SEM analysis of CuO NPs powder.

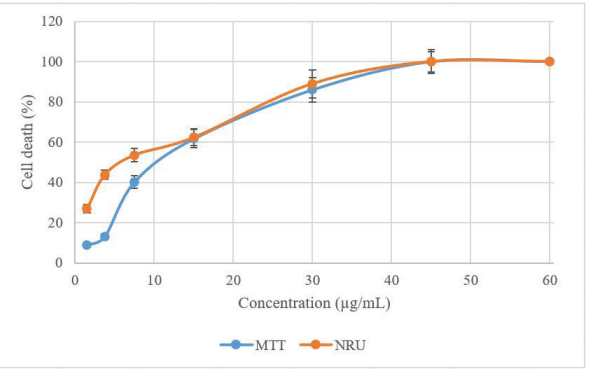

Figure 3. Cytotoxicity of CuO NPs in HeLa cell line at 1.5-60 µg/mL exposure concentrations.

DNA damage-inducing potential: Copper (II) Oxide NPs induced significant DNA damage at all exposure concentrations (Figure 4). The damage depended on concentrations. At the highest exposure concentration (12.5 µg/mL), the increase in the tail moment was 3.85-fold, while they were 2.5-fold and 1.3-fold in 6.25 µg/mL and 3.125 µg/mL, respectively, compared to the negative control group ($p < 0.05$).

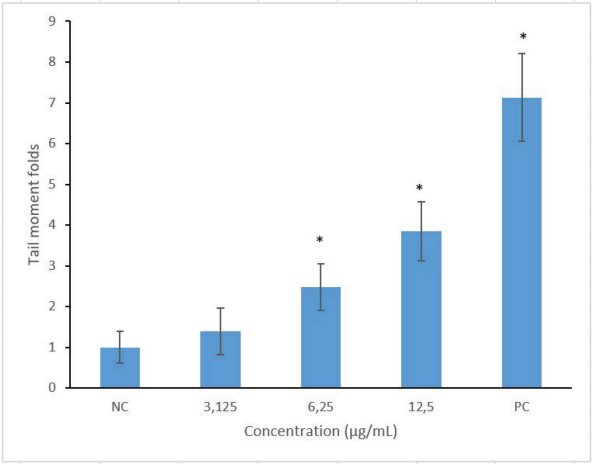

Figure 4. The DNA damage in HeLa cells exposed to CuO NPS. Cells were exposed to 3.125, 6.25, or 12.5 µg/mL CuO NPs for 24 hours. The unexposed cells and cells exposed to H_2O_2 (100 µM) for two hours were accepted as negative and positive controls, respectively. One-way ANOVA, post hoc, and Dunnett test (SPSS version 28.0) were used for the statistical analysis. *p< 0.05 was considered significant.

DISCUSSION

The cellular uptake of nanoparticles was reported by different research groups as well as the research in our lab (Abudayyak et al., 2016a, 2016b, Abudayyak, Altincekic Gurkaynak, & Özhan, 2016c; Abudayyak, Guzel, Özhan, 2017; 2020). Previous data have shown that the exposure to CuO NPs and other Cu-based NPs for 24 hours induced cytotoxic effects in different cell lines such as breast cancer (MCF-7) (Alishah, Pourseyedi, Ebrahimi-pour, Mahani, & Rafiei, 2017; Mahmoud, Mohamed, Ahmed, & Akhtar, 2020), lung (A549) (Karlsson, Cronholm, Gustafsson, & Möller 2008; Wang et al 2012), hepatic cancer (HepG2) (Abudayyak et al., 2020; Siddiqui et al., 2013; Wang et al., 2011), intestinal (Caco-2) (Abudayyak et al., 2020; Piret et al., 2012), Rat kidney (NRK-52E) (Abudayyak et al., 2016b), keratinocytes (HaCaT), embryonic fibroblasts (BALB3T3) (Akhtar et al., 2012), airway epithelial (HEp-2) (Farshori et al., 2022), neuroblastoma (SH-SY5Y) (Abudayyak et al., 2016a; Chen et al., 2008), neuro-glioma (H4) (Chen et al., 2008), mouse neuroblastoma (N2A) (Perreault et al., 2012), cardiac microvascular endothelial cells (Sun et al., 2011), and primary culture of channel catfish hepatocytes cells (Wang et al., 2011). In the cell lines, the reported IC_{50} was less than 16.4 µg/mL, except for N2A cells where the cell viability was about 37% at 400 µg/mL.

Recently, the synthesis of NPs as CuO NPs using plants, yeast, and bacteria in what is called biosynthesis or green synthesis has increased dramatically. Rani & Saini (2022) reviewed more than 25 studies using herbs only, While Dadure et al. (2022) summarized 45 studies related to biosynthesized Cu-based NPs. The green synthesized CuO NPs were evaluated for their anticancer activity using MTT assay in different cell lines, HCT-116 human colon cancer cells (IC_{50}: 40 µg/mL) (Gnanavel, Palanichamy, & Roopan, 2017), AMJ-13 breast cancer cells (IC_{50}: 1.47 µg/mL), and SKOV-3 ovarian cancer cells (IC_{50}: 2.27 µg/mL) (Andleeb et al., 2021). In another study using A549 cells, the IC_{50} value for biogenic CuO NPs was 200 µg/mL (Sankar, Maheswari, Karthik, Shivashangari, & Ravikumar, 2014). The studies carried out using A549, Hep-2, MCF-7, MDA-MB-231 (human breast cancer cells), NHDF (normal human dermal fibroblast cells), and HeLa cell lines were also used to evaluate the anticancer/cytotoxic effect of green CuO NPs. The studies reported that IC_{50} values were between 18.1 µg/mL and 45.3 µg/mL, while for HeLa cells specifically were 20.3 - 45.3 µg/mL (Rehana et al., 2017). Nagajyothi et al. (2017) used HeLa cells to evaluate their green synthesized CuO NPs, after 24 hours of exposure. The IC_{50} level was higher than 500 µg/mL, besides, they noticed the ability of CuO NPs to inhibit the colony of cancerous cells (Nagajyothi, Muthuraman, Sreekanth, Kim, & Shim, 2017). Oza et al. (2020) reported no cytotoxicity of their biosynthesized CuO NPs, as they reported 80% viability at 100 µg/mL for 72 hours of exposure. The variation in the reported potential of cytotoxic effect, and so, the IC_{50} depends on different factors such as the shape, the size, the porous state, the synthesis method, and importantly the used cell line. Hela cell is one of the famous cell lines that are used for anticancer research and one of the oldest immortalized cell lines. The wide use of HeLa in different biomedical and biochemical research gives the opportunities to compare the results with the other chemicals and also with other labs (Masters 2002; Verma & Hansch 2006), however, the effect of pure, chemical, or physically synthesized, CuO NPs were not evaluated previously. For that, in the present study, CuO NPs purchased from Sigma Aldrich with known properties and wide use in research is also preferred, which gives the chance to compare the results among the cell lines. The results of this study show that CuO NPs caused cell death with IC_{50} calculated to be 10.7 µg/mL and 6.73 µg/mL by MTT and NRU assays, respectively. This indicated that HeLa cells are more sensitive toward the chemically synthesized CuO NPs compared to green NPs in the previous studies (Nagajyothi et al., 2017; Oza et al., 2020; Rehana et al., 2017). And also, HeLa cells are more sensitive than other cells used previously (Abudayyak et al., 2016a; 2016b; 2020; Akhtar et al., 2012; Alishah et al., 2017; Chen et al., 2008; Dadure et al., 2022; Farshori et al., 2022; Karlsson et al., 2008; Mahmoud et al., 2020; Perreault et al., 2012; Piret et al., 2012; Rani & Saini 2022; Sankar et al., 2014; Siddiqui et al., 2013; Sun et al., 2011; Wang et al., 2011; Wang et al 2012), except AMJ-13 and SKOV-3 cell lines (Andleeb et al., 2021).

The mechanism of cellular death induction was the topic of different studies. The elevation of reactive oxygen species (ROS) and the disruption of oxidative status inside the cells, the cell arrest, the induction of apoptosis, the damages in genetic materials, and the effects on inflammatory pathways were reported in the cells exposed to CuO NPs (Maksoudian et al., 2020; Tuli et al., 2015). According to Ingle et al. (2013), Cu NPs can degrade the cellular DNA even in the absence of H_2O_2 or other outer factors necessary for oxidative reactions that make those NPs good for targeted therapy (Ingle, Duran, & Rai, 2013). In the present study, Comet assay results reveal the NPs dose-dependently increased in the DNA damage. The tail moment was 1.3-fold at 3.125 µg/mL, 2.5-fold at 6.25 µg/mL, and 3.8-fold at 12.5 µg/mL. These results confirm the results of different previous studies, it was noticed that the same nanoparticles for the same exposure period at concentrations arranged between 5 and 50 µg/mL caused DNA damage with induction of oxidative stress in NRK-52E and SHSY-5Y cells

where the DNA damages were 1.85- to 8.4-fold and 2.57- to 7.09-fold, respectively (Abudayyak et al., 2016a; 2016b). Similar results were noticed after exposure to the same NPs for the same period at concentrations between 5-20 µg/mL in Caco-2 and HepG2; In these cells, the DNA damages were between 1.2- to 7.6-fold in HepG2 cells and 5.89- to 9.6-fold in Caco-2 cell line. (Abudayyak et al., 2020).

Perreault et al. (2012) reported the induction of genotoxicity by CuO NPs at concentrations higher than 12.5 µg/mL (24 hours exposure) using a micronucleus assay in Neuro-2A cells. Similar results were reported in A549 cells after 24 hours of exposure, Akhtar et al. (2016) reported that the tail moment was 27% at 15 µg/mL, compared to 5% in the control group and Wang et al., (2012) reported an increase in the tail moment at 15 mg/L with 4.5-fold compared to the control (Akhtar et al., 2016; Wang et al., 2012). Ahamed et al. (2010) concluded that CuO NPs, at 50 µg/mL for 24 hours of exposure, can induce genotoxicity in A549 cells via oxidative stress pathway by up-regulating the expression of proteins important in DNA damage repair and cell cycle as p53 Rad51 and MSH2 (Ahamed et al., 2010). Previous data together with our results confirm the high cytotoxicity of CuO NPs in HeLa cancerous cells through damaging its genetic material, in addition to the ability to use these NPs for targeted therapy, these NPs could be used for killing those cancerous cells.

CONCLUSION

The previous data reported different results related to CuO NPs-induced toxicity in cancerous cells. This difference could be due to the variation in the cell lines that are used or could be related to the type, shape, and size of NPs, and especially the method of synthesis. The results show that CuO NPs caused cell death at relatively low concentrations (exposure concentration 1.5-60 µg/mL, $IC_{50} \leq 10.7$ µg/mL). The increase in DNA damage in the exposed cells indicates that DNA damage is one of the mechanisms of cell death in HeLa cells exposed to CuO NPs. The present results confirm the previous data that Cu-based NPs in general and CuO NPs specifically have the chance to be developed for use in targeting cancerous tissue and killing these cells. However, there is a need for further *in vitro* and *in vivo* studies to a better understanding of the mechanisms underlying CuO NPs and to approve or disapprove the safety of these NPs and their ability to be developed for use in cancer treatment.

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

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Original Article

Nanoemulsion formulation containing carbamazepine and levetiracetam: Development and *in vitro* characterization

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ABSTRACT

Background and Aims: Epilepsy is one of the most disabling and most common neurological disorders, affecting approximately 65 million people of all age groups worldwide. When there is no response to monotherapy in the treatment of epilepsy, the combined use of Carbamazepine (CARBA) and Levetiracetam (LEV), which have different mechanisms of action, can be additive/synergistic and may be useful in the clinic. The aim of our study is to develop a fixed-dose combination (FDC)-nanoemulsion (NE) formulation containing CARBA and LEV for the treatment of epilepsy.
Methods: Blank NE (BLNK-NE) and CARBA+LEV-FDC-NE formulations were prepared and carried out the *in vitro* characterization studies [morphological analysis, centrifugation test, droplet size (DS), polydispersity index (PDI), zeta potential (ZP), viscosity and pH measurements, FT-IR analysis, the percent entrapment efficiency (EE%), and *in vitro* release study].
Results: The DS, PDI, pH, and ZP values for the BLNK-NE formulation were found to be 117.63±3.82 nm, 0.240±0.014, 4.62±0.03, and (-)26.07±3.04 mV, respectively. For the CARBA+LEV-FDC-NE formulation, the DS, PDI, pH, and ZP values were determined as 137.56±3.11 nm, 0.225±0.013, 4.60±0.06, and (-)21.62±0.29 mV, respectively. The EE% values obtained for CARBA+LEV-FDC-NE were 97.33±0.56% (for CARBA) and 96.73±0.91% (for LEV).
Conclusion: The CARBA+LEV-FDC-NE formulation was successfully prepared. This formulation had suitable *in vitro* characterization results.
Keywords: Carbamazepine, epilepsy, fixed-dose combination, levetiracetam, nanoemulsion

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INTRODUCTION

Epilepsy, one of the most disabling and most common neurological disorders affecting approximately 65 million people of all age groups worldwide, is characterized by an enduring predisposition to produce recurrent and unprovoked seizures or electrical disturbances in the brain and associated psychological, cognitive, and social consequences (Devinsky et al., 2018; Kanner & Bicchi, 2022; Patel & Parikh, 2020). The sudden appearance of temporary signs and symptoms caused by excessive and abnormal or synchronized neuronal activity in the brain is defined as an epileptic seizure (Kanner & Bicchi, 2022). There are three diagnostic levels (seizure type, epilepsy type, and epilepsy syndrome) in "the International League Against Epilepsy 2017 Classification". It is also emphasized that etiology and comorbidities should be considered at each level (Wirrell, Tinuper, Perucca, & Moshé, 2022). The most common types of epilepsy are focal and generalized epilepsies (Kanner & Bicchi, 2022). Epilepsy treatment approaches include using antiepileptic drugs (AEDs), a special diet (ketogenic diet), vagus nerve stimulation, or surgery (Green, Nguyen, Kaalund-Hansen, Rajakulendran, & Murphy, 2020). AEDs should be selected according to epilepsy and seizure types, epilepsy syndrome, and drug-related adverse effects. The main goal of epilepsy treatment with AEDs is to eliminate seizures while minimizing AED-related adverse effects (Kanner & Bicchi, 2022).

Carbamazepine (CARBA), a derivative of dibenzazepine used for the treatment of epilepsy, trigeminal neuralgia and pain associated with other neurological disorders, is a sodium channel blocker and also modulates other voltage-gated ion channels (e.g., voltage-gated calcium channels) (Beydoun et al., 2020; Maan, Duong, & Saadabadi, 2022; Uzunović, Vranić, & Hadžidedić, 2010). Dizziness, nausea, vomiting, ataxia, and drowsiness are the most common side effects associated with using CARBA. Also, a few severe skin reactions are rarely seen (Maan et al., 2022). CARBA is a potent cytochrome P-450 enzyme inducer and is significantly more prone to drug-drug interactions (Beydoun et al., 2020). It is a BCS (The Biopharmaceutics Classification System) Class II active substance (low solubility and high permeability), and its oral bioavailability is limited by its dissolution rate. Therefore, the solubility and dissolution rate of CARBA are critical determinants of its oral bioavailability (Uzunović et al., 2010).

Levetiracetam (LEV) is a second-generation established AED, approved by the FDA in 2000 for adjunctive therapy in treating myoclonic seizures, focal seizures, and primary generalized seizures (Kumar, Maini, & Kadian, 2022; Lyseng-Williamson, 2011). It is thought that LEV exerts its effect by binding to a unique synaptic vesicle protein 2A (SV2A is found on synaptic vesicles and some neuroendocrine cells), thereby regulating the release of neurotransmitters, including excitatory amino acids and ultimately suppressing epilepsy discharge (Yi et al., 2018). LEV is very soluble in water and shows high permeability. Therefore, LEV is a BCS Class I active substance (Petruševska et al., 2015). LEV is rapidly absorbed and has a very high oral bioavailability (96%). The main metabolic pathway for LEV is the enzymatic hydrolysis of the acetamide group. LEV does not show a significant drug-drug interaction (pharmacokinetically) due to the fact that the hepatic cytochrome P450 system plays a minimal (2.5%) role in its metabolism (Kumar et al., 2022; Lyseng-Williamson, 2011). The most common side effects associated with LEV use are headaches, fatigue, depression, mood swings, sedation, agitation, irritability, confusion, nausea, vomiting, anorexia, abdominal pain, etc. (Kumar et al., 2022). In the treatment of epilepsy, when seizures cannot be controlled with monotherapy, combination therapy can be used for the patient (Sarhan, Walker, & Selai, 2016). There are two approaches to drug combination therapy: 1. the combined use of two or more drugs, which are used separately in the treatment, and 2. the use of a single dosage form containing at least two active substances (known as FDC) (Kim & Weon, 2021; Rahman et al., 2020). The primary purpose of treatment with FDCs is to increase therapeutic efficacy and improve patient compliance through synergistic effects while minimizing adverse effects/toxic effects (Kim & Weon, 2021). Although the efficacy of the combination therapy is supported by the data obtained, there is a possibility of increased side effects (Sarhan et al., 2016). In combination therapy, drug interactions (pharmacokinetic and pharmacodynamic interactions) should also be considered (Park, Kim, & Lee, 2019). For these reasons, patients should be followed carefully during combination therapy. In a review on the effect of antiepileptic drugs in combination therapy, it was reported that the combinations of LEV-oxcarbazepine or CARBA-LEV or lacosamide-LEV were the most common dual AEDs combinations (Mäkinen, Rainesalo, Raitanen, & Peltola, 2017).

In this study, we aimed to develop an FDC-NE formulation containing CARBA and LEV (CARBA+LEV-FDC-NE) for the combination therapy of epilepsy and carried out *in vitro* characterization studies of the NE formulations [BLNK-NE (without active substances) and CARBA+LEV-FDC-NE].

MATERIALS AND METHODS

LEV, CARBA, and Labrasol were generous gifts from DEVA Holding A.Ş. (Türkiye), Biofarma İlaç San. Tic. A.Ş. (Türkiye) and Gattefosse (France), respectively. Ethyl oleate and Tween 80 were obtained from Merck (Germany).

Methods

Preparation of BLNK-NE and CARBA+LEV-FDC-NE

The preparation method developed by Sigward et al. (2013) was modified and used in our study. For the preparation of FDC-NE formulations containing CARBA and LEV, the oil phase was prepared by mixing ethyl oleate (40%), surfactants [Tween 80 (35%) and Labrasol (15%)], and CARBA (200 mg) by first using a magnetic stirrer followed by Ultra-Turrax (T10, IKA, Germany). Besides, the solution of LEV (50 mg) in ultrapure water (as the aqueous phase) was prepared. The prepared aqueous phase was added to the oil phase to obtain the primary emulsion while mixing at high speed (27500 rpm). The primary emulsion [W/O(water/oil)] was added to ultrapure water (1:2 ratio) under magnetic stirring (750 rpm) and mixed until W/O/W (water/oil/water) NE containing CARBA and LEV (CARBA+LEV-FDC-NE) was obtained.

The procedure mentioned above was used for the preparation of BLNK-NE without active ingredients.

In vitro characterization of BLNK-NE and CARBA+LEV-FDC-NE

Centrifugation test

To assess the stability of BLNK-NE and CARBA+LEV-FDC-NE under stress conditions, we centrifuged the formulation samples (5 g each) at 3500 rpm (15 min.). After centrifugation, we evaluated the NE formulations for phase separation, creaming, or active substance precipitation.

The morphology of CARBA+LEV-FDC-NE

The CARBA+LEV-FDC-NE formulation was diluted 100-fold. Later, it was placed on a copper grid and dried at room temperature for 24 h. Then, we obtained the transmission electron microscope (TEM) images of the CARBA+LEV-FDC-NE using a Hitachi TEM HT7700 (Hitachi HighTech, Japan) operated at an accelerating voltage of 120 kV.

Measurements of the DS, PDI, ZP, pH and viscosity of BLNK-NE and CARBA+LEV-FDC-NE

The DS, PDI, and ZP values of BLNK-NE and CARBA+LEV-FDC-NE were determined at room temperature using ZetaSizer Nano ZSP (Malvern Inc., UK) at a scattering angle of 173°. The NE formulations were diluted 100-fold before the measurement.

In addition, the pH and viscosity values of these formulations were determined at room temperature using a pH meter (Thermo Scientific, Orion 3 StarTM, USA) and a viscometer (Brookfield RV DV2T, USA), respectively.

FT-IR analyzes of the active substances, BLNK-NE and CARBA+LEV-FDC-NE

We recorded the FT-IR spectra (4000-400 cm^{-1}) of CARBA, LEV, BLNK-NE and CARBA+LEV-FDC-NE using the Shimadzu IRSpirit-T model FT-IR spectrophotometer (Japan).

Determination of EE% for CARBA+LEV-FDC-NE

CARBA+LEV-FDC-NE formulation (0.25 g) was weighed, and the volume was made up to 10 mL with methanol in a volumetric flask (n=6). The mixture was stirred under a magnetic stirrer for 15 min. Then, the mixture was filtered through a membrane filter [PTFE, 0.45 µm (Agilent Captiva)]. The CARBA and LEV contents in the filtrate were analyzed using the HPLC method developed and validated in our previous study (Kandilli, Uğur, Çetin, & Miloğlu, 2018).

In vitro release study

In vitro release study was performed in two different release media (HCl pH 1.2 or phosphate buffer pH 6.8) using the dialysis bag method. The NE formulation (1 mL)-containing dialysis bag (MWCO 14.000 Da) was placed in a colored vial containing 100 mL release medium in a horizontal shaking water bath (37±0.5 °C, 50 rpm). 1 mL of sample was taken from the release medium at the specified time intervals (0.5, 1, 2, 3, 4, 8, 12, and 24 h), and the same volume of fresh release medium (warmed at 37±0.5°C) was added to maintain the constant volume (to maintain "Sink condition"). The samples were filtered through a membrane filter [PVDF, 0.45 µm, Isolab]. The CARBA and LEV contents in the filtrate were analyzed using the HPLC method developed and validated in our previous study (Kandilli et al., 2018).

Statistical analysis

We used the independent-t test (SPSS Statistics 22.0; SPSS Inc., Chicago, USA) to compare the results obtained from our study and found the difference between the two independent groups. $p<0.05$ was considered "statistically significant".

RESULTS AND DISCUSSION

NEs composed of water, oil, and surfactant/s are kinetically stable systems (Nastiti et al., 2017; Ugur Kaplan et al., 2019). Due to their small droplet size, usually in the range of 20-500 nm (Gupta, 2020), NEs show long-term physical stability (Rehman, Akram, Seralin, Vandamme, & Anton, 2020). NE has drawn increasing attention for its great potential to enhance the solubility of poorly water-soluble active substances and improve their oral bioavailability. In addition, it is a beneficial dosage form that combines different advantages such as ease of preparation, being prepared to contain both hydrophilic and hydrophobic active substances, and protecting the relevant active substance/s from environmental conditions (Rosso et al., 2020; Sabjan, Munawar, Rajendiran, Vinoji, & Kasinathan, 2020). There are studies on the preparation of NEs for the combinational delivery of two active substances, but these are commonly for cancer treatment (Alkhatib, Bawadud, & Gashlan, 2020; Alkreathy et al., 2020; Mahajan & Patil, 2021; Pangeni, Choi, Jeon, Byun, & Park, 2016).

In the literature, there are studies on the NE formulations containing CARBA (Echeverri et al., 2020; Kelmann, Kuminek, Teixeira, & Koester, 2007; Prokhorov et al., 2014) and a microemulsion formulation containing LEV (Djekic et al., 2021). However, there is no study on a NE formulation containing LEV and CARBA (in combination).

In our study, an FDC-NE formulation containing CARBA and LEV (active substances, one hydrophobic and the other hydrophilic) was developed, and *in vitro* characterization studies were carried out. In this way, we aimed to use two active substances with different action mechanisms in combination and to achieve an improvement in the solubility and oral bioavailability of CARBA.

In the centrifugation test, phase separation, creaming, or active substance precipitation was not observed in the NE formulations. The TEM images of CARBA+LEV-FDC-NE are shown in Figure 1. The DS, PDI and ZP values of BLNK-NE and CARBA+LEV-FDC-NE are given in Table 1.

DS, PDI, and ZP measurements are essential to assess the physical stability of NEs. In NE, which is a colloidal dispersion, the Brownian motion of small droplets contributes to resistance to physical destabilization caused by creaming, gravitational separation, and coalescence (Mahamat Nor, Woi, & Ng, 2017; Santos, Trujillo-Cayado, Carrillo, López-Castejón, & Alfaro-Rodríguez, 2022). Also, smaller droplets have a larger surface area, which provides a larger contact area with the intestinal mucosa, and as a result, NEs increase the absorption of the active substance (Hu, Xie, Zhang, Qi, & Li, 2021). In our study, the DS values of the NE formulations were in the nano-range (Table 1). The TEM images of CARBA+LEV-FDC-NE also supported this

result (Figure 1). Moreover, the droplets of CARBA+LEV-FDC-NE were approximately spherical in the TEM images (Figure 1). There was a significant difference ($p<0.05$) between the DS values of the BLNK-NE and CARBA+LEV-FDC-NE. The presence of active substances caused a slight increase in the DS of NE. The PDI values of the NE formulations were <0.3 (Table 1). This shows that the droplet size distributions of the NEs are in a narrow range and acceptable (Uğur Kaplan et al., 2019). The ZP values of BLNK-NE and CARBA+LEV-FDC-NE were about (-)26 mV and (-) 22 mV, respectively (Table 1). The difference between the ZP values of these formulations was significant ($p<0.05$). The change in the ZP value could be due to the chemical structure of CARBA and LEV. In a study, the effects of active substances with different physicochemical properties on the nanoemulsion formulation were investigated and compared to blank nanoemulsion with neutral ZP. It was stated that the ZP value of the nanoemulsion formulation containing cyclosporine A, which has an amine group in its structure, shifted slightly to positive, while the nanoemulsion containing curcumin with a hydroxyl group had a negative value (Wik, Bansal, Assmuth, Rosling, & Rosenholm, 2020). ZP values greater than or equal to (+) 30 mV and less than or equal to (-) 30 mV provide good physical stability for emulsions (Mahamat Nor et al., 2017). However, ZP values around (+/-) 20 mV can provide sufficient physical stabilization in the formulations where electrostatic stabilization is combined with steric stabilization. Non-ionic surfactants such as Tween 80 contribute to the physical stabilization of NEs by decreasing interfacial tension and providing a steric barrier against coalescence (Ugur Kaplan et al., 2019).

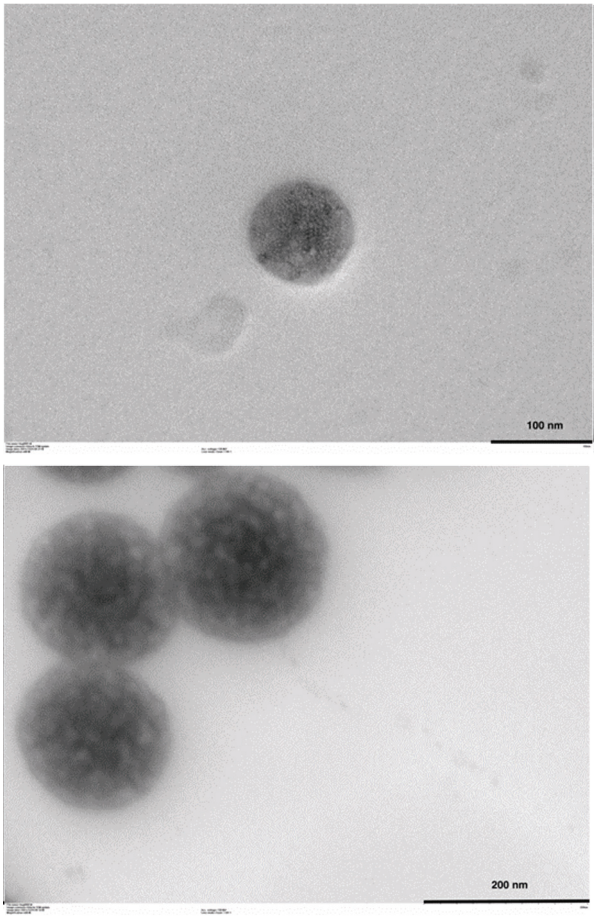

Figure 1. The TEM images of CARBA+LEV-FDC-NE.

pH is a critical parameter to be determined during the preparation of aqueous liquid formulations because it can affect the solubility and activity of the active substance, as well as the stability and biological tolerability of the formulation (Vázquez-Blanco, González-Freire, Dávila-Pousa, & Crespo-Diz, 2018). In a study, it was determined that CARBA was more stable under acidic conditions (Rajadhyaksha, Jain, & Amin, 2007). In another study, it was reported that LEV formulations prepared in the "Ora-Sweet and Ora-Plus" vehicle could remain stable for 91 days when they were stored at either 25°C (at pH 4.25) or 4°C (at pH 4.34) (Ensom, Decarie, & Rudolph, 2011). In our study, the pH value for both BLNK-NE and CARBA+LEV-FDC-NE was about 4.6 (Table 1). The difference between the pH values of both formulations was not significant ($p>0.05$).

FT-IR analysis can be performed to determine the presence of any interaction between the active substance/s and other formulation components. Therefore, we carried out FT-IR analysis in our study and gave the FT-IR spectra of active substances and the NE formulations in Figure 2. In this study, the reobtained FT-IR spectra for the active substances (CARBA and LEV; Figure 2) are compatible with the FT-IR spectra of CARBA, and LEV presented in our previously published paper (Kandilli et al., 2020). Moreover, the FT-IR spectra of BLNK-NE and CARBA+LEV-FDC-NE formulations given in Figure 2 were similar, and the peaks related to CARBA or LEV did not appear in the FT-IR spectrum of CARBA+LEV-FDC-NE. Consequently, there was no interaction between the formulation components with the active substances (CARBA and LEV). Also, CARBA and LEV dispersed in NE formulation at the molecular level.

The EE% values obtained for CARBA+LEV-FDC-NE were found to be 97.33±0.56% (for CARBA) and 96.73±0.91% (for LEV).

Figures 3-a and 3-b show the *in vitro* release profiles of CARBA and LEV from CARBA+LEV-FDC-NE in the two different release media (HCl pH 1.2 and phosphate buffer pH 6.8). In HCl pH 1.2,

Table 1. The DS, PDI, ZP and pH values of BLNK-NE and CARBA+LEV-FDC-NE (Mean±SD; n=9).

Formulation	DS (nm)	PDI	ZP (mV)	pH
BLNK-NE	117.63±3.82	0.240±0.014	(-)26.07±3.04	4.62±0.03
CARBA+LEV-FDC-NE	137.56±3.11	0.225±0.013	(-)21.62±0.29	4.60±0.06

SD: Standard deviation; BLNK-NE: Blank nanoemulsion; CARBA+LEV-FDC-NE: CARBA and LEV-containing fixed-dose combination nanoemulsion

the percent of CARBA released from NE formulation at 30 min and 24 h were about 9% and 99%, while the percent of LEV released from NE formulation at 30 min and 2 h were about 80% and 100%, respectively (Figure 3-a). In phosphate buffer (pH 6.8), the percent of CARBA released from NE formulation at 30 min and 24 h were about 9% and 97%, while the percent of LEV released from NE formulation at 30 min and 2 h were about 81% and 100%, respectively (Figure 3-b). According to these results, the pH of the release medium has no effect on the release of CARBA and LEV from the NE formulation. LEV, a hydrophilic active substance, was released more rapidly than the NE formulation, but CARBA, a hydrophobic active substance, was released more slowly than the NE formulation. This result obtained for CARBA may be due to its limited diffusion into the aqueous phase (external phase) of the emulsion (Echeverri et al., 2020).

Formulation components (such as water, oil, and surface-active agent/s) and their concentrations affect the viscosity of NE formulations. For example, when the surfactant content in the formulation decreases, the viscosity of the formulation increases, while increasing the water content in the formulation can cause a decrease in the viscosity (Lovelyn & Attama, 2011) Table 2 and Figure 4 show the viscosity values and rheograms of the BLNK-NE and CARBA+LEV-FDC-NE formulations, respectively. The difference between the viscosity values of the BLNK-NE and CARBA+LEV-FDC-NE formulations was significant ($p<0.05$). The presence of active substances (CARBA and LEV) in the formulation caused a slight increase in the viscosity of the NE formulation (Table 2).

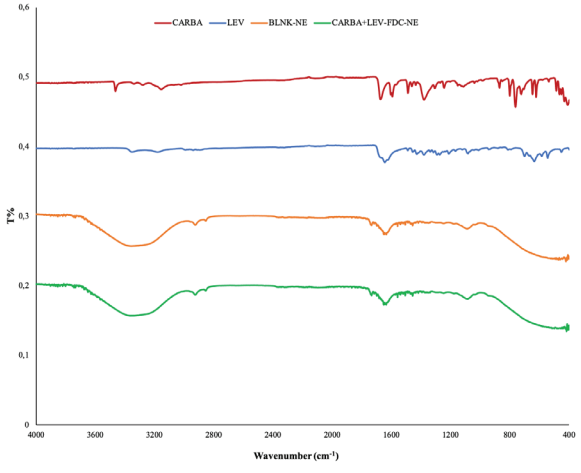

Figure 2. The FT-IR spectra of CARBA, LEV, BLNK-NE and CARBA+LEV-FDC-NE.

Prokhorov et al. (2014) prepared the CARBA-NE formulation and evaluated the anticonvulsant activity of the CARBA-NE against maximum electric shock- or picrotoxin-induced seizures in mice. They reported that the CARBA-NE formulation has more anticonvulsant effect than pure CARBA against seizures.

Echeverri et al. (2020) prepared NE formulation containing CARBA using an ultra-high-pressure homogenization method. The DS, PDI, ZP, pH, and viscosity values of freshly prepared NE formulation were 320.90 nm, <0.3, (-)11.29 mV, 5.9, and 2.27 cP, respectively. The *in vitro* release study for NE formulation was performed in phosphate buffer pH 7.4 using a dialysis bag (MWCO 14 kDa). They reported that the release of CARBA from NE formulation was pretty low. In *in vivo* study, they found

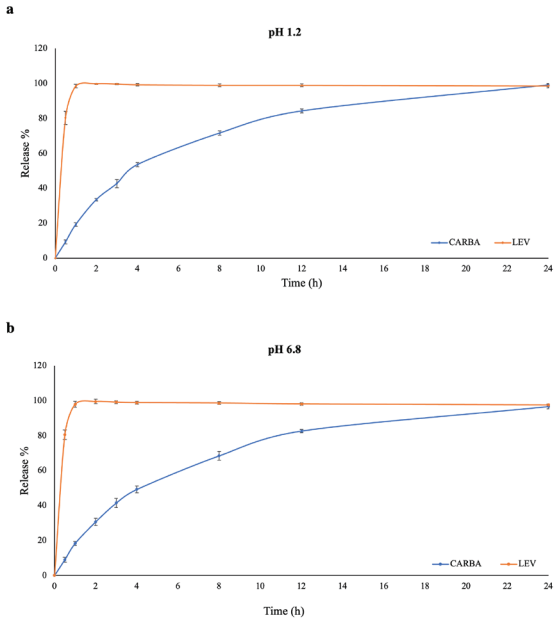

Figure 3. The release profiles of CARBA and LEV from the NE formulation in the different release media (Mean±SD; n=3).

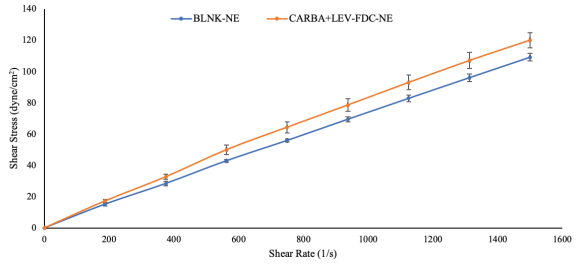

Figure 4. The rheograms of the BLNK-NE and CARBA+LEV-FDC-NE formulations (Mean±SD; n=3).

Table 2. The viscosity values of the BLNK-NE and CARBA+LEV-FDC-NE formulations (Mean±SD; n=3).

Formulation	Viscosity (cP) (at shear rate: 1500 s^{-1})
BLNK-NE	7.28±0.17
CARBA+LEV-FDC-NE	8.01±0.32

SD: Standard deviation; BLNK-NE: Blank nanoemulsion; CARBA+LEV-FDC-NE: CARBA and LEV-containing fixed-dose combination nanoemulsion

that the time to the maximum plasma concentration ($t_{max)}$ for the CARBA-NE formulation or the coarse emulsion containing CARBA administered orally to New Zealand breed rabbits was 45 min and 12 h, respectively.

In another study, an *in vitro* release study for the CARBA-containing NE formulation (The characteristics of this formulation= DS:148.47 nm, PDI: 0.211, ZP: (-)39.68 mV, and CARBA content: 95.25%) was performed in phosphate buffer pH 7.4 using the dialysis bag method (MWCO: 100000 Da). About >90% of CARBA was released from NE formulation within 11 h (Kelmann et al., 2007).

In our study, for oral administration in the treatment of epilepsy, the CARBA+LEV-FDC-NE formulation was successfully prepared. This formulation had suitable *in vitro* characterization results. As a result of the study, it was seen that the droplet size of CARBA+LEV-FDC-NE was 137.56 nm with a narrow size distribution (PDI <0.3), and the ZP of the formulation was sufficient for physical stability. Also, high EE% values were obtained for both active substances. CARBA+LEV-FDC-NE formulation might be beneficial in the treatment of epilepsy.

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Original Article

Development and statistical optimization of carvedilol floating beads for chronotherapeutic drug delivery

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ABSTRACT

Background and Aims: The aim of the present research work was to develop and optimize statistically carvedilol floating beads for chronotherapeutic drug delivery.

Methods: Multiple unit floating pulsatile beads of carvedilol were prepared by simple ionotropic gelation method intended for chronotherapy of hypertension. Pectin and sodium alginates were used as matrix forming, polymer and sodium bicarbonate was used as floating agent. A 2^3 full factorial design was applied to investigate the combined effect of three independent formulation variables namely amount of sodium alginate, sodium bicarbonate and calcium chloride on the dependent variables as % entrapment efficiency, floating lag time and drug release percentage.

Results: The formulation was optimized and tested based on its drug release pattern which presented minimum drug release in 0.1 N HCl and after 6 h lag time period and showed maximum drug release in 6.8 pH phosphate buffer by burst release within 45 mins. Surface response plots were presented graphically to represent the effect of independent variables on floating lag time, entrapment efficiency and drug release in 0.1 N HCl. The generated mathematical model for each response was validated and checked by formulating three extra-design checkpoint batches. There were no significant changes in drug content, floating lag time, entrapment efficiency and drug release of the formulation following its stability studies at 40 ^{0}C and 75% relative humidity.

Conclusion: It was concluded that the floating beads were successfully formulated for chronotherapy of hypertension giving site- and time specific release of drug.

Keywords: Floating beads, drug release, chronotherapy, ionotropic gelation, hypertension

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INTRODUCTION

Hypertension is one of the common cardiovascular disorders of modern times. Hypertension is the state of the body in which systolic blood pressure is 140/90 mmHg or more. One third of men and two fifth of women over 40 years of age are hypertensive because of age progression (Tripathi, 2002). Blood pressure is not steady over a 24 hours period, and also it is fluctuating according to a circadian pattern (Sajan, Cinu, Chacko, Litty, & Jaseeda, 2009). The chronobiology deals with the observation of each metabolic event goes through rhythmic changes in time that will be measured from seconds to seasons. During the chronotherapeutic treatment system in vivo drug has been timely available according to cyclic pulsing of drug related biological phenomenon to create maximum benefit and minimizing the harm. Biological rhythms at the level of cellular and sub cellular can raise the significant dosing-time differences in the pharmacodynamics of medications that are unrelated to their pharmacokinetics mechanism. This phenomenon is termed chronesthesy. Rhythms in receptor number or conformation, second messengers, metabolic pathways, or free-to bound fraction of medications are useful to elucidate this phenomenon. Blood pressure reaches its lowest point around 3 a.m. by gradual declination throughout the day and especially during sleep (Smolensky, & Peppas, 2007). This is because of many factors like the time of awakening, rise in physical activity, serum cortisol level and catecholamine levels which all increase blood pressure, heart rate and myocardial contractility. A pathophysiologic explanation for the myocardial infarction, sudden cardiac death and angina pectoris in the early morning hours is explained by the above factors (Sajan et al., 2009). Currently, chronotherapeutic calcium channel blockers are available in the market for the management of certain cardiovascular diseases. When administered at bedtime, these provide a peak effect coinciding with the rise in blood pressure and heart rate in the critical time period of 6:00 a.m. to noon, and trough concentrations during sleep (Singhai, Chopra, Nagar, Gautam, & Trivedi, 2010; Shan, & Kawashima, 2012).

Carvedilol has a beta-adrenergic receptor blocking ability and decreases the rate of heart, myocardial contractility, and myocardial oxygen requirement. Carvedilol is used to decrease systemic vascular resistance with its alpha-adrenergic receptor blocking properties. Its absorption after oral administration is rapid and extensive with an absolute bioavailability of approximately 25 % to 35 % due to the significant degree of first pass metabolism. Conventional dosage forms of carvedilol in the case of hypertension have several disadvantages like less bioavailability, more dose requirement, non-compliance of patient and other toxic adverse effects (Tanwar, Chauhan, & Sharma, 2007). Therefore, the present study of multiple unit dosage forms in the form of floating beads offers more reliability and flexibility than single-unit dosage forms.

Floating drug delivery system (FDDS) is helpful for drugs which have an absorption window in the stomach or in the upper small intestine. It is also helpful for drugs that act locally in the proximal part of gastrointestinal (GI) tract in case of antibiotic administration for *Helicobacter pylori* eradication in the treatment of peptic ulcer and for drugs that are poorly soluble or unstable in the intestinal fluid. A disadvantage of distinct unit floating systems like tablets and capsules is the high unpredictability of the GI transit time due to their all-or-nothing emptying processes (Patel, Dalvadi, & Shah, 2011). On the other hand, the multiple-unit dosage forms may be an attractive alternative since they have been shown to reduce inter and intra subject variability in drug absorption as well as lowers the possibility of dose dumping (Pongjanyakul, & Puttipipatkhachorn, 2007).

Therefore, this study investigated floating beads to treat hypertension by offering chronotherapeutic effect of carvedilol.

MATERIAL AND METHODS

Materials

Carvedilol was obtained as a gift sample from Sun Pharma Ltd., Vadodara, Gujarat. Low methoxy pectin with high molecular weight (6.08×105 Daltons or higher) was procured from Pecti Chem Industry, Jodhpur. Pectin and calcium chloride were purchased from Sulab Laboratory, Vadodara. Sodium bicarbonate was purchased from Chemdyes, Corporation, Baroda. Acetic acid was purchased from Sisco Research Laboratory, Pvt. Ltd. All the other chemicals and solvents used were of analytical grade.

Methods

Drug excipient compatibility study

Drug excipient compatibility was studied using Bruker alpha T FTIR spectroscopy (Bruker Optik GmbH, Germany). The FTIR studies were performed using the pressed Pellet technique with a KBr press. Potassium bromide (100 mg) was taken and kept in a hot air oven for two hours to remove any moisture if present. The drug powder and excipient mixture sample (10 mg) was mixed by using dried KBr crystals, and the mixture was pressed to form pellets with KBr press. The prepared pellet was placed in the sample holder and kept in the instrument to record the peaks (Ammanage, Rodriques, Kempwade, & Hiremath, 2020).

Preparation of preliminary trial batches for selection of the cross-linking medium, polymer concentration and sodium bicarbonate concentration

Preliminary trial batches of floating beads were formulated by varying concentrations of polymers, gas generating agent and calcium chloride as shown in Table 1. Cross linking solution was made by dissolving different concentrations of calcium chloride in the deionized water. For the selection of optimum polymer concentration, floating beads of various polymer concentrations were formulated and analyzed for morphology and drug entrapment efficiency (Table 2).

Preparation of floating beads

The beads are commonly prepared using ionotropic gelation technique. In this technique, an aqueous dispersion of negatively charged polymer together with a gas generating agent ($CaCO_3$ or $NaHCO_3$) was added drop-wise into acidic gelation medium consisting of divalent cations such as Ca^{2+}. As the droplet immerses into the acidic gelation medium, CO_2 is

Table 1. Preliminary trial batches for selection of concentration of sodium alginate (T1 to T4), sodium bicarbonate (T5 to T7) and calcium chloride along with curing time (T8 to T16).

Batch No.	Amount of drug (mg)	Sodium alginate (mg)	Concentration of calcium chloride (%)	Ratio of sodium alginate: Pectin (mg)	pH of cross linking solution	Concentration of sodium bicarbonate	Curing time
T1	5	50	2		1.2		
T2	5	100	2		1.2		
T3	5	150	2		1.2		
T4	5	300	2		1.2		
T5	5		2	300:300	1.2	100	
T6	5		2	300:300	1.2	300	
T7	5		2	300:300	1.2	500	
T8	5		2	300:300	1.2		15
T9	5		3	300:300	1.2		15
T10	5		4	300:300	1.2		45
T11	5		2	300:300	1.2		45
T12	5		3	300:300	1.2		30
T13	5		4	300:300	1.2		30
T14	5		2	300:300	1.2		30
T15	5		3	300:300	1.2		45
T16	5		4	300:300	1.2		15

Table 2. Selection of pH of the cross linking medium (T1 to T4), polymer concentration (T5 to T8), sodium bicarbonate (T9 to T11), calcium chloride and (T12 to T20) along with obtained FLT, FT and entrapment efficiency.

Batch code	Polymer Concentration (mg)	pH of the media	Morphology	Floating Lag time (mins)	Floating time (Hours)	Entrapment efficiency (%)
T1	300	6.2				34.01
T2	300	6.4				26.78
T3	300	1.4				56.34
T4	300	1.4				68.98
T5	50		Uneven			34.97
T6	100		Disc shaped			49
T7	150		Spherical			75.07
T8	300		Spherical			89
T9				10	4	76.7
T10				1	8	86
T11				<1	9	67
T12						61
T13						76.75
T14						78.06
T15						63.21
T16						61.56
T17						70
T18						63.45
T19						86
T20						79.42

released due to the gas-generating agent which is then captured in the gel matrix making it lighter than stomach fluids, and concurrently Ca^{2+} present in gelation medium interacts with anionic groups on polymer molecule resulting immediate formation of strong aggregation of pairs of helices giving strong bead shaped gel structures (Verma, Sharma, Verma, & Pandit, 2013).

Varying quantities of pectin and sodium alginate were dissolved in deionized water along with carvedilol with varying amounts of sodium bicarbonate as shown in Table 1. All these were mixed uniformly. The sonicated dispersion free of air bubbles was dropped via a 23-gauge syringe needle (0.65 mm internal diameter) into different concentrations of calcium chloride solution having 10 % acetic acid. The contents were stirred at 100 rpm with magnetic stirrer (Remi Instrument Ltd., Mumbai, India) for 15 mins. The beads were then filtered, washed three times with distilled water and subsequently dried in oven at 50 ^{0}C for 4h (Reddy, & Reddy, 2017; Abduljabbar, Badr-Eldin, & Aldawsari, 2015; Torre et al., 1998; Patil, Indikar, & Umarji, 2015).

Evaluation of floating beads

Flow property

The floating beads were evaluated for particle size, bulk density, tapped density, Carr's compressibility index, Hausner's ratio and angle of repose (Aulton, 2002; Lachman, Lieberman, & Kanig, 1991; Patrick, 2006).

Particle size determination

The particle sizes of formulations were measured by using an optical microscope fitted with an ocular and stage micrometer. In all measurements at least 100 particles were examined, and each experiment was carried out in triplicate (Khonsari, Zakeri-Milani, & Jelvehgarid, 2014).

Surface analysis by scanning electron microscopy (SEM)

Morphology and surface characteristics of the floating beads were performed by using a scanning electron microscope (SEM) JSM 840 [Jeol, Tokyo, Japan] **(**Patil, Indikar, & Umarji, 2015; Lachman, Lieberman, & Kanig, 1991**)**. For assessment of the internal structure of the beads, they were cut into a half with a steel blade.

Determination of entrapment efficiency

Carvedilol content in the floating beads was determined at 332 nm by a UV-spectrophotometric method. (UV 1800 Shimadzu) (Abbas, & Alhamdany, 2020; Gupta, & Pathak, 2008). The drug entrapment efficiency was determined using following equation

Drug Entrapment Efficiency % = (Actual drug content/ Theoretical drug content) x 100.

Floating study

Floating properties of beads were evaluated in a dissolution vessel of USP type II dissolution Tester (Electrolab TDT-08L, Electrolab (India) Pvt. Ltd.) using 500 ml of simulated gastric fluid (pH 1.2). Paddle rotation speed of 0 and 100 rpm were tested maintaining the temperature at 37 ± 0.2 ° C. Fifty beads were placed in the media, and floating time was measured by visual observation (Aulton, 2002).

Dissolution study

The dissolution study of the beads equivalent to 10 mg of carvedilol was performed using a USP rotating basket apparatus (Electrolab TDT-08L). The drug release study was performed in 0.1N hydrochloric acid primarily for 2 or 9 hours depending on the floating characteristics of the beads followed by dissolution in phosphate buffer pH 6.8 for two hours. Each 900 ml of dissolution medium was maintained at 37±0.5 0 C and agitated at 100 rpm. Periodically, the samples were withdrawn and filtered through Whatman filter paper, and the concentration of carvedilol was measured using UV spectrophotometer at 332 nm. Withdrawn volume of dissolution medium was replaced by adding fresh dissolution medium to maintain sink condition (Lachman, Lieberman, & Kanig, 1991; Aulton, 2002). All the batches F1-F8 were subjected to dissolution study in simulated gastric fluid without enzymes initially for 2-9 h based on floating time (T80) of the beads followed by dissolution in phosphate buffer for two hours. Chronotropic drug delivery systems require drug release as soon as possible after the predetermined lag time for lowering hypertension in the early morning. Predetermined lag time for relieving hypertension in the early morning is 6 h.

Further drug release mechanism was studied using power law equation (Siepmann & Peppas, 2001; Zhang, Zhang, & Wu, 2003).

$$Mt/M\infty = K(t-T)^n \quad (1)$$

Where Mt and M∞ are the absolute cumulative amount of drug released at time t and infinite time, respectively; K is a constant, T is lag time and n is the release exponent, indicative of the mechanism of drug release.

Design of experiment for optimization of formulations

A 3^2 factorial design was performed with Design-Expert (8.0.7.1 Trial Stat Ease Inc., Minneapolis, USA). It was used for exploring quadratic response surfaces and constructing polynomial models. In this three factors X1, X2 and X3 were evaluated at two levels, and experimental trials were carried out at all eight possible combinations. The factors were selected based on the trial batches. The concentration of sodium alginate (X1), concentration of sodium bicarbonate (X2) and concentration of calcium chloride (X3) were selected as independent variables. The entrapment efficiency (Y1) in pH 6.8 phosphate buffers, floating lag time (Y2) and drug release (Y3) in 0.1N HCl were selected as dependent variables (Dhoranwala, Shah, & Shah, 2015).

Statistical analysis and mathematical model fitting

The targeted response parameters were statistically analyzed by applying one-way ANOVA (analysis of variance), and the significance of the model was estimated using the Design Expert software. The individual parameters were evaluated using F test, and mathematical relationship was generated between the factors (independent variables) and the responses (dependent variables) using multiple linear regression analysis

for determining the level of factors which yield optimum dissolution responses (Menini, Furalanetto, Maestrelli, Pinzauti, & Mura, 2008).

Validation of mathematical model

To find the reliability of the developed mathematical model, all of three responses were checked for three additional random check point batches (C1, C2 and C3) covering the entire range of experimental domain. For each of these test runs, responses were estimated by use of generated mathematical model and by the experimental procedure.

Stability studies

The studies were performed at 40±2 ^{0}C and 75±5 % relative humidity (RH) in the sealed glass chambers with saturated salt solution for up to 1 month. A visual inspection, drug content, floating study *and in vitro* drug release studies were carried out every 15 days for the entire period of stability study (EMA, 2003).

RESULTS AND DISCUSSION

Drug excipient compatibility study

FTIR spectra of pure drug (carvedilol) showed the characteristic peak at 1594.26 cm^{-1} (-C-C- stretching), 3343.76 cm^{-1} (-N-H- bending), 3058.50 cm^{-1} (-O-H stretching), 1214.18 cm^{-1} (-C-O-C stretching), 3058 cm^{-1} (C-H stretching). IR spectra of formulation showed the characteristics peak at 1586.83 cm^{-1} (-C-C- stretching), 3410.69 cm^{-1} (-N-H- bending), 2933.51 cm^{-1} (-O-H stretching),1218.40 cm^{-1} (-C-O-C stretching), 2933.91 cm^{-1} (C-H stretching). The FTIR spectra of formulation of the drug with excipients and peaks present in IR spectra of formulation were nearly similar to the frequency of principle peaks present in IR spectra of pure drug which confirmed the absence of any chemical interaction between them. The results are revealed in Figure 1.

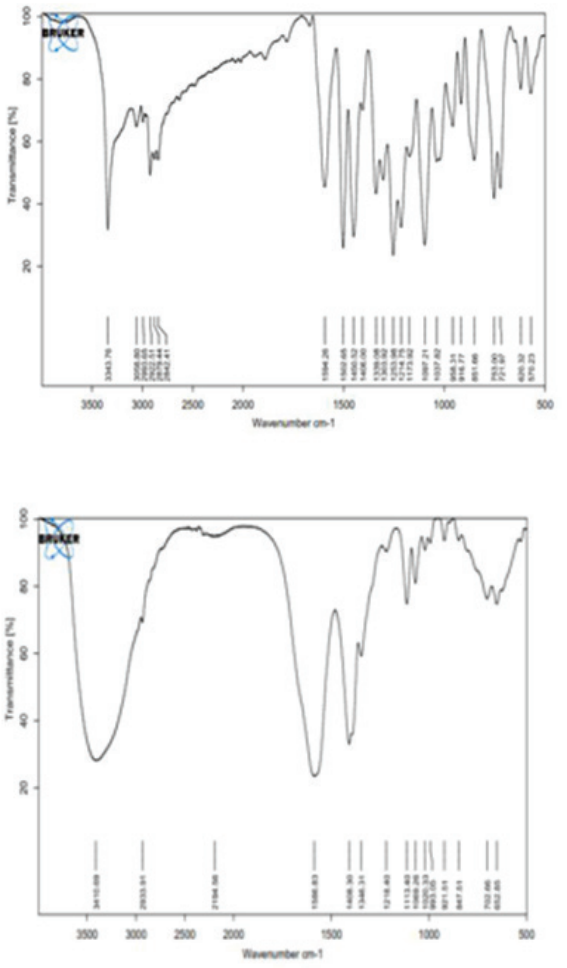

Figure 1. IR spectra of pure Carvedilol and floating beads formulation.

Preparation of preliminary trial batches for selection of the cross linking medium, polymer concentration and sodium bicarbonate concentration

It was observed from the preliminary trial batches that beads prepared by aqueous calcium chloride solution were having very low entrapment efficiency. It was found that at low polymer concentration, beads were of disc shaped and had a weak gelling capacity. Low gelation capacity resulted in low entrapment efficiency. It was found from the trial batches that as polymer concentration increased, the shapes of the beads were shifted towards spherical, and drug entrapment efficiency was also found to increase up to optimum level. This might be due to the increase in the polymer concentration; there is a greater availability of active calcium binding sites in the polymeric chains and greater degree of cross linking. On the other hand, the increase in the polymer concentration may also reduce the loss of the drug in the curing medium due to formation of dense matrix structure.

The beads prepared from 50 mg alginate solution had entrapment efficiency of 34.97%, while the beads prepared from 100 mg and 150 mg alginate solution were having entrapment efficiency 75.07% and 89%, respectively, which showed good entrapment. By increasing the polymer concentration, viscosity was also increased, and the shape of the beads was spherical, and the size was also uniform. The results are shown in Table 2.

From the results, it was found that as the concentration of sodium bicarbonate was increased floating efficiency, and floating time was also increased and floating lag time was decreased. By decreasing the concentration simultaneously, the beads were ruptured, and so entrapment efficiency was decreased, but at optimum concentration, entrapment efficiency was found to increase. In these trial batches, T9 batch was found to have 76.7% entrapment efficiency, but time of floating was decreased, and floating lag time was more than 10 mins which is undesirable. In the batch T10 entrapment, efficiency and floating time was 86% and 8 h, respectively. But, at higher concentration in T11 batch due to fast generation of carbon dioxide beads were ruptured, and hence it is concluded that higher concentration decreased the entrapment efficiency as revealed in Table 2.

Selection of calcium chloride and curing time

From the results, it was found that as the concentration of calcium chloride increased cross linking increased, and hence entrapment efficiency was also increased. As curing time increased, the entrapment efficiency was increased, but it was insufficient. In Batch T14, at higher concentration of calcium

chloride and at more curing time the drug entrapment, efficiency was increased. In Batch T12, it was found less (Table 2).

From the results of the preliminary trial batches, two different levels of sodium alginate, sodium bicarbonate and calcium chloride were selected to optimize the formulation using 2^3 factorial design. The formulations batches using factorial design are shown in Table 3.

Evaluations of floating beads

Flow property

As shown in Table 4, as the concentration of sodium bicarbonate was increased, the size of the particles was found to increase. Batches F1, F3, F5, F8 have the particle size greater than the other batches. The results of bulk density and Carr's index for all the batches F1-F8 were found to comply the suggested range. The angle of repose for all the formulation was found 27.20^0 ±0.87 to 30.27^0 ±0.77, which indicated good flow property.

The results showed that all the formulations batches had the good flow properties.

Entrapment efficiency

It was observed that with the increase in the polymer concentration entrapment, efficiency was found to increase (Table 4). It was affected by the polymer concentration and calcium chloride concentration. Increased in the calcium chloride concentration, the cross-linking ability was also increased because of the more availability of the spare chloride ions. Thus, Batch F1 has highest entrapment efficiency of 89.41 %, which was more than other batches.

Floating study

The results of the floating study showed that with the increase in concentration of sodium bicarbonate, carbon dioxide gas generation was increased, and the beads were found to float for longer period of time. Floating lag time was decreased as the sodium bicarbonate increased. Thus, F1, F3, and F8 have less floating lag time than the other batches (Table 4).

Surface analysis by SEM

The SEM study of the floating beads showed that the beads were having uniform spherical shape and presence of entrapped gas bubbles. The surface examination of uniform spheres under higher magnification showed smooth surface. The results are revealed in Figure 2.

Dissolution study

All the batches of the beads released 1.1±0.45 % to 24.0±0.37 % of the drug in 0.1 N HCl. Thus, the floating beads showed

Table 3. Formulation of floating beads using 2^3 factorial designs.

Batch No.	Sodium Alginate concentration (mg)	Sodium bicarbonate concentration (mg)	Calcium chloride (%)
F1	300(+)	225(+)	4(+)
F2	300(+)	115(-)	4(+)
F3	300(+)	225(+)	3(-)
F4	0(-)	115(-)	3(-)
F5	0(-)	225(+)	3(-)
F6	0(-)	115(-)	4(+)
F7	300(+)	115(-)	3(-)
F8	0(-)	225(+)	4(+)

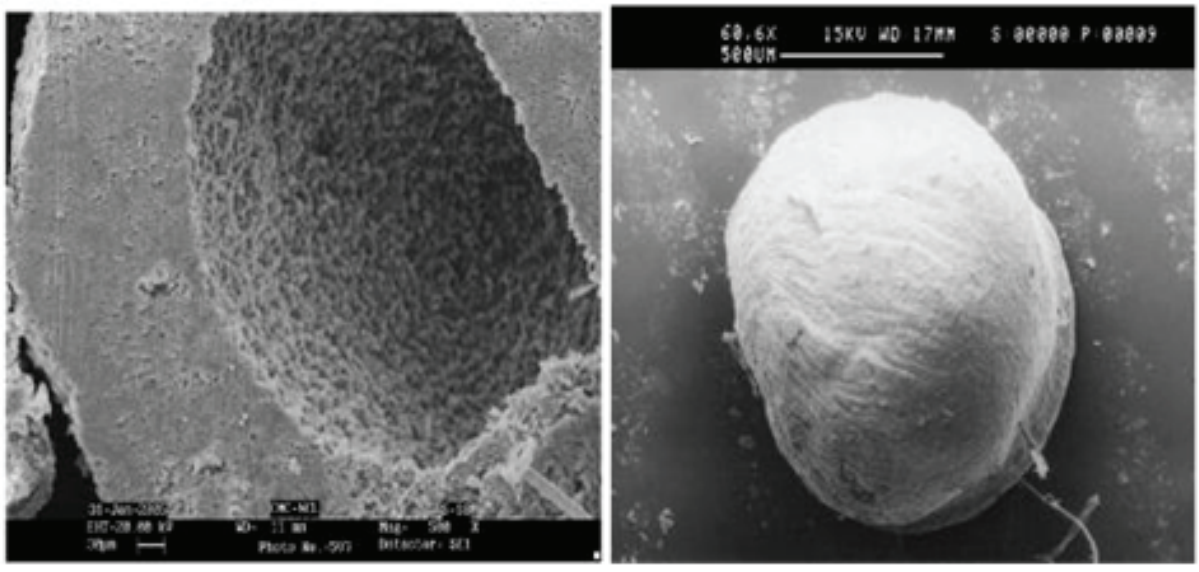

Figure 2. SEM images for surface analysis of floating beads (Optimized batch C3).

excellent lag time in drug release at acidic pH. All the batches of the floating beads released 84.00±0.75 % to 98.0±0.20 % cumulative release in pH 6.8 phosphate buffer. The results are revealed in Figure 3.

The exponents were calculated by using equation $Mt/M\infty = K(t-T)^n$. R^2= 0.7383 and n= 0.27, indicating that swelling-controlled drug release mechanism is involved in the drug release. Thus, chronotherapeutic release of the drug in the morning for the treatment of hypertension was achieved by preparation of floating beads.

Table 4. Evaluation of floating beads.

Batch No.	Particle size* (mm)	Bulk Density* (g/cm³)	Carr's Index (%)	Entrapment efficiency* (%)	Floating Lag time (mins)	Angle of Repose* (degrees)
F1	1.97± 0.09	0.320±0.02	10.36	89.41±0.02	1	27.20 ±0.87
F2	1.43± 0.05	0.485±0.02	2.53	82.52±0.02	6	28.65 ±1.65
F3	1.82± 0.09	0.658±0.01	3.99	86.98±0.03	1	30.20 ±0.85
F4	1.41± 0.05	0.699±0.10	1.92	76.79±0.03	7	30.33 ±0.75
F5	1.92± 0.08	0.481±0.03	9.10	85±0.02	1.5	28.24 ±0.54
F6	1.38± 0.05	0.357±0.07	8.98	81.5±40.02	6.5	28.98 ±0.93
F7	1.45± 0.05	0.645±0.06	4.89	80.27±0.03	8	29.59± 1.65
F8	1.86± 0.08	0.627±0.11	5.76	86.98±0.03	1	30.27 ±0.77

*Mean±SD;n=3

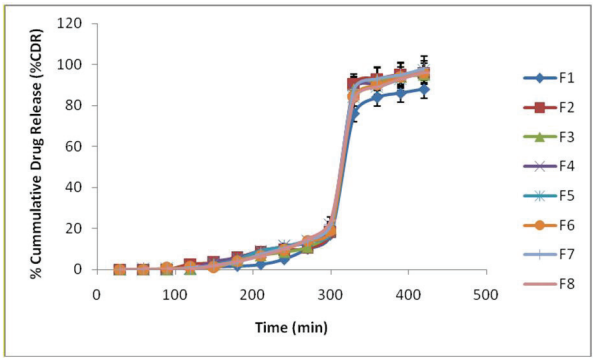

Figure 3. Dissolution study of batches F1-F8.

Optimization of formulation using 2^3 factorial designs

Batches F1 and F4 were found to release the drug almost completely within 40 mins in 6.8 pH phosphate buffer, while Batches F5 and F8 were found to release the drug 97.00±0.63 and 96.060±0.87, respectively within 40 mins. So, cumulative drug release in pH 6.8 phosphate buffer within 40 mins was selected as one of the response parameters. The formulation should also have sufficient floating time in the stomach to release the drug specifically to the small intestine, and the formulation was supposed to have minimum drug release in gastric acid condition to provide a lag time.

Statistical analysis and mathematical model fitting

The F-value 60.62 of entrapment efficiency implied the model was significant. The ratio of 20.853 indicated an adequate signal.

% Entrapment efficiency (Y1) = 62.615+6.620X1+0.0598X2+3.0825X3

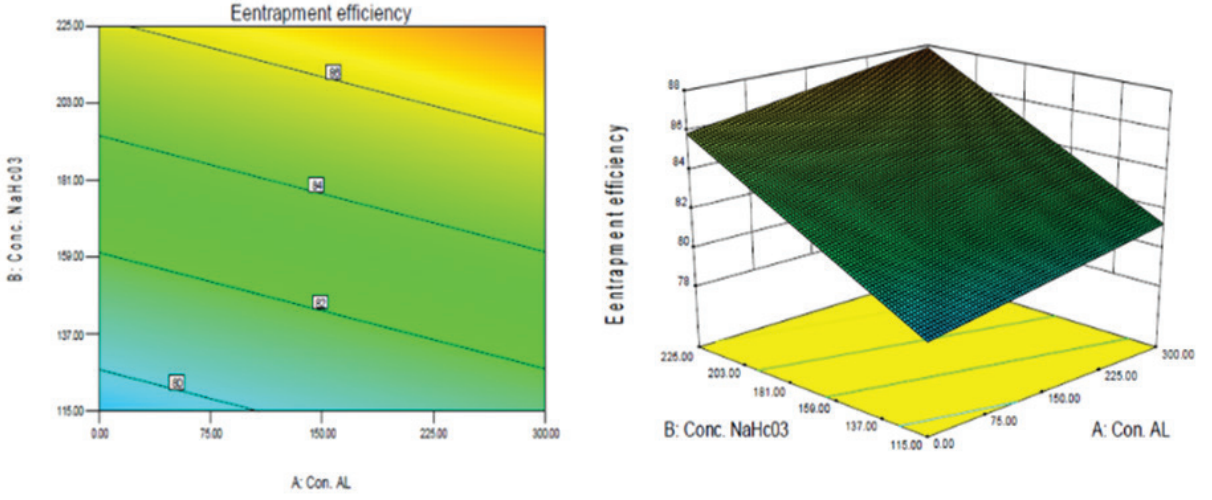

Figure 4. Countour plot and response surface plot for (%) entrapment.

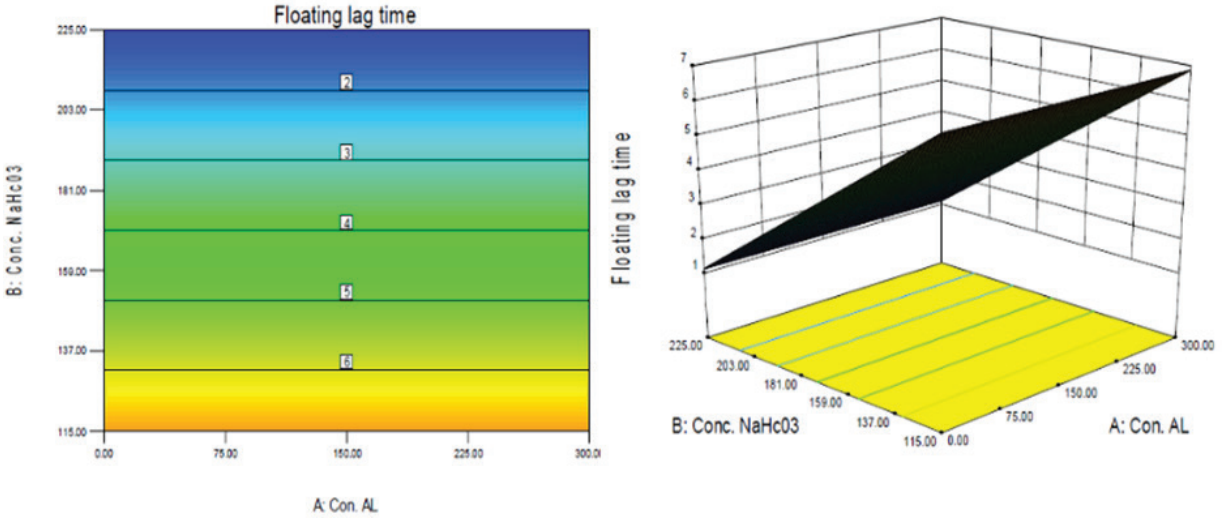

Figure 5. Countour plot and response surface plot for Floating Lag time (Y2).

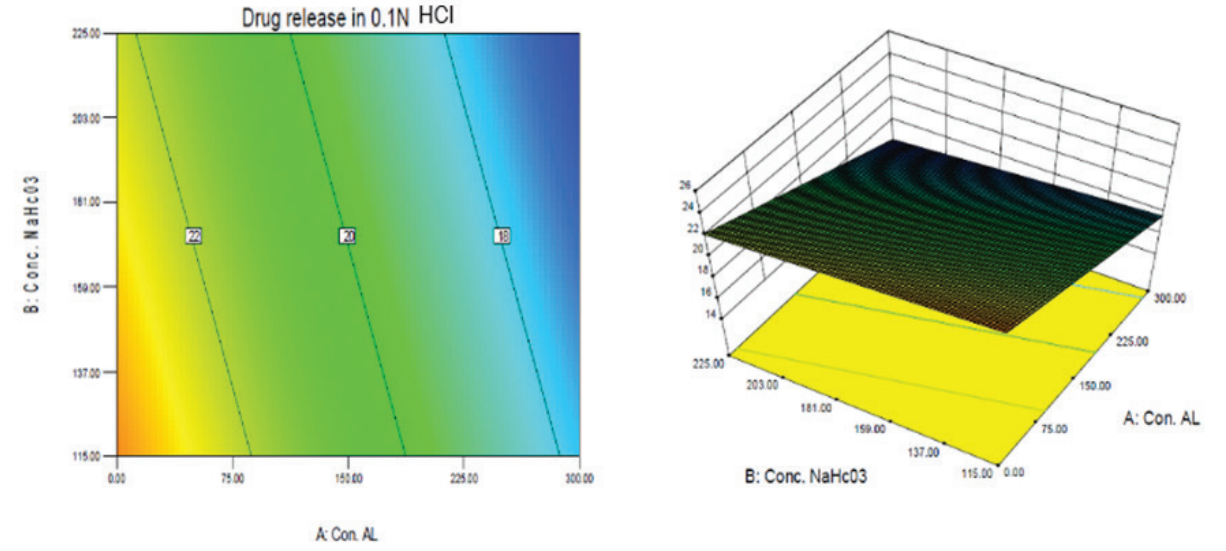

Figure 6. Countour plot and response surface plot for Drug Release in 0.1N HCl (Y3).

In this equation, coefficients of X1, X2 and X3 showed positive signs, so increase in concentration of sodium alginate, sodium bicarbonate and calcium chloride increased the entrapment efficiency. The results are revealed in Figure 4.

The Model F-value of 71.73 for floating lag time implied the model was significant. The ratio of 16.44 indicated an adequate signal.

Floating lag time (Y2) = +15.51-3.140X1-0.0523X2-0.750X3

In this equation, coefficient of X1 showed a negative sign, so increase in concentration of sodium alginate increased floating lag time. The coefficient of X2 showed a negative sign, so increase in sodium bicarbonate concentration decreased the floating lag time, and coefficient X3 showed a positive sign, so increase in calcium chloride concentration increased entrapment efficiency. The results are revealed in Figure 5.

The Model F-value 14.67 of entrapment efficiency implied the model was significant. The ratio of 8.552 indicated an adequate signal.

Drug Release % in 0.1N HCl (Y3) =27.06878-0.02X1-0.0136X2-0.50X3

In this equation, coefficient of X1 showed a negative sign, so increase in concentration of sodium alginate decreased drug release in 0.1N HCl. The coefficient of X2 showed a negative sign, so increase in sodium bicarbonate concentration decreased the drug release in 0.1N HCl, and the coefficient X3 showed a negative sign so increase in calcium chloride concentration decreased drug release in 0.1N HCl. The results are revealed in Figure 6.

Validation of mathematical model

The close similarity between the observed and predicted response value assessed the robustness of predictions. These

values designate the validity of generated model. The results are revealed in Table 5.

Table 5. Validation of mathematical model.

Batch Code	Composition			Responses*	Predicted Value	Experimental value	%Error
	X1	X2	X3				
F1	300	225	4	Y1	89.40	89.41	0.013
				Y2	0.75	1	0.25
				Y3	16	16	0.00
C1	300	200	3	Y1	83.61	84.35	0.74
				Y2	2.36	1.50	0.86
				Y3	16.83	17.00	0.17
C2	200	225	3	Y1	84.40	81.03	-3.37
				Y2	1.11	1.00	0.11
				Y3	18.44	18	0.44
C3	300	200	4	Y1	86.90	85.07	1.83
				Y2	1.79	1.50	0.29
				Y3	16.52	17.00	0.48

***** Y1= (%) Entrapment efficiency, Y2= Floating lag time, Y3= (%) Drug release in 0.1N HCl.

Stability studies

No significant change in drug entrapment efficiency, floating time (T80) and drug release during storage indicated that the developed floating beads formulation of carvedilol were stable.

CONCLUSION

The present work was undertaken to formulate the floating beads of carvedilol for the chronotherapy of hypertension. In preliminary trial batches, various concentrations of sodium alginate, pectin and calcium chloride were used for preparation of carvedilol containing floating beads. It has been found that the increase in the concentration of sodium alginate increased - the entrapment efficiency. Increased in the concentration of sodium bicarbonate decreased the floating lag time. From the results of the preliminary trial batches, 2^3 factorial designs were employed for optimization of the formulation by selecting dependent variables as entrapment efficiency, floating lag time, drug release in 0.1N HCl and independent variables as concentration of sodium alginate, concentration of sodium bicarbonate and concentration of calcium chloride. The formulated floating beads were evaluated for micrometrics, scanning electron microscopy, entrapment efficiency, floating study and dissolution study. F1 batch found to have 1.97±0.09 mm particle size, 1min. floating lag time, 89.41±0.02 % entrapment efficiency, 15.5±0.1 % drug release in 0.1N HCl and 90.5±0.002 % drug release in pH 6.8 phosphate buffer. The optimized formulation F1 was selected on the basis of its good entrapment efficiency, less floating lag time and less drug release in 0.1 N HCl. The results of all F1-F8 batches justified by regression equation derived for responses like entrapment efficiency, floating lag time, and drug release in 0.1 N HCl by 2^3 factorial design. The response surface model and contour plots gave an idea about the effect of different concentrations on entrapment efficiency, floating lag time and drug release in 0.1 N HCl. The stability studies of an optimized batch showed no significant change in terms of the drug entrapment efficiency, floating lag time and drug release in 0.1 N HCl following storage at 40±2 ^{0}C and 75±5 % RH.

Thus, it can be concluded that the present work can be considered as one of the promising formulation techniques for preparing multi-particulate floating pulsatile drug delivery of carvedilol in the form of floating beads and can be effectively used in chronotherapeutic management of hypertension by opening a new therapeutic dimension to an existing drug molecule.

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

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Original Article

Electrochemical behavior and differential pulse voltammetric determination of budesonide in suspension ampoules

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ABSTRACT

Background and Aims: Budesonide (BUD) is a broad-spectrum anti-inflammatory and anti-allergic glucocorticosteroid agent. It is used in the treatment of chronic obstructive pulmonary disease (COPD), Crohn's disease, and ulcerative colitis. The aim of the study was to investigate the electrochemical properties of BUD for the first time and to develop a sensitive, easy, and selective new differential pulse voltammetry (DPV) method for its determination in drug formulation.

Methods: The electrochemical behavior of BUD was investigated using cyclic voltammetry (CV) and differential pulse voltammetry (DPV) with a carbon paste electrode (CPE) in various electrolyte and buffer solutions with pH ranging from 2 to 9. An irreversible, well-defined reduction current peak of about -859 mV was obtained. A DPV method was developed and validated for the determination of BUD in suspension ampoules using a CPE electrode in a 0.1 M HCl electrolyte solution containing 13% KCl and 8% methanol.

Results: The cathodic peak was found to be adsorption-controlled. The calibration curve was linear between 1.65- 35.35 µg/ml. The limit of detection (LOD) and limit of quantification (LOQ) values were found to be 0.52 µg/mL and 1.57 µg/mL, respectively. The developed method offered an effective capability for the determination of BUD in suspension ampoules, with a recovery rate of 98.47%.

Conclusion: The DPV method developed in this study could be used for routine quantitative analysis of BUD in pharmaceutical preparations due to its fast, accurate, inexpensive, and environmentally friendly nature.

Keywords: Budesonide, determination, pharmaceutical preparation, validation, voltammetry

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INTRODUCTION

Budesonide [BUD, 16,17-Butilidenebis(oxy)-11,21-dihydroxy-pregna-1,4-diene-3,20-dione, Figure 1], a potent glucocorticoid, is an anti-inflammatory agent. BUD mainly treats asthma, COPD, Crohn's disease, ulcerative colitis, catarrh, and inflammatory conditions of the upper respiratory tract and intestines by preventing and reducing swelling and inflammation. Prolonged use of high doses of corticosteroids may cause hypercriticism and suppression of the adrenal axis. BUD is commercially available in inhalation, tablet, nasal spray, and rectal forms.

Patients given BUD should be monitored for symptoms and respiratory function to ensure effective therapy and dose adjustment. Since BUD is mainly metabolized in the liver, it may cause impaired hepatocyte function and accumulation in the blood. In addition, the simultaneous use of thiazide and thiazide-like loop diuretics may increase the risk of developing hypokalemia. Medication, therefore, needs to be monitored (Hofer 2003; Szefler 2001).

For all these reasons, easy, cheap, fast, and reliable detection methods are needed for the determination of BUD. Various high-performance liquid chromatography (HPLC) methods utilizing ultraviolet (Hryniewicka, Starczewska, & Gołębiewska, 2019; Peng et al., 2022), fluorescence (Ahmed & Atia, 2019), mass spectrometry, and tandem mass spectrometry (Gazzotti et al., 2016; Rower et al., 2019; Szeitz, Manji, Riggs, Thamboo, & Javer, 2014), as well as gas chromatography-mass spectrometry (Krzek, Czekaj, Rzeszutko, & Jończyk, 2004; Matabosch et al., 2012) and spectrophotometric (Prasad 2006; Sanap, Sisodia, Patil, & Janjale, 2011) methods have been reported for the determination of BUD in human body fluids and pharmaceutical formulations. While these methods offer sufficient sensitivity and selectivity, they are often expensive, time-consuming, and require multi-step processes such as derivatization and pre-separation, along with excessive use of organic solvents. Therefore, there is still demand for new analytical methods that allow selective, accurate, inexpensive, and environmentally friendly determination of BUD from pharmaceutical and biological samples. According to the literature search, no electrochemical method has been reported for the determination of BUD so far. Electrochemical methods, especially voltammetric methods, have been increasing in recent years because they are easy, fast, sensitive, and selective in the determination of drug analysis. The low capacitive current of the differential pulse voltammetry (DPV) greatly increases the sensitivity of the method. Also, the pulse technique with small step sizes in DPV assists in symmetrical sharp voltammetric peaks, which increases the selectivity of the DPV method (Scott & Yu, 2015).

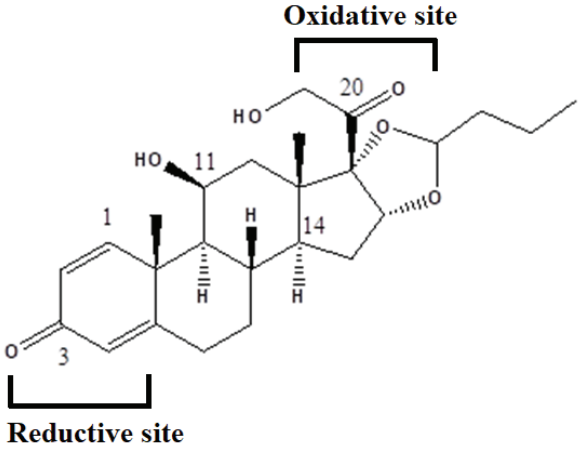

Figure 1. Structure of the budesonide.

Therefore, there is still a need for new analytical methods that offer selective, accurate, inexpensive, and environmentally friendly determination of BUD from pharmaceutical and biological samples. To our knowledge, no electrochemical method for the determination of BUD has been reported so far. In recent years, electrochemical methods, particularly voltammetric methods, have become increasingly popular because of their ease of use, speed, sensitivity, and selectivity in drug analysis. The DPV method is especially advantageous due to its low capacitive current, which significantly enhances its sensitivity. Furthermore, the pulse technique with small step sizes in DPV leads to symmetrical sharp voltammetric peaks, which increases the selectivity of the DPV method (Scott & Yu, 2015)

In this study, the electrochemical behavior of BUD on a simply prepared, unmodified carbon paste electrode (CPE) was investigated, and a fast and accurate DPV method was developed and validated for the determination of BUD in inhalation preparations.

MATERIALS AND METHODS

Apparatus and reagents

Electrochemical measurements were performed using a computer-controlled BASi Epsilon-EC version 2 potentiostat system (Bioanalytical Systems, Inc., West Lafayette, IN) and a three-electrode system with a BASi C-3 Cell Stand. Ag/AgCl (saturated KCl) and platinum wire were used as reference and auxiliary electrodes, respectively. The working electrode was a carbon paste electrode (electrode body BASi CF-1010 carbon paste). pH measurements were employed with a pH ion meter (Mettler Toledo) and pure water was obtained by an ultra-pure water device (Purelab Option).

BUD was kindly provided by DEVA Holding pharmaceutical company. Methanol and sodium hydroxide (NaOH) were purchased from Riedel de Haen. *Hydrochloric acid* (*HCl*), sulfuric acid (H_2SO_4), glacial acetic acid (CH_3COOH), boric acid (H_3BO_3), orthophosphoric acid (H_3PO_4), and potassium chloride (KCl), were used as electrolyte solutions, and were purchased from Merck. All the reagents were of analytical purity. Graphite powder (< 20 µm) was obtained from Sigma-Aldrich. Multi-walled carbon nanotubes (MWCNTs) and graphene were obtained from the Nanografi Company (Turkiye).

BUD was accurately weighed, and 1.0 mg/mL stock solutions were prepared by dissolving it in methanol. The standard solutions at 0.1 µg/mL and 0.01 µg/mL were made by diluting BUD stock solution with methanol. The stock and standard solutions were stored at +4 °C and remained stable for at least one month.

To prepare the 0.1 M phosphate buffer solutions (PBS), phosphoric acid was used to create solutions with pH values between 2.0 and 4.0, while disodium hydrogen phosphate and sodium dihydrogen phosphate were used to create solutions with pH values between 5.0 and 9.0. Britton Robinson (BR) buffer solutions (0.1 M) at pH values between 2 and 9 were prepared using a mixture of phosphoric acid, boric acid, and acetic acid. The pH of the solutions was adjusted to the desired values using NaOH and phosphoric acid solutions.

Preparation of electrode

To obtain a homogeneous carbon paste electrode, 70% graphite and 30% silicone oil were continuously mixed in a small mortar for approximately 10 minutes. The resulting carbon paste was then filled into a hole (0.3 cm deep, 0.7 cm diameter) at the end of a 7.5 cm electrode body. The surface of the paste was smoothed and polished by rotating it on a slippery paper surface. Before measurement, the paste was removed from the electrode cavity and regenerated with fresh carbon paste.

Measurement procedure

A mixture of 0.1 M HCl solution containing 13% KCl and 8% methanol was used in the measurements. The solutions of BUD prepared in 5 different concentrations between 1.65 and 35.35 µg/mL with a final volume of 10 mL were taken into an electrochemical cell. Nitrogen gas was passed for 2 minutes before the measurements. In each series, voltammograms of the empty solutions were recorded first. A new surface was created before each measurement set. differential pulse voltammograms at CPE (against Ag/AgCl) were recorded in the potential range from 0.0 (initial) to -1400 mV(final) (scanning rate =20 mV/s; pulse amplitude =50 mV; pulse width = 50 ms; step E= 4 mV) (Aydoğmuş, Aslan, Yildiz, & Senocak, 2020). Well-defined reduction peak currents at a potential of about -859 mV were recorded in the DPV analysis. In constructing the calibration curve, at least six serial runs for each concentration were performed. A calibration curve was created by plotting the current values measured by DPV against the corresponding concentration, and the regression equation was calculated.

Determination in drug formulation

The plastic PULMICORT® Nebulizer Suspension ampoules containing 1 mg/2 mL of BUD were used to apply the developed DPV method. 0.2 mL of the suspension was taken directly from the ampoules using an automatic pipette to achieve a final concentration of 10 µg/mL and analyzed according to the "measurement procedure" section. Three separate analyses were performed using 2 different suspension ampoules, and the averages were calculated. The concentration of BUD in the drug formulation was determined by substituting the obtained current values in the regression equation prepared for the standard substance.

RESULTS AND DISCUSSION

Selection of the working electrode and electrochemical behavior of BUD

Carbon paste electrodes (CPEs) have high surface activity, and their surface can easily form bonds with various functional groups such as hydrogen, hydroxyl, and carboxyl groups. CPEs are widely used in drug analysis because they are easy and fast to prepare, have low construction costs, can be regenerated, have a wide potential range, have low residual currents, and can contain many electrode materials at the same time. In addition, CPEs can be easily modified to improve their selectivity and sensitivity toward specific analytes (Speranza 2019).

Carbon-based CPE, GCE, 10% graphene-modified CPE, and 10% MWCN-modified CPE electrodes were tested as working electrodes for the sensitive and selective determination of BUD by CV and DPV methods (Figure 2). Initially, CV and DPV analyses were carried out in BR buffers with pH values of pH=2 and pH=7, selected as acidic and basic electrolyte solutions, using a 10 µg/mL standard BUD solution. The electrochemical behavior of BUD at the tested electrode surfaces showed a reduction peak at approximately -900 mV potential in voltammograms taken in the potential range of 0.0 to -1400 mV (Figure 2). The same measurements revealed no peaks in the reverse scan, indicating an irreversible reduction process of the BUD solution. The study found that the reduction peak of BUD was not significantly different between the modified and unmodified CPE electrodes in terms of obtaining a sharp, highly sensitive peak. Therefore, unmodified CPE was chosen as the working electrode since it does not require any additional modification steps and is simpler to use.

Selection of electrolyte solution and pH effect

Cyclic and differential pulse voltammograms were recorded in BR and phosphate buffers (pH 2 - 9), 0.1 M HCl, and 0.1 M H_2SO_4 solutions in the potential range from 0.0 to -1400 mV to select the optimum electrolyte solution in the BUD analysis and investigate the effect of the pH of the buffer solution on the electrochemical process. Since BUD has very low solubility in water, 1.0 mL of methanol was added to each tested electrolyte solution. Depending on the pH, an irreversible reduction peak between -854 and -1088 potentials was obtained in electrolyte solutions. Analysis results showed that BUD solution at CPE gave the highest current peak in 0.1 M HCl solution, very slightly at pH 5, and did not give any reduction peak at pHs above 5.0. Then, in order to increase the intensity of the peak current, 0.1 M KCl *supporting electrolyte* and methanol were added in certain proportions to the 0.1 M HCl solution

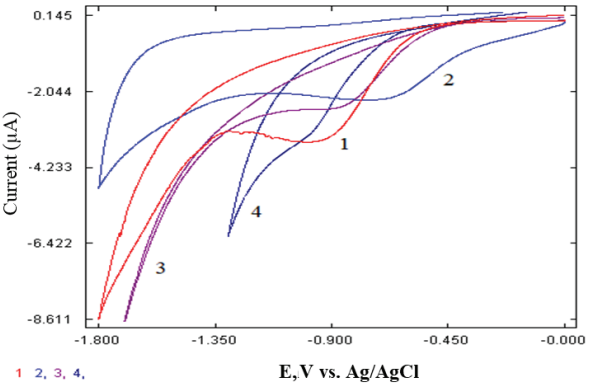

Figure 2. CV voltammograms (100mV/s) of 10 µg/mL BUD with CPE (1), GCE (2), 10% MWCN modified CPE (3), and 10% Graphene modified CPE (4).

and investigated by CV. Experiment results exhibited that the reduction peak of the drug increased significantly in 0.1 M HCl solution containing 13% 0.1 M KCl electrolyte solution and 8% methanol, and further studies were continued with this solution (Figure 3).

Investigation of the pH effect on the peak potential and peak current of BUD was evaluated in the selected 0.1 M HCl solution (pH ~1) containing 0.1 M KCl-methanol, in BR buffer (pH 2-4), and in phosphate buffers (pH 2-5) separately using the CV technique with 10 µg/mL BUD (Figure 3). The shift of the reduction peak potential to a more negative value with increasing pH indicates that the BUD reduction on CPE is pH dependent and protons are involved in the electrode reaction.

The regression equation of the graph drawn between the peak currents (Ip) and the peak potentials (Ep) obtained in the BR buffer system was found to be Ep (pH 1-4) = -52.2pH - 841.5 mV versus Ag/AgCl with a correlation coefficient R^2 = 0.9194. In the phosphate buffer system, the regression equation was found as Ep (pH 1- 4) = [-76.6pH - 772.5] mV versus Ag/AgCl with a correlation coefficient of R^2=0.9904. The negative Ep-pH slopes obtained in two different buffers were found to be -52,2 pH and -76.6 pH, respectively. These slope values are very close to the theoretical 59 mV/pH value at 25 °C, indicating that the number of protons and electrons involved in the reduction reaction is equal (Alimohammadi, Kiani, Imani, Rafii-Tabar, & Sasanpour, 2019).

Effect of scanning rate

The effect of the scanning rate on the reduction peak of the current of BUD (10 µg/mL) on the CPE surface was investigated by CV in the selected solution in the range of 20-200 mV/s. It was observed that the reduction peaks current of BUD increased with increasing scanning rate, and its potential shifted towards a more negative scale (Figure 4). In order to construe whether the electrochemical reaction of the drug is adsorption and diffusion-controlled, calibration curves namely, the logarithm of peak current versus the logarithm of scan rate (log v – log I_p) and peak current versus logarithm square root of the scan rate (I_p - $\upsilon^{1/2}$) values were prepared using the scanning rate and related current values, and the regression equations were log I_p = 1.0412logυ - 1.2531 (R^2 = 0.9887) and I_p(µA) = 1.198 $\upsilon^{1/2}$- 4.6946 (R^2 = 0.9952), respectively (Figures 5). Here,

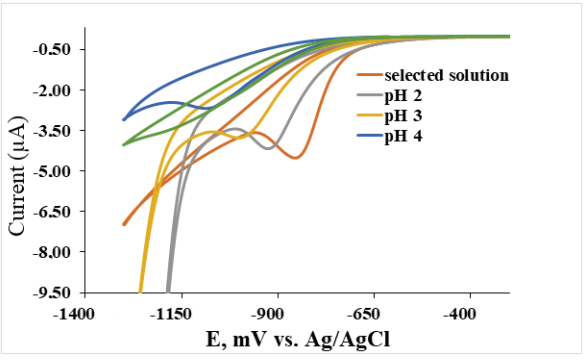

Figure 3. CV-associated voltammograms of 10 µg/mL BUD solution in mixtures of HCl solution (pH~1) and in phosphate buffers of pH 2.0-5.0.

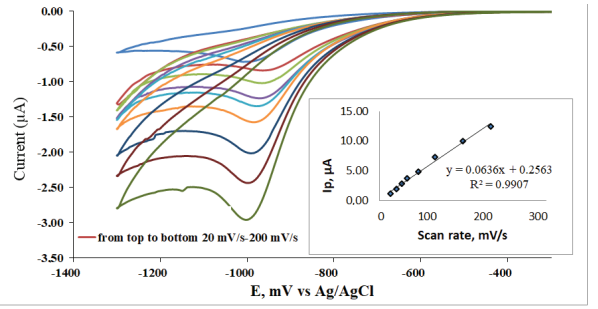

Figure 4. CV of 10 µg/mL BUD at CPE in selected solution in various scan rates. From top to bottom: blank, 20, 30, 40, 50, 70, 100, 150, and 200 mV/s. Inset: Plot of Ip vs. υ.

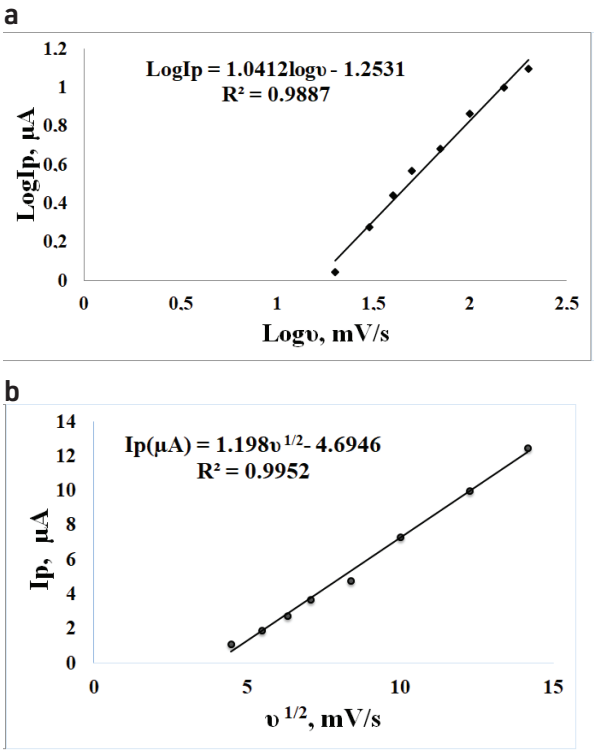

Figure 5. Curve of change of logarithm of peak current versus logarithm of scan rates (logIp - log υ)(a); the curve of variation of the peak current versus the square root of the scan rates (Ip - $\upsilon^{1/2}$) (b).

the slopes of log v – log I_p and I_p - $v^{1/2}$ are between 1.04 and 1.19, indicating that the electrochemical reaction is strongly adsorption controlled. For the irreversible process, Ep can be defined by the Laviron equation (Laviron 1979) given below:

Ep (mV/s) = E^0 + (2.303RT/ αnF) log (RTk^0/ αnF) + (2.303RT/ αnF) logυ (mV/s),

Where α is the electron transfer coefficient, k^0 is the standard heterogeneous rate constant, υ is the scan rate, n is the number of electrons transferred per molecule, Ep is the peak potential and E^0 is the formal potential that can be found from the intercept of the Ep vs scan rate (υ) curve, by extrapolating to the vertical axis at υ = 0. (T = 298 K, R = 8.314 J/K mol and F = 96.485 C/mol) (Aydoğmuş, Aslan, Yildiz, & Senocak, 2020). The regression equation of the linear curve between the peak

potential and the logarithm of the scan rate was obtained as Ep(mV) = -42.385logυ(mV/s) - 905.45 (R^2 = 0.9718). The value of αn is calculated from the slope of Ep against the log v plot by Laviron's equation (Laviron 1979). It was calculated as 1.39 (slope=2.303RT/αnF). In the non-reversible electrode process, α is considered to be between 0.3 and 0.7 (Bond 1980; Guidelli et al., 2014). Assuming α is 0.7, the number of electrons in the reaction was found to be 1.98≈2.

In addition, in the pH-dependent BUD/CPE reaction, the n value was calculated from the equation [E_p-$E_{p/2}$ = (47.7/ αn) mV, 25°C], where E_p is the peak potential and $E_{p/2}$ is the half-wave potential where the current is half of its peak current using CV at 100 mV/s [Sartori, Clausen, Pires, & Salamanca-Neto, 2017). From this equation, αn was calculated as 1.41 and the n value was found to be 2.01≈2 when α is taken as 0.7. These two n-value calculations confirmed each other and were in good agreement with the values reported in the reduction reaction of some corticosteroids (Alimohammadi et al., 2019; Hammam 2007; Vedhi, Eswar, Prabu, & Manisankar, 2008).

Possible reaction mechanism

Although there is no research on the electrochemical properties of BUD in the literature, there are some voltammetry methods developed for the determination of corticosteroid drugs with the molecular skeleton of BUD, such as betamethasone (Alimohammadi et al., 2019; Ghoneim, El-Attar & Ghoneim, 2009; Goyal, Chatterjee & Rana, 2010), triamcinolone acetonide (Goyal, Gupta & Chatterjee, 2009; Hammam 2007; Vedhi et al., 2008), and prednisolone (Rezaei & Mirahmadi-Zare, 2011). In these studies, glassy carbon electrode (GCE) (Vedhi et al., 2008), modified GCE (Alimohammadi et al., 2019), edge-plane pyrolytic graphite electrode (Goyal, Chatterjee & Rana, 2010; Goyal, Gupta & Chatterjee, 2009), hanging mercury drop electrode (HMDE) (Ghoneim, El-Attar & Ghoneim 2009; Hammam 2007), and molecularly imprinted polymer-multiwalled carbon nanotube paste electrode (Rezaei & Mirahmadi-Zare, 2011) have been used as working electrodes.

In general, corticosteroids have two electroactive sites that act separately as reducing and oxidative (Figure 1). Nevertheless, in a few studies, corticosteroids have been found to be reduced from carbonyl groups at unconjugated C-20, which are activated by neighboring hydroxyl groups at C-17 and C-21 (Alimohammadi et al., 2019; Goyal, 2009). In the other studies, it was shown that the C-3 carbonyl group adjacent to the double bonds in drug molecules was reduced more easily than the C-20 carbonyl group. Also, some studies have shown that these molecules can be oxidized depending on the electrode and pH (Rezaei & Mirahmadi-Zare, 2011). For the reduction process, studies exhibited that two hydrogens (+2H) and two electrons (2e-) were added to the C=O groups in drugs, which is consistent with the data found for BUD in the present work. Considering the literature and data from the currently proposed DPV study, it is predicted that BUD is reduced by adding +2H and 2e- to a carbonyl group at C-3 or C-20 under selected acidic analysis conditions.

Method validation

The developed method has been validated with regard to linearity, linear range, detection and detection limits, accuracy, selectivity, and stability.

Linearity and sensitivity

The determination of BUD was performed on a simple, unmodified CP electrode at about -859 mV (against Ag/AgCl) with the DPV method, which is much more sensitive and has a lower background current than CV. The calibration curve was obtained by plotting the peak currents of the BUD against the concentration under the determined optimum conditions. The calibration curve for BUD was determined to be linear between 1.65 and 35.35 µg/mL and the corresponding regression equation was calculated as Ip (µA) = 0.4957C(µg/mL) + 7.3563 (Figure 6). The correlation coefficient (R^2) value of this equation was found to be 0.9999, indicating perfect linearity (Table 1).

The limit of detection (LOD) and limit of quantification (LOQ) values were calculated using the equations LOD = 3.3 SD/m and LOQ = 10 SD/m) (Guideline, ICH Harmonised Tripartite

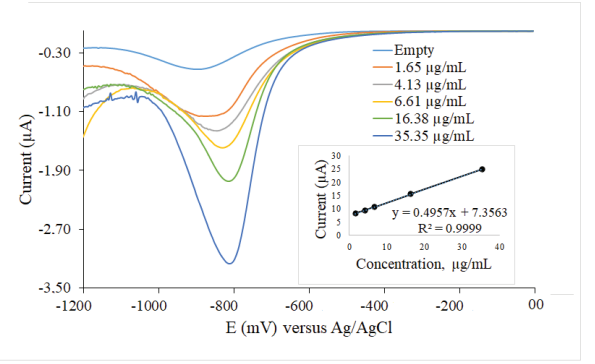

Figure 6. DPV of BUD at various concentrations (blank solution, 1.65, 4.13, 6.38, 16.38 and 35.35 µg/mL). Inset: Calibration curve for BUD from these data.

Table 1. Statistical parameters for analysis of BUD by DPV in standard solution.

Parameter	Value
Measured potential, mV	- 859
Linearity range, µg/mL[a]	1.65-35.35
Regression equation, y = mc + b	I_p (µA) = 0.4957C (µg/mL) + 7.3563
Slope	0.4957
Intercept	7.3563
Coefficient of determination (R^2)	0.9999
SD[b] of m	0.003
SD of b	0.078
LOD, µg/mL	0.52
LOQ, µg/mL	1.57

[a] Average of six determinations for the drug in standard solution (n = 6); 0.1 M HCl containing 13% KCl and 8% methanol medium; working electrode: CPE; potential window: between 0.0 and -1400 mV (amplitude: 50 mV, pulse width: 50 ms and scan speed: 20 mV/s.
[b] Standard deviation.

2005), respectively. Here, SD and m were the standard deviation of the intercept and the slope of the calibration curve, respectively. The LOD and LOQ of BUD were 0.52 µg/mL and 1.57 µg/mL, respectively (Table 1).

Accuracy and precision

In order to evaluate the intraday and interday accuracy and precision of the developed method, three different concentrations of BUD solution (3.0, 10.0, 20.0 µg/mL) were tested under selected conditions. Analyses were performed on the same day (intraday) and on five different days (interday) within two weeks. Five separate analyses were performed for each concentration. The concentration was found by substituting the peak current values in the regression equation obtained for the standard BUD solution. Accuracy was given as the percent recovery values of the concentrations found, while precision was expressed as the relative standard deviation (%RSD) from the determined concentrations. The mean intraday and interday recovery values between 100.38% and 100.05% (SD = 0.18 - 0.09) showed that the accuracy of the method was excellent. The mean relative standard deviation (%RSD) values of the experiments performed intraday and interday were found to be between 1.31-3.18% and 0.33-3.38%, respectively (Table 2).

Selectivity and effect of excipients

The prepared electrolyte solution was analyzed by CV and DPV under conditions determined in the presence and absence of BUD and drug samples. The blank solution gave a current peak at the reducing potential of the drug well below the LOD, indicating that the method is selective. In addition, the potential interaction of excipients conventionally found in pharmaceutical preparations or biological fluids in the determination of BUD with the developed DPV method was investigated. The substances that may cause interference were added 100 times to the BUD solution (30 µg/mL) and it was analyzed whether it caused interference with the developed DPV method under the optimized same analysis conditions. The currents were recorded by making 3 readings before and after adding the substance whose interference effect was examined, and these currents were compared and the % current difference values were calculated separately for each substance. Results given in Table 3 exhibited that a hundred-fold excess of hydroxypropyl methylcellulose, citric acid, lactose, saccharose, and Na^+ did not show any significant interaction in DPV current response. However, ascorbic acid and glucose negatively affected the DPV current response by 14.76% and 7.2%, respectively.

Electrode stability and reproducibility

The stability of the CPE was investigated using three freshly prepared CPEs to determine BUD (10 µg/mL) using CV. These electrodes showed good stability, with a relative standard deviation of 5.6% as a result of voltammograms recorded once a week for 1 month. Prepared CPE was stored in tightly sealed glass containers at 25°C and stayed stable for at least 3 months.

Determination of BUD in ampoules of inhalation suspension

The applicability of the developed DPV method was tested to determine BUD in nebulizer suspension plastic ampoules containing 1 mg/2 mL of Pulmicort Respules (BUD inhalation suspension). Samples corresponding to 10 µg/mL were taken and studied as described in the "measurement procedure" sections (Figure 7). Sample contents were calculated using the measurement curve equation prepared for the standard substance. The recovery was between 91.1% and 100.7% (mean= 98,47),

Table 2. Inter-day and intra-day and accuracy and precision of BUD determination by DPV method (*n* = 5).

	Intraday		Interday	
Concentration (µg/mL)	**Recovery (%)[a] ± SD[b]**	**% RSD[b]**	**Recovery (%)[a] ± SD[b]**	**% RSD[b]**
3.0	100.70 ± 0.10	3.18	99.33 ± 0.10	3.38
10.0	99.80 ± 0.13	1.31	100.30 ± 0.11	1.08
20.0	100.65 ± 0.31	1.56	100.51 ± 0.07	0.33
Mean	100.38 ± 0.18	0.34	100.05± 0.09	1.60

[a]Mean of five determinations (n = 5). [b]SD is standard deviation and RSD is relative standard deviation.

Table 3. Influence of potential excipients on the voltammetric response of 30 µg/mL budesonide.

Excipients	Ip of BUD in the absence of Excipient	Ip of BUD in the presence of Excipient	Signal change (%)
Na+	22.01	22.49	2.19
Glucose	21.61	20.05	-7.2
Ascorbic acid	21.48	18.31	-14.76
Citric acid	21.51	20.90	-2.86
Saccharose	20.64	20.32	-1.52
Lactose	20.89	20.43	-2.2
Hydroxypropyl methylcellulose	21.62	21.52	-0.47

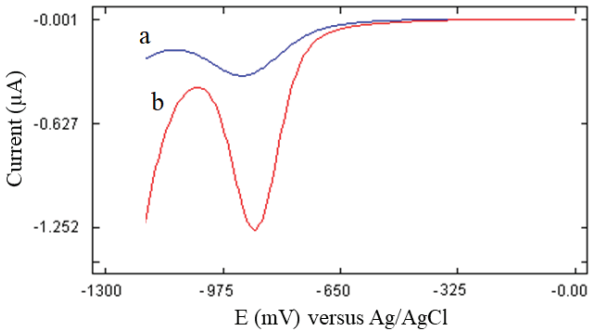

Figure 7. Empty electrolyte solution (a) and BUD inhalation suspension (10 µg/mL) (b).

and the RSDs were found to be 3.73% on average. This shows that the developed method is sensitive and selective enough.

Comparison of the proposed method with some reported methods

In this study, the determination of BUD was performed by the DPV method using unmodified carbon paste electrodes. Various HPLC and spectrophotometric methods have been published for the determination of BUD (Kolsure, Daniel, & Bhat, 2021). However, an electrochemical method has not been reported. Table 4 presents a comparison of some of these methods for determining substance concentration in terms of linear range and LOD with previous findings reported in the literature. The current approach is simple and does not require the use of pretreatment procedures or time- and chemical-consuming reactions such as derivatization. In addition, although the sensitivity and separation power is high in analyses with HPLC or HPLC-mass spectrometry instruments, they are expensive, often requiring very time-consuming processes such as derivatization and requiring the use of large amounts of solvents. However, as can be seen in the Table, the newly developed voltammetric method provides superiority to some HPLC studies in terms of both linear range and sensitivity. Compared with previously published studies on the determination of BUD, the current voltammetric procedure offers a sufficiently wide linear range for drug determination. Working with unmodified CPE is a very inexpensive, simple, fast, and selective method. These evaluations showed that the method developed in this study will be an important alternative to other published methods in terms of a wide linear dynamic range, relatively low detection limit, selectivity, and excellent reproducibility in the determination of the substance.

CONCLUSION

In the study, the electrochemical properties of BUD were investigated and developed a novel, efficient, and reliable new DPV method for its determination in pharmaceutical samples. The effect of scanning rate and pH were investigated to obtain the highest response for DPV analysis of BUD. Electrochemical studies show that the reaction of BUD on CPE was irreversible and adsorption-controlled, involving the transport of two protons and two electrons. The experiments were conducted using 0.1 M HCl solution containing 13% KCl and 8% methanol as the supporting electrolyte with a pH of about 1.0 for CPE. The dynamic linear range was between 1.65 and 35.35 µg/mL with

Table 4. Comparison of linear range and detection limits for BUD with published methods.

Method	Analysis Conditions	Linear range (µg/mL)	LOD (µg/mL)	Application	Ref.
HPLC-UV	C18, 0.05 M Sodium acetate buffer/ acetonitrile (40:60, v/v)	0.5- 50.0	0.187	Inhaler medicine	Salem et al., 2017
HPLC-UV	Hypersil C18, Ethanol/acetonitrile phosphate buffer pH 3.4; 25.6mM) (2:30:68, v/v/v)	2.5 - 25.0	0.30	Pulmicort Turbuhaler	Hou, Hindle, & Byron,2001
HPLC-UV	Kromasil C8 (150 mm x 4.6 mm) Acetonitrile/ phosphate buffer (pH 3.2-0.025 M) (55:45 v/v)	1-50	0.1	Pharmaceutical form	Gupta, & Bhargava,2006
HPLC-UV	Bondapak RP- C18 Acetonitrile/ monobasic potassium phosphate (55:45, pH 3.2) (28)	1-20	0.05	Drug formulation	Varshosaz et al., 2011
UV Spectrophotometry	pH 6.8 buffer	1.4 - 25	0.01	Drug formulation	Bharti et al., 2011
DPV on CPE	0.1 M HCl solution containing 13% KCl and 8% methanol	1.65 - 35.35	0.52	Inhalation preparations	Current study

a low LOD value of 0.52 µg/mL.

The use of unmodified CPE as the working electrode has made the method more accessible and cost-effective, while the wide linear range and low detection limit make it suitable for both quality control and research applications. The validation of the method has also shown its accuracy, selectivity, and stability, making it a promising alternative to existing analytical methods for BUD determination. In addition, the developed voltammetric can be considered a green chemistry approach to drug analysis as it avoids the use of hazardous reagents or solvents that may pose risks to human health or the environment. The use of unmodified CPE is also advantageous in terms of cost-effectiveness and simplicity, making it a promising alternative to other more complex and expensive methods for routine analysis of BUD.

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

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Original Article

Protective effect of curcumin against perfluorooctane sulfonate induced oxidative stress

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ABSTRACT

Background and Aims: The potential effects of perfluorooctane sulfonate (PFOS) on the environment and human health have aroused great concerns in recent years. To our knowledge, there are limited studies related with the effect of curcumin on PFOS induced damage in the literature. The existing studies are focused on DNA damage. No study has been found examining the effect on the antioxidant system. We planned this study to investigate the impact of curcumin on the antioxidant defense system response in rats exposed to PFOS.

Methods: The animals were divided into six groups, with the first group used as control. Groups II to VI were orally treated with curcumin (80 mg/kg), PFOS (1.25 mg/kg), PFOS (1.25 mg/kg) + curcumin, PFOS (2.5 mg/kg), and PFOS (2.5 mg/kg) + curcumin daily for 30 days, respectively. For oxidative stress, liver, kidney, and brain samples were homogenized. The activities of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) activities, and malondialdehyde (MDA) content were determined. Data from the experiments were statistically analyzed by SPSS 11.0 program.

Results: Our data showed that PFOS increased MDA level, while the activities of SOD and CAT decreased. It was observed that the application of curcumin together with PFOS decreased the MDA level and increased the antioxidant enzyme activities.

Conclusion: As an antioxidant, curcumin plays an important protective role against oxidative damage and inhibits PFOS-induced lipid peroxidation.

Keywords: Curcumin, perfluorooctane sulfonate, antioxidant, oxidative stress, lipid peroxidation.

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INTRODUCTION

Perfluorooctane sulfonate (PFOS, $C_8F_{17}SO^{3-}$) is an associate of the class known as fluorochemical compounds (PFCs). PFOSs are compounds that are synthesized in industry and are highly resistant to degradation (Endirlik & Gürbay, 2018). These compounds have a wide range of use in many industrial and commercial areas, such as carpets, leather, and food (repellent coatings), various surfactants, as well as cosmetics and firefighting foams (Dorts et al., 2011). People can be exposed to PFOS through consumption of contaminated food and drinking water, breathing air, and contaminated environments (Pachkowski, Post, & Stern, 2019). After exposure, PFOS enters the cells of various organs and then interacts with macromolecules such as protein and DNA, causing direct oxidative damage and a range of cytotoxic effects such as cell death (Zhang et al., 2015; Beesoon & Martin, 2015). After 24 h exposure to PFOS, increased reactive oxygen species (ROS) and significant DNA damage are observed in the human hepatoma cell line (HepG2) (Wielsøe, Long, Ghisari, & Bonefeld-Jorgensen, 2015).

It has been reported that PFOS causes a decrease in fertility in women, a decrease in sperm quality in men, low birth weight, hyperactivity, an increase in total and LDL cholesterol levels, and changes in thyroid hormone levels (Ceccatelli et al., 2018; Grasl-Kraupp et al., 2020). In addition to the above-mentioned disorders, it has been reported that there is an increased risk of developing prostate and bladder cancer in those working in the production facilities of such chemicals (Tsuda, 2016).

Aerobic organisms affect oxygen consumption due to cell growth that leads to the formation of reactive oxygen species (ROS). According to reports, the generation of ROS is a significant apoptotic signal. These ROS comprise radicals, like excited oxygen species, superoxide anion radicals, hydroxyl radicals, hydrogen peroxide, and singlet oxygen. Free radicals that occur during physiological events are kept with antioxidant activity. The levels of these reactive oxygen metabolites increase when antioxidant activity is inadequate or antioxidant activity is insufficient. Reactive oxygen metabolites can attack and damage macromolecules, including DNA. Furthermore, lipid peroxidation products formed by oxidative degradation of polyunsaturated fatty acids can cause cellular damage. DNA damage may cause changes in cell function. A large proportion of such DNA damage leads to carcinogenic events (Comporti, 1989).

Curcumin is an important polyphenolic compound obtained from the rhizomes of Curcuma longa L. (turmeric) (Bright, 2007). The medicinal properties of curcumin are due to the curcuminoids in its structure and the main component in the rhizome containing curcumin (diferuloylmethane)–(1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5,dione (Figure 1) (Maheshwari, Singh, Gaddipati, & Srimal, 2006). Curcumin, used as a traditional medicinal plant, has various therapeutic properties, which include anti-inflammatory, antibacterial, and anticancer (Rathore et al., 2020). Its antioxidant property is controlled by different enzymes like catalase (CAT), superoxide dismutase (SOD), and glutathione peroxide (GPx) (El-Hack et al., 2021).

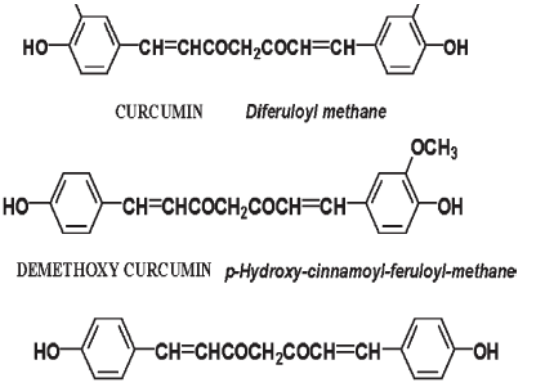

Figure 1. Chemical structure of curcumin. Curcuminoids, demethoxycurcumin, and bisdemethoxycurcumin curcumin which show anti-inflammatory and antioxidant properties[5].

Curcumin has more antioxidant effects than the antioxidant vitamins C and E (Mughal, 2019). Previous research has shown that curcumin has a defensive effect in various tissues against toxic agents (Hosseini & Hosseinzadeh, 2018). It has been also shown the ability of curcumin to protect against liver damage from some xenobiotics (Farzaei et al., 2018). Curcumin has been proven to be efficient in protecting against liver damage and fibrogenesis by reducing oxidative stress in the presence of carbon tetrachloride (CCl4) (Reyes-Gordillo et al., 2007; Fu, Zheng, Lin, Ryerse, & Chen, 2008). In addition, curcumin has been shown in various animal models to inhibit lipid peroxidation (Maheshwari, et al., 2006). The potential environmental and human health threats of exposure to PFOS have aroused great concerns in recent years. As with many environmental pollutants, in vivo and in vitro animal experiments are the main way to determine the human health risks and PFOS's toxic effects (Ceccatelli et al., 2018; Solan & Lavado, 2020).

In laboratory animal studies, exposure to PFOS has been reported to be associated with hepatotoxicity (Xu, Jiang, Liu, Liu, & Gu, 2017), neurotoxicity (Long et al., 2013), reproductive toxicity (Lopez-Doval, Salgado, Pereiro, Moyano, & Lafuente, 2014), immunotoxicity (Zheng, Dong, Jin, & He, 2009), pulmonary toxicity (Qin et al., 2017) and renal toxicity (Tang, et al., 2022). in numerous in vitro human cell lines.

Eke & Çelik (2016), showed that DNA damage in mouse peripheral blood using single-cell gel electrophoresis/comet test and a micronucleus test in vivo and showed that all PFOS concentrations increased the micronucleus frequency and curcumin reduced DNA damage induced by PFOS. These findings, and the associated epidemiological studies, confirm the damaging consequences of PFOS on human health (Ceccatelli et al., 2018; Qin, Ren, Zhao, & Guo, 2022). Although the toxic effect of PFOS has been investigated, more research is needed and as far as we know, but there are limited studies on the effect of curcumin on PFOS in the literature. These studies focused on DNA damage, and no studies examining the effect on the antioxidant system were found. In this study, it was aimed to determine whether curcumin substance has a protective effect against PFOS-induced oxidative damage.

MATERIALS AND METHODS

Chemicals

PFOS (CAS No.: 1763-23-1) and curcumin (chemical purity >99%) were purchased from Sigma (St. Louis, MO, USA). All other chemicals and reagents were purchased from Sigma-Aldrich, Merck and all were analytical grade.

Animals groups and treatment

In this study, 36 adult male Wistar albino rats weighing 180-200 g were used. All experimental protocols were approved by the Mersin University Animal Experiments Local Ethics Committee (2010/ HADYEK/67). The rats were given standard rat chow and pipe water, with a 12 h light/12 h dark rhythm, in a room at a temperature (22±2 °C). The animals were divided into six groups, with the first group used as control. Groups II to VI were orally treated with curcumin (80 mg/kg), PFOS (1.25 mg/kg), PFOS (1.25 mg/kg) + curcumin, PFOS (2.5 mg/kg), and PFOS (2.5 mg/kg) + curcumin daily for 30 days, respectively.

All the concentrations of PFOS and curcumin used in the current study were selected based on earlier studies (Eke & Çelik, 2016).

The liver, kidney, and brain tissues of rats were isolated under anesthesia (ketamine 200 mg/kg i.p.) Then, the tissues were homogenized with phosphate buffer (25 mM, pH = 7.4) to make approximately 10% w/v homogenates. The obtained homogenates were centrifuged at +4 °C for 10 minutes at 13,000 rpm. Up until analysis, the supernatant portion was kept at -20 °C. The MDA levels, SOD, and CAT activities were examined in homogenized tissues.

Determination of SOD enzyme activity

This method is based on the reduction of nitroblue tetrazolium by superoxide radicals formed through the xanthine/xanthine oxidase (Sun, Oberley, & Li, 1988). The color (blue-violet) formed as a result of the reaction was measured spectrophotometrically at 560 nm. Specific activity was expressed as U/mg protein.

Determination of CAT enzyme activity

The CAT activity was assessed spectrophotometrically by the process described by Aebi (Aebi, 1984). The principle of the method is the catalytic degradation of the H_2O_2 substrate at 240 nm. The enzyme activities are given in U/mg protein.

Determination of lipid peroxidation

The MDA determination as the final product of lipid peroxidation was measured according to the process reported by Yagi, Nishigaki, & Ohama (1968). A total of 750 µL of thiobarbituric acid was added to 50 µL of tissue homogenate. Then, the obtained mixture was incubated at 95 °C for 30 min and then centrifuged at 3500 rpm for 15 min. Absorbance was measured at 560 nm by a spectrophotometer. The results were expressed as nmol/mg of protein.

Protein determination

Using a method created by Lowry, Rosebrough, Farr, & Randall (1951), the protein concentrations of the tissue homogenates were examined to identify the specific activity of the antioxidant enzymes. Bovine serum albumin was used as a reference protein. The total protein content of the samples was determined by measuring with a spectrophotometer at a wavelength of 750 nm.

Statistical analysis

The SPSS 11.0 statistical program was used for all statistical evaluations. The one-way analysis of variance test (ANOVA) with Tukey's post-hoc test was used to determine the statistical difference between groups. The reported data were presented as mean ± standard deviation, with values having p-value <0.05 considered to be significant.

RESULTS

Figure 2 presents the SOD antioxidant enzyme activity in the liver, kidney, and brain tissues of rats belonging to the control, PFOS and PFOS+curcumin groups. Significant decreases ($p < 0.05$) in SOD enzyme activity were observed in all experimental groups, except for the curcumin group, when compared to the control group (group I). Compared to the group I, 1.6, 1.6, and 1.7-fold change for liver, kidney, and brain tissues were observed in group III, respectively. In group V, 1.7, 1.7, and 1.9-fold change for liver, kidney, and brain tissues compared to the group I were obtained. The curcumin group displayed higher SOD activity in all tissues than the control group. Additionally, group IV and group VI exhibited higher SOD activities compared to group III and group V. A mean 1.6-fold change in liver, kidney, and brain tissues was observed when comparing group IV with group III, and a mean 1.8-fold change was observed for all of these tissues when comparing group VI with group V.

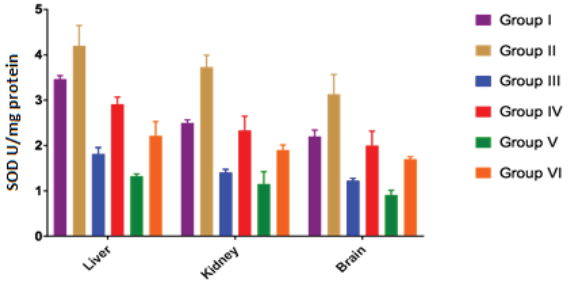

Figure 2. Activities of SOD in control, PFOS and PFOS+ Curcumin groups in liver, kidney, and brain tissues of rats.
Groups I: control, Groups II: curcumin (80 mg/kg), Groups III: PFOS (1.25 mg/kg), Groups IV: PFOS (1.25 mg/kg) + curcumin, Groups V: PFOS (2.5 mg/kg), Groups IV: PFOS (2.5 mg/kg) + curcumin

The findings of the CAT activity in the liver, kidney, and brain tissues are illustrated in Figure 3. No significant difference was observed between the control group and the curcumin group (group II) in all tissues ($p > 0.05$). However, when comparing the other groups (groups III-V) with the control group, CAT enzyme activity significantly decreased ($p < 0.05$). Group III exhibited a 0.5-fold change in liver tissue, a 0.4-fold change in kidney tissue, and a 0.3-fold change in brain tissue compared to the control group (group I). In group V, a 0.2-fold change was observed for all the tissues studied compared to group I. CAT activity declined in animals treated with low-dose PFOS (group III) and high-dose

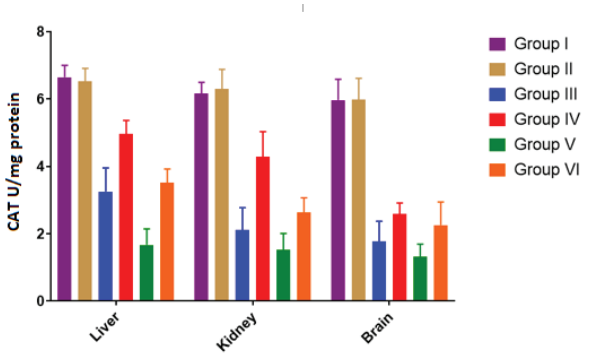

Figure 3. Activities of CAT in control, PFOS and PFOS+Curcumin groups in liver, kidney, and brain tissues of rats.
Groups I: control, Groups II: curcumin (80 mg/kg), Groups III: PFOS (1.25 mg/kg), Groups IV: PFOS (1.25 mg/kg) + curcumin, Groups V: PFOS (2.5 mg/kg), Groups IV: PFOS (2.5 mg/kg) + curcumin.

PFOS (group V), while a significant increase in CAT enzyme activities was observed in the groups exposed to PFOS+curcumin (group IV, group VI). Comparing group IV with group III, a mean 1.7-fold change was observed for liver, kidney, and brain tissues, while a mean 1.9-fold change was observed for all these tissues when comparing group VI with group V.

The levels of MDA in the tissues are given in Figure 4. A comparison between the results of the control and curcumin groups showed no significant difference. However, lipid peroxidation, as indicated by MDA levels, significantly increased in the PFOS groups compared to the control group (group I) ($p < 0.05$). Compared to the control group (group I), 2.5-fold change for liver tissue, 5-fold for kidney tissue, and 9.4-fold for brain tissue were observed in group III. In group V, 4.7-fold change for the liver, 6-fold for the kidney, and 10.5-fold for the brain were detected. The highest increase was defined in the group exposed to high-dose PFOS. Treatment with curcumin after PFOS exposure significantly decreased the MDA levels. In the comparison of group IV with group III, a mean 0.34-fold change was observed for liver, kidney and brain tissues. Furthermore, when group VI was compared with group V, a mean 0.45-fold change was discerned for all of these tissues.

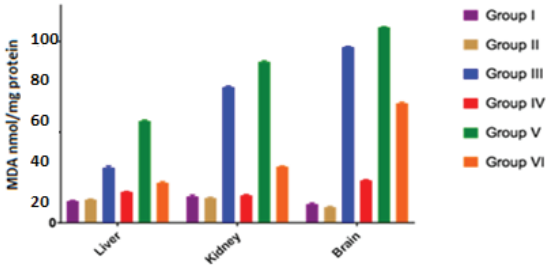

Figure 4. Level of MDA in control, PFOS and PFOS+ Curcumin groups in liver, kidney, and brain tissues of rats.
Groups I: control, Groups II: curcumin (80 mg/kg), Groups III: PFOS (1.25 mg/kg), Groups IV: PFOS (1.25 mg/kg) + curcumin, Groups V: PFOS (2.5 mg/kg), Groups IV: PFOS (2.5 mg/kg) + curcumin

DISCUSSION

Oxidative stress occurs as a result of an imbalance between free radicals and the antioxidant defense system. This can lead to the loss of the functions of these basic biomolecules and the formation of many diseases (Xu, Meng, Li, Gan, Li, & Li, 2018).

Curcumin is widely used as colorant and spice. This bright yellow curcuminoid includes various functional antioxidant groups, such as the β-diketo group, carbon-carbon double bonds, and phenyl chains. Due to these properties, curcumin converts to a phenoxy radical by removing lipid radicals in the cell membrane. Therefore, this compound is accepted as a very effective natural antioxidant. It was also found that curcumin inhibited lipid peroxidation and neutralized reactive oxygen species (superoxide, hydroxyl radicals) (Farzaei et al., 2018).

PFOS is a member of the Per- and Polyfluoroalkyl Substances chemical class (PFAS). The exceptional resistance of PFOS to environmental degradation is due to the strong chemical bond between carbon and fluorine atoms. The motility, toxicity, and bioaccumulation possibility of this chemical cause potential adverse effects on the environment and human health (Torres, Redko, Limper, Imbiakha, Chang, & August, 2021; Endirlik & Gürbay, 2018). A thorough research of the potentially harmful effects on living things has become more important as a result of the increasing usage of PFOS in industries and cosmetic applications. (Dhore & Murthy, 2021).

There is no study in the literature about the effects of curcumin with antioxidant properties on oxidative damage caused by PFOS in rat liver, kidney, and brain tissues. In this study, we investigated whether oxidative damage induced by PFOS on the liver, kidney, and brain tissues of rats and curcumin have a possible protective effect against this oxidative damage or not. Our study's findings demonstrated that PFOS administered at different concentrations in rat liver, kidney, and brain tissues caused oxidative damage. Furthermore, a reduction in SOD and CAT enzyme activities was detected in the PFOS treated tissues. There was a significant increase in the MDA levels. Moreover, our findings correlate with previous studies showing that the toxicity of PFOS is closely related to ROS production and the induction of oxidative stress (Xu et al., 2013; Wang et al., 2020) Xing et al. (2016) treated liver tissue homogenates of adult male C57BL/6 mice with PFOS at different doses (2.5, 5 or 10 mg PFOS/kg BW/day) for 30 days and examined the SOD, CAT and MDA levels. They found that SOD and CAT activities in the liver decreased significantly depending on the increased PFOS dose, while MDA levels were significantly higher compared to the control (Xing et al., 2016).

Mandour et al. (2022) exposed adult male albino rats to PFOS (20 mg/kg/day) for 28 days and determined that the antioxidant enzyme levels (SOD and CAT) in the liver tissue of the rats decreased significantly and MDA levels increased (Mandour, Maher, Abd El, & Moawad, 2022). Treatment with 100 µM PFOS for 24 hours has been reported to induce ROS production in renal tubular cells (Lee et al., 2022). It was determined that the levels of MDA, a critical marker for oxidative stress, in the kidney tissues of rats exposed to PFOS (20 µM

and 60 μM) for 24 hours were significantly higher than the control group (Tang, Yu, Zhuge, Chen, Zhang, & Jiang, 2022). In one study, it was determined that PFOS (100 μM) exposure for 1, 3, 6 and 24 hours significantly increased ROS production in renal tubular cells (Wen, Chen, Lee, Ko, Chou, & Juan, 2021). Antioxidant enzymes like SOD and CAT are crucial in mammalian cells for blocking superoxide and hydroxyl ions (Sankar, Telang, & Manimaran, 2018). It has been shown that PFOS causes oxidative stress in a dose-dependent manner, not only by producing ROS, but also by reducing levels of antioxidants like SOD and CAT. This has been shown to cause a weakening of the antioxidant defense systems and induce tissue damage. In addition, in this study, the increase in lipid peroxidation value shows impairment of the membrane foundation. These changes in the MDA, CAT, and SOD values were more strong in the high dose PFOS-exposed rats as compared to rats treated with low dose PFOS. The response of the antioxidant system to oxidative stress shows variations among species (Adonaylo & Oteiza, 1999). In our study, the highest decrease in SOD and CAT enzyme activities and increase in MDA levels were detected in the brain tissue treated with PFOS. The brain was found to be more vulnerable to oxidative damage than the liver and kidney. The brain, which contains large amounts of polyunsaturated fatty acids, may be particularly sensitive to oxidative damage. When the natural structure of these organs is disturbed by toxicants or other stimulants, all of the essential physiological processes may get imbalanced. Their toxicities are fundamental biosafety assessment markers for novel medicines (Kanwal et al., 2019).

In our study, the antioxidant enzyme activities increased while there was a significant decrease in the MDA levels in the PFOS+curcumin treated group compared to the group treated with PFOS. The antioxidant mechanism of curcumin interacts with the oxidative cascade and can neutralize free radicals. Curcumin inhibits MDA production to improve antioxidant levels in PFOS-treated sensitive to oxidative stress.

Studies have shown that curcumin can directly remove free radicals and prevent ROS production (Tapia et al., 2014). In a study conducted by reducing the amount of curcumin oxidative stress, inflammation, and apoptosis, the colistin-induced nephrotoxicity and neurotoxicity was determined to be decreased (Edrees, Galal, Monaem, Beheiry, & Metwally, 2008). Curcumin has been found to have an important hepatoprotective effect against liver damage induced by ochratoxin A, CCl_4, sodium florid, and heavy metals in adult rats (Damiano et al., 2021; Park, Jeon, Ko, Kim, & Sohn, 2000; Moghaddam et al., 2015; García-Niño &

Pedraza-Chaverrí, 2014). In a previous study, rats treated with PTZ (pentylenetetrazole) for 35 days were given different doses of curcumin (50, 100, 200 mg/kg) and a dose-dependent reduction in MDA levels in brain tissue (Agarwal, Jain, Agarwal, Mediratta, & Sharma, 2011). In a study performed by AL-Harbi et al., curcumin application (60 mg/kg) was found to have hepatoprotective effects on oxidative stress induced by sodium fluoride, and it also decreased hepatotoxicity and liver enzyme activities (AL-Harbi, Hamza, & Dwary, 2014). In a previous work, it has been shown that Turmeric reduces cell viability in the L-02 human fetal hepatocyte cell line, prevents oxidative stress, and inhibits SOD activities and GSH levels. (Dai, Tang, Li, Zhao, & Xiao, 2015). In another study, curcumin administered to rats exposed to cadmium, a common environmental heavy metal pollutant, has been shown to have the capability to reduce lipid peroxidation and increase GSH levels (Eybl, Kotyzová, & Bludovská, 2004). Singh et al. stated that curcumin can be a powerful protective agent on lindane-induced hepatotoxicity (Singh & Sharma, 2011).

In conclusion, our study demonstrates that curcumin has a protective effect against oxidative damage caused by PFOS in the liver, brain, and kidney tissues of rats. These protective effects of curcumin are basically related to its antioxidant properties. Our findings highlight the toxicity of PFOS exposure and indicate that careful consideration should be given to PFOS use because it may have detrimental effects on humans.

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Original Article

Antioxidant, cytotoxic and anti-inflammatory properties of *Anthemis tricolor* Boiss. through a series of cellular assays and inhibition of Turkish *Macrovipera lebetina obtusa* venom induced inflammation in rat

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ABSTRACT

Background and Aims: Venom of *Macrovipera lebetina* obtusa (Viperidae), Turkey's biggest viper, is highly toxic to humans and causes inflammation. In Anatolian traditional medicine, many *Anthemis* L. species are used in wound healing and against inflammatory diseases. In the present study, antioxidant, cytotoxic and anti-inflammatory activities of different endemic *A. tricolor* Boiss. extracts were evaluated by *in vitro* assays. Besides, the anti-inflammatory activities of chloroform extract against carrageenan and snake venom induced-edema were investigated in rats.
Methods: Antioxidant and anti-inflammatory activities were evaluated by DCFH-DA, iNOS, NF-κB inhibitions and NAG-1 gene activation, while WST-1 assay was preferred for cytotoxic activity. Besides, the hind paw edema test was used for *in vivo* studies.
Results: Chloroform extract exhibited the strongest NAG-1-inducing activity. In addition, this extract showed potent iNOS and NF-κB inhibition (IC_{50}:14.0 and 10.75 µg/mL, respectively) and cytotoxic effect against human osteosarcoma (U2OS; IC_{50}: 15.18 µg/mL) and human cervical cancer (HeLa; IC_{50}: 18.3 µg/mL) cell lines. Moreover, chloroform extract had stronger anti-inflammatory effects against both carrageenan and snake venom induced-edema formation than indomethacin. Fifty and 100 mg/kg extracts reduced the paw edema to 1-3% at 4 h after the snake venom injection. On the other hand, 50, 25 and 12.5 mg/kg of the extract completely inhibited inflammation induced by carrageenan.
Conclusion: This is the first report on the antioxidant, cytotoxic and anti-inflammatory effects of endemic *A. tricolor* by *in vitro* studies and snake venom-induced paw edema in rats. The plant exhibits strong potential for treating local tissue damage in snake bites.
Keywords: *Macrovipera lebetina obtusa, Anthemis tricolor,* cytotoxicity, antioxidant, anti-inflammatory, *in vivo*

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INTRODUCTION

Snake bite is a worldwide problem that can lead to serious injuries and even death (Chippaux, Williams, & White, 1991). In these cases, some local effects, such as necrosis, bleeding, pain and edema, which develop rapidly after injury, and usually cause a wound have been reported (Ohsaka, 1979; Otero et al., 2002; Rosenfeld, 1971; Sawai, 1980). Anti-venom treatment is the primary medical application in the therapy of bitten people by venomous snakes. However, studies have shown that anti-venom therapy is not effective in symptomatic treatment (Melo, Habermehl, Oliveira, & Nascimento, 2005; Morais, De Freitas, Yamaguchi, Dos-Santos, & DaSilva, 1994; Picolo, G., Chacur, Gutierrez, Teixeira, & Cury, 2002).

Most snake venom cases in Turkey are caused by viper bites. The blunt-nosed viper *Macrovipera lebetina* (Linnaeus, 1758) (Viperidae) is quite a poisonous species and is spread widely from the west and central Asia to northern Africa (Nilson & Andrén, 1988). In Turkey, *M. l. obtusa* (Dwigubsky, 1832) is distributed in East, Southeast and Northeast Anatolia. Venom of this species has cytotoxic, antitumoral and antimicrobial activities (Ghazaryan et al., 2015; Ozen, Igci, Yalcin, Gocmen, & Nalbantsoy, 2015; Suzergoz et al., 2016). Also, anti-inflammatory effects of *Centaurea calolepis* Boiss. and its major compound cnicin against *M. l. obtusa* venom have been investigated by our team previously (Demiroz, Albayrak, Nalbantsoy, Gocmen, & Baykan, 2018).

The genus *Anthemis* L. (Asteraceae) comprises of nearly 210 species distributed widely across South-West Asia, North and North-East Africa and Europe. Fifty-one species are present in Turkish flora (Boukhary, Aboul-ElA, & El-Lakany, 2019; Ghafoor, 2010). Generally, *Anthemis* species are known as "papatya, Alman papatyası" in Anatolia and capitulums (flowers) are used to treat urinary inflammation, dysmenorrhea, gastrointestinal disorders, hemorrhoids, hypertension and wounds in Anatolian traditional medicine (Baytop, 1999; Boukhary et al., 2019; Cakilcioglu, Khatun, Turkoglu, & Hayta, 2011; Tetik, Civelek, & Cakilcioglu, 2013). Cytotoxic, antimicrobial, anti-inflammatory, enzyme inhibition (elastase, α-amylase, α-glucosidase, anticholinesterase and tyrosinase inhibition), antidepressant, sedative, anti-diuretic and antioxidant activities of different *Anthemis* species have been reported previously (Acquaviva et al., 2012; Baltaci, Kolatan, Yilmaz, & Kivçak, 2011; Boukhary et al., 2019; Chemsa et al., 2018; Conforti et al., 2012; Hajdú, Zupkó, Réthy, Forgo, & Hohmann, 2010; Quaerenghi, Tereschuk, Baigori, & Abdala, 2000; Rossi, Melegari, Bianchi, Albasini, & Vampa, 1988; Sarikurkcu, 2020; Vučković, Vujisić, Klaas, Merfort, & Milosavljević, 2011). Phytochemical investigations have shown sesquiterpene lactones (nobilin, tatridin A, 1-epi-tatridin B), phenolics (caffeoylquinic acid derivatives, ferulic, gallic and 3-hydroxybenzoic acids) and flavonoids (salvigenin, apigenin, quercetin, naringenin, pectolinarigenin, eupatilin, rutin) are mainly secondary metabolites of this genus (Emir & Emir, 2020; Gönenç, Akkol, Süntar, Erdoğan, & Kıvçak, 2014; Pavlovic, Kovacevic, Couladis, & Tzakou, 2006; Staneva, Todorova, & Evstatieva, 2008; Sut et al., 2019; Todorova, Trendafilova, Danova, & Dimitrov, 2011).

Anthemis has 10 taxa in Cyprus flora and *A. tricolor* Boiss. is one of the two endemic species of this genus in Cyprus. The major compounds of *A. tricolor* essential oil have been reported as germacrene D (5.5%), β-caryophyllene (9.1%) and muurola-4,11-diene (20.2%) (Karaalp et al., 2014). There are also two studies on the anti-inflammatory and antibacterial effects of this species (Demirkan, Özçınar, & Kıvçak, 2019; Gulsoy Toplan, Tuysuz, Mat, & Sariyar, 2017). In this study, cytotoxic and antioxidant activities of *A. tricolor* were reported in detail. Moreover, the anti-inflammatory effects of this species against the standard inductive agent carrageenan and snake venom have been investigated.

Edema is a clear sign of inflammatory response and is due to mediators that cause local vasodilatation, such as histamine, bradykinin, serotonin, prostaglandin E2 and I2 (De Toni et al., 2015). Inflammation is one of the major symptoms not only for snake bite but also cancer, obesity and metabolic disorders. The NAG-1 gene, which is responsible for the apoptotic elimination of cancer cells, is related to the anti-inflammatory action of NSAIDs and the induction of NAG-1 is a COX-independent mechanism (Baek, Wilson, Lee, & Eling, 2002). Another important gene associated with the expression of several proinflammatory genes, such as cytokines and inducible enzymes, is nuclear factor kappa B (NF-κB). Activation of NF-κB plays a central role in initiating and promoting the inflammatory response. Nitric oxide (NO) is a pleiotypic inflammatory mediator, which is produced by inducible nitric oxide synthase (iNOS). Under pathological conditions, macrophages can significantly increase NO production. Thus, iNOS and NO inhibition are important goals of anti-inflammatory drug discovery (Gosslau, Li, Chi-Tang, Chen, & Rawson, 2001).

Reactive oxygen species (ROS) are mediators of oxidative stress that can damage all cell structures. It has a major role in several diseases such as cancer, impaired immune system and increased risk of infectious diseases, diabetes, cardiovascular disorders, etc. Exogenous antioxidants (natural products) can prevent, or repair injuries caused by ROS and enhance the immune defense against cancer and degenerative diseases (Alexieva, Markova, Nikolova, Aragane, & Higashino, 2010). It is still urgent to identify new anticancer agents with selective toxicity, although there have been advances in cancer research. Natural products, mainly originated from plants, are used clinically as anti-cancer agents (Cragg & Newman, 2005). Plant extracts are the starting point of investigation of leading anticancer compounds from natural sources.

In this study, we aimed to understand the antioxidant, cytotoxic and anti-inflammatory activities of *A. tricolor* and to show the effects of its different extracts on the substances involved in the inflammation mechanism both *in vitro* and *in vivo*. For this purpose, the anti-inflammatory effects of the extracts were investigated by *in vitro* cell-based tests and *in vivo* in rats against different inflammation inducers such as *Macrovipera lebetina obtusa* venom and carrageenan.

MATERIALS AND METHODS

Reagents

The cells were obtained from ATCC (Rockville, MD, USA). DCFH-DA, RPMI-1640 and DMEM/F12 media were purchased from Invitrogen (Carlsbad, CA). The NF-κB reporter construct contained two copies of the element from the immunoglobulin

K promoter (p BIIXLUC) and was a gift from Dr. Riccardo Dalla-Favera (Columbia University, New York, USA). The Sp-1 reporter plasmid (pGL3-promoter) was from Promega. The luciferase constructs containing NAG-1 promoter were a gift from Dr. Elling, NIH, North Carolina. The Luciferase Assay kit was from Promega (California, USA). All chemicals and reagents were purchased from Sigma (St. Louis, MO, USA).

Snake venom

The lyophilized venom has obtained from pooled venom extracted for previous studies from two adult *M. l. obtusa* males which were from Osmaniye province in Turkey (Ozen et al., 2015). The lyophilized venom was dissolved in isotonic saline and sterilized by filtration through a 0.22 µm filter. The protein concentration of crude venom was determined by the BCA assay (Thermo Scientific, USA). Measurements were performed at 562 nm by a spectrophotometer (Thermo Scientific, USA).

Plant material and extraction

From our previous study, *A. tricolor* was collected from Taşkent Village, Cyprus during the flowering period (April 2010) (NEUN Herbarium no. 01337) (Karaalp et al., 2014).

For extraction, aerial parts of the *A. tricolor* (20 g) were dried and powdered. Then, *n*-hexane, chloroform and methanol extracts were prepared with an ultrasonic bath (3x 200 ml, 4 h for each) and evaporated to dryness under reduced pressure at 40°C (Demiroz, Nalbantsoy, Kose, & Baykan, 2020).

***In vitro* experiments**

To prepare a stock solution, lyophilized extracts were dissolved in DMSO at a concentration of 10 mg/mL. The concentrations of the extracts were 0.5, 5 and 50 µg/ml in DCFH and iNOS inhibition and NAG-1 activation assays. The final concentration of DMSO was adjusted to be less than 0.5%.

Inhibition of Reactive Oxygen Species (ROS) generation

Inhibition of cellular oxidative stress was measured by the DCFH method according to Reedy et al. (Reddy, Gupta, Jacob, Khan, & Ferreira, 2007). Briefly, myelomonocytic HL-60 cells (1 x 10^6 cells/mL) cultured in RPMI 1640 medium were treated with test compounds (0.5, 5, 50 µg/mL) for 30 min. Then, cells were stimulated with 100 ng/mL phorbol 12-myristate-13-acetate (PMA, Sigma) for 30 min as previously described by our team (Demiroz et. al., 2020). DCFH-DA (Molecular Probes, 5 µg/mL) was added and cells were further incubated for 15 min. Levels of DCF were measured on a PolarStar plate reader with excitation wavelength at 485 nm and emission at 530 nm. The antioxidant activity of test samples was determined in terms of % decrease in DCF production compared to the vehicle control. Trolox was used as the positive control.

Activation of nonsteroidal anti-inflammatory drug (NSAID) activated gene-1 (NAG-1)

Human chondrosarcoma cells (SW1353) cultured in DMEM/F12 were transfected with 25 µg of luciferase plasmid construct (pcDNA3.1-NAG-1) containing a full-length NAG-1 cDNA by electroporation at 160 V and one 70-ms pulse using a BTX Electro Square Porator T 820 8BTX IN (San Diego, CA). Transfected cells were plated in 96 well plates at 1x10^5 cells/200 µl/well in DMEM/F12 supplemented with 10% FBS. After 24 h, the cells were treated with different concentrations of test compounds (0.5, 5, 50 µg/mL) for 24 h (Nalbantsoy et al., 2012). Luciferase activity was measured using the Luciferase Assay kit (Promega, USA). Light output was detected on a SpectraMax plate reader and fold activation of NAG-1 activity was calculated in comparison to the vehicle control (Nalbantsoy et al., 2012). Diclofenac was used as the positive control.

Inhibition of iNOS (Inducible Nitric Oxide Synthase) activity

Mouse macrophages (RAW264.7) were cultured in phenol red-free RPMI medium with 10% bovine calf serum. The assay cells were seeded in 96-well plates (50,000 cells/well) and incubated for 24 h. The assay was performed as previously described (Demiroz et al., 2020). The cultured cells were treated with different concentrations of the extracts (0.5, 5, 50 µg/mL). The level of nitrite in the medium was measured by using Griess reagent. The percent inhibition of nitrite production by the samples was calculated in comparison to the vehicle control. IC_{50} values were obtained from dose curves.

Cytotoxicity assay

In vitro cytotoxic activities of extracts of *A. tricolor* were evaluated using WST1 reagent (Roche, Mannheim, Germany) by analyzing the number of viable cells by the cleavage of tetrazolium salts added to the culture medium. Three cancer cell lines, U2OS (human osteosarcoma cell line), A549 (human lung cancer cell line), HeLa (human cervical cancer cell line) and one non-cancer cell line, 293 HEK (human embryonic kidney cell line) were cultured in DMEM supplemented with L-glutamine (2 mmol/L), 100 U/mL penicillin, 100 µg/mL streptomycin and 10% fetal bovine serum. Cells in the exponential growth phase were seeded in 96-well plates to make 5,000 cells/wells and 24 h later, samples were added at various concentrations (100, 50, 25, 10, 5 and 2 µg/mL). After 48 h drug exposure, cell viability was measured using WST-1 cell proliferation reagent. Absorbance at 450 nm and a reference wavelength at 620 nm were determined by microplate reader (Versamax, Molecular Devices, Sunnyvale, California, United States). The absorbance of the culture medium with supplemented WST-1 in the absence of cells was used as the background control. All measurements were performed in triplicate (Parlar et al., 2018).

***In vivo* experiments**

Acute toxicity of A. tricolor extracts

Animals used in the venom and carrageenan-induced paw edema experiments were observed for 48 h and cases of morbidity or mortality were documented for each group at the end of the observation period.

Experimental animals

The experimental protocol was approved by Ege University's Local Ethical Committee of Animal Experiment (Date: 21.10.2015, number: 2015-078). Male/Female Wistar albino rats weighing 150– 200 ± 20 g were purchased from the Experimental Animal Center of Ege University (Izmir, Turkey). Rats were maintained under standard conditions of temperature 22 ± 1°C with a regular 12 h light: 12 h dark cycle and were allowed free access to standard laboratory food and water.

***M. l. obtusa* venom and carrageenan-induced inflammation**

M. l. obtusa venom (75 µg/paw) and carrageenan 1% (10 mg/kg) were used as challenge doses to create edema, as stated in our previous study (Demiroz et al., 2018). The venom was dissolved in isotonic saline and injected sub-plantarly into the left hind paw of the rats. Control and indomethacin groups (against carrageenan and venom) were tested at the same time as a previous study performed by our team to reduce the use of animals due to ethical regulations. Therefore, the results in Table 4, Figure 1 and Figure 2 include the values determined in the previous study.

Assessment of anti-inflammatory activity

Rats were deprived of food overnight. Male and female rats were divided randomly into 11 groups (n=6). Twenty-five, 50 and 100 mg/mL of *A. tricolor* extracts against *M. l. obtusa* venom including 75 µg/paw *M. l. obtusa* venom+Tween 20 (5%) as a negative control group and 2.5, 12.5, 25 and 50 mg/mL *A. tricolor* extracts against carrageenan including carrageenan+Tween 20 (5%) as a negative control group were tested to compare (n=6). Chloroform extracts were dissolved in Tween 20 (5%) and administrated orally. Thirty minutes after administration, 0.1 mL of inductive agent (*M. l. obtusa* venom

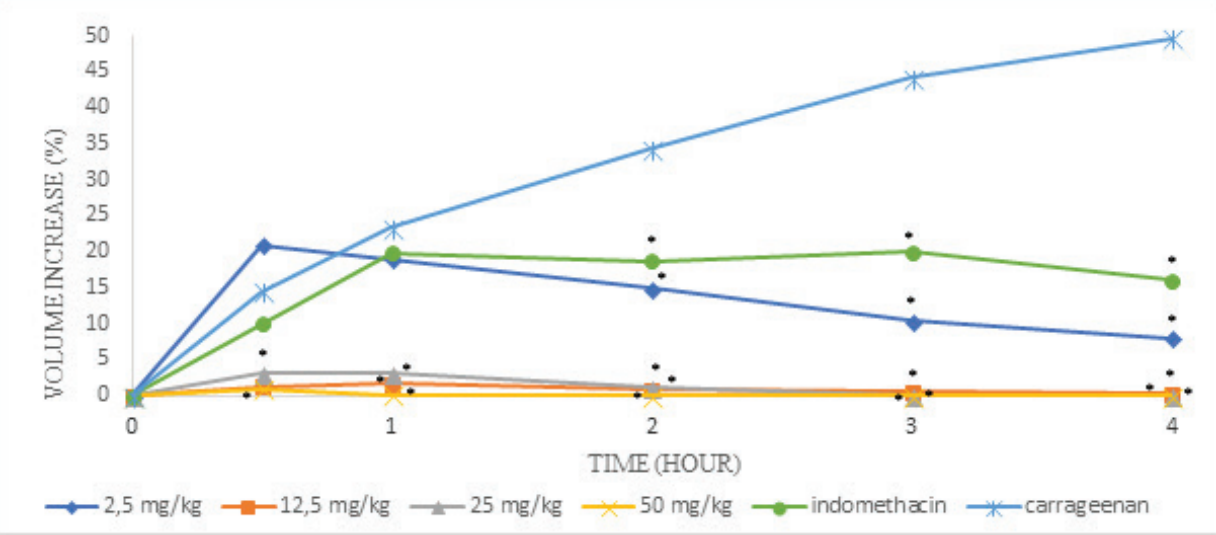

Figure 1. Anti-inflammatory potential effect of *A. tricolor* extract (2.5, 12.5, 25, 50 mg/kg) and indomethacin (10 mg/kg) against carrageenan-induced edema in rats. (*p<0.001 compared to carrageenan) carrageenan-induced edema.

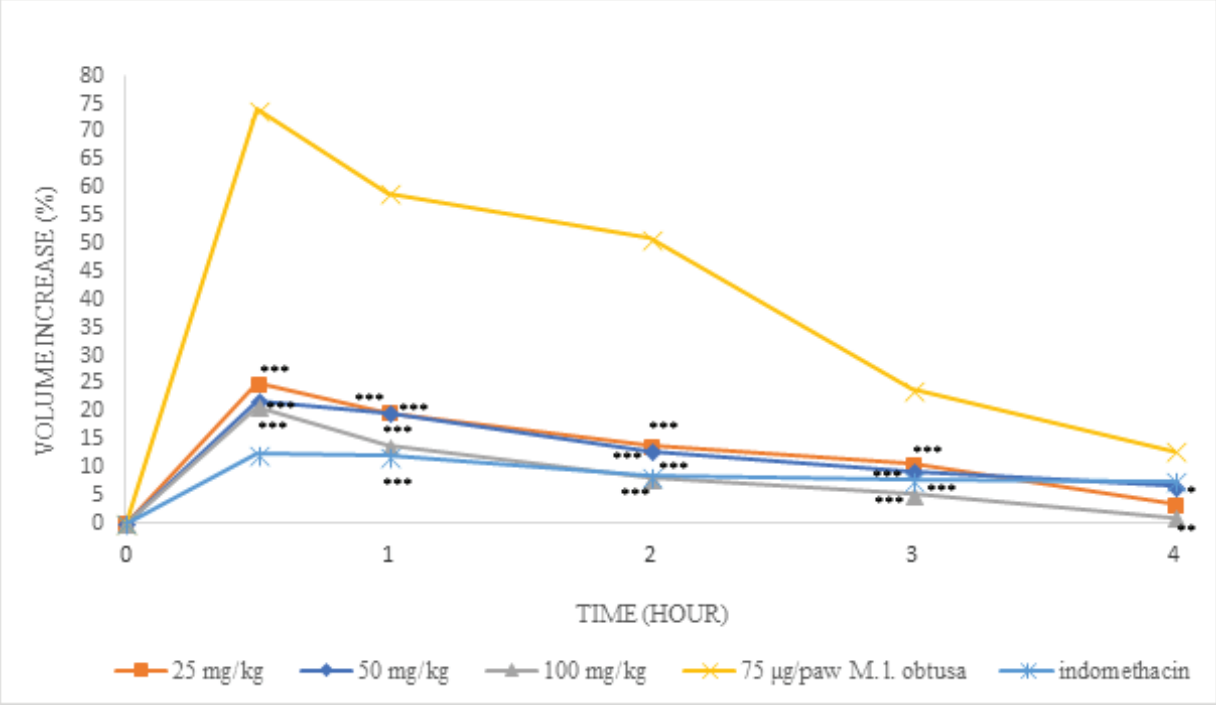

Figure 2. Anti-inflammatory potential effect of *A. tricolor* extract (25, 50, 100 mg/kg) and indomethacin (10 mg/kg) against 75 µg/paw of *M. l. obtusa* venom (*p < 0.05, **p < 0.01, ***p < 0.001 compared to *M. l. obtusa* venom).

or carrageenan) dissolved in isotonic saline was injected subplantar into the left hind paw. Isotonic saline (0.1 mL) was injected in the same way into the contralateral hind paw as a negative control group (n=6, for each group). Both paw volumes were measured by using a hydroplethysmometer (UGO BASILE, 21020 Monvalle VA, Italy) at 0.5, 1, 2, 3 and 4 h following inflammatory agent injection. Indomethacin (10 mg/kg) was used as the positive control anti-inflammatory agent (Sigma Chemical Co, St, Louis, USA) (Demiroz et al., 2018). The data obtained from the different groups was reported as the mean ± SD and % edema increase was calculated by the following formula:

$$Inhibition\ of\ edema\ (\%) = (Vcontrol - Vtreated)/Vcontrol$$

Data analysis

Each experiment was performed in triplicate for cytotoxic activities and anti-inflammatory experiments. Values were presented as mean ± standard error of the mean (SEM). IC50 calculation, and variance analysis (standard deviation calculation) were performed with Graph Pad Prism. Data were analyzed using the Student t-test, ANOVA, or nonparametric tests. Differences between extract or drug treated and control groups in vivo tests were also evaluated using Dunnetts t-test. The mean and SD of n = 6 were calculated. A probability value of $p \leq 0.05$ was considered statistically significant.

RESULTS

In present study, aerial parts of *A. tricolor* were extracted with *n*-hexane, chloroform and methanol. Then, antioxidant, cytotoxic and anti-inflammatory activities of the extracts were evaluated in *in vitro* and *in vivo* conditions. Yields of *n*-hexane, chloroform and methanol extracts were found as 1.44, 0.72 and 7.24% (288, 143 and 1447 mg), respectively.

***In vitro* experiments**

In vitro anti-inflammatory activities were evaluated over cellular mechanisms such as NF-κB, iNOS and ROS inhibition and induction of NAG-1. Results are shown in Table 1. While hexane and chloroform extracts showed moderate inhibition of iNOS (IC_{50}:16.5 ± 2.1 µg/mL and 14.0 ± 1.4 µg/mL respectively) and NF-κB (IC_{50}:15.0 ± 4.2 µg/mL and 10.75 ± 1.7 µg/mL respectively), methanol extract of the plant was inactive towards both targets. Moreover, hexane and chloroform extracts exhibited a weak inhibitory effect on Sp-1 dependent luciferase expression. Inhibition of cellular oxidative stress was measured by the DCFH method. The chloroform extract demonstrated the highest effect (IC_{50}: 4.45 ± 0.88 µg/mL), followed by methanol extract (IC_{50}: 36.0 ± 9.90 µg/mL) and finally hexane extract was found to be inactive in the inhibition of the ROS generation mechanism.

Dose response of NAG-1 induction by the extracts is given in Table 2. All extracts exhibited significant activity. The chloroform and hexane extracts showed the highest activity with about 7-8-fold induction at 50 µg/mL, followed by the methanolic extract (approximately 5-fold). At the doses of 5 µg/mL and 0.5 µg/mL, activation by all extracts was lower than the standard drug diclofenac (4.7-fold at 20 µM).

The cytotoxicity of the extracts against cancerous U2OS (human osteosarcoma), A549 (human lung cancer), HeLa (human cervical cancer) and non-cancerous HEK293 (human embryonic kidney) cell lines were evaluated and results are summarized in Table 3. Both hexane and chloroform extracts inhibited proliferation of the non-cancerous HEK293 cells. However, the strongest cytotoxic effect was observed in the chloroform extract against U2OS (IC_{50}:15.18 µg/mL) and HeLa (IC_{50}: 18.3 µg/mL). The hexane extract showed less toxicity against U2OS cell

Table 1. ROS, NOS, NF-κB and Sp-1 inhibition activities of *A. tricolor* extracts (IC_{50}, µg/mL).

	ROS	iNOS	NF-κB	Sp-1
Hexane extract	-	16.5 ± 2.1	15.0 ± 4.2	36.5 ±1.6
Chloroform extract*	4.45 ± 0.88	14.0 ± 1.4	10.75 ± 1.7	45.5 ± 4.9
Methanol extract	36.0 ± 9.9	-	-	-
Trolox	0.22 ± 0.02	N.A.	N.A.	N.A.
Parthenolide	N.A.	3.2 ± 0.7	0.9 ± 0.1	6.25 ± 1

IC_{50} values were presented as mean ± SEM. Dash means no activity; N.A.: Not applicable. (*p< 0.5 for Chloroform extract compared to other extracts in all of activities).

Table 2. *A. tricolor* extracts on NAG-1 fold induction.

	50 µg/mL	5 µg/mL	0.5 µg/mL	20 µg/mL
Hexane extract	6.8 ± 0.8	3.4 ± 1.0	2.5 ± 0.2	N.A.
Chloroform extract*	7.9 ± 2.1	4.1 ± 0.1	4.4 ± 0.9	N.A.
Methanol extract	5.2 ± 0.6	4.1 ± 1.1	2.9 ± 0	N.A.
Diclofenac	N.A.	N.A.	N.A.	4.7 ± 0.1

Values were presented as mean ± SEM. N.A.: Not applicable. (*p < 0.5 for Chloroform extract compared to other extracts).

line than chloroform extract (IC_{50}: 33.25 µg/mL), it was ineffective against HeLa and A549. In addition, the methanol extract had no activity against any of the cell lines tested.

In vivo experiments

In vivo studies were performed with chloroform extract, which had the highest effect in the *in vitro* anti-inflammatory studies. Also, the extract had no effect, inducing no obvious acute toxicity at all, in the exposure doses in the rats.

Carrageenan-induced inflammation

Percentages of paw volume increase were tested for the 2.5, 12.5, 25 and 50 mg/kg doses of the *A. tricolor* extract against carrageenan-induced edema. Percent edema (Edema %) increases at all doses can be seen in Table 4. The results were compared with the carrageenan and indomethacin results in our previous study, which was performed by our group concurrently with the current study. According to the previous study, at 4 h post induction of carrageenan, the percentage paw volume peaked around 49.64%. After indomethacin application, a plateau was observed between 1 and 3 h (81-82% inhibition) and finally a 16.01% edema increase was determined at 4 h (Demiroz et al., 2018). The current study showed all doses of extracts inhibited edemas at 0.5 h after the carrageenan injection. Moreover, *A. tricolor* extracts had much stronger anti-inflammatory activity than indomethacin against carrageenan-induced edema. At the first half hour, 1-3% volume increases were observed with 12.5, 25 and 50 mg/kg extracts and then the edemas were inhibited approximately 100% at end of 4 h. As an exception, an approximate 21% increase was observed 0.5 h after 2.5 mg/kg extract application. However, it had a similar effect to indomethacin at the end of 4 h (92.04 % inhibition) (Figure 1).

Snake venom-induced inflammation

In the previous study, 75 µg/paw of the venom was chosen as the challenge dose. The percentage volume of the paw measured peaked at 73.5% at 0.5 h after venom injection and reduced to 13.5%, the lowest percentage of paw volume was at 4 h. After 10 mg/kg indomethacin application, the percentage paw volume increase induced by *M. l. obtusa* venom were

Table 3. Cytotoxic activities of *A. tricolor* extracts on different cell lines (IC_{50}, µg/mL).

Extract/Cell Line	A549	U2OS	HeLa	HEK293
Hexane	-	33.25 ± 1.72	82.10 ± 5.65	34.38 ± 1.06
Chloroform*	40.85 ± 2.23	15.18 ± 0.84	18.30 ± 0.98	12.26 ± 0.71
Methanol	-	-	-	-

IC_{50} values were presented as mean ± SEM. Dash means no activity. (*p < 0.5 for Chloroform extract compared to other extracts

Table 4. Anti-inflammatory effect of *A. tricolor* extract against *M. l. obtusa* venom and carrageenan-induced edema in rats.

Group	Agent	Dose (mg/kg)	Edema Increase (%)				
			0.5 h	1 h	2 h	3 h	4 h
Control	Car.*	100 µL (%1 Car.)/paw	14.43±3.42	23.34±2.57	34.35±3.93	44.2±4.9	49.64±4.33
	*M. l. obtusa**	75 µg /100 µl/paw	73.5±3.39	58.67±3.39	51.17±1.35	23.5±1.45	13.5±1.11
A. tricolor	Car.	2.5	20.91±0.05	19.02±0.09	**14.84±0.13**	**10.39±0.11**	**7.96±0.09**
		12.5	**1.18±0.02**	**1.65±0.04**	**0.82±0.03**	**0.47±0.02**	**0.23±0.01**
		25	**3.12±0.05**	**3.03±0.05**	**1.12±0.02**	**0**	**0**
		50	**1.0±0.02**	**0.12±0**	**0**	**0**	**0**
A. tricolor	*M. l. obtusa*	25**	22.0±0.17	19.86±0.18	12.94±0.17	9.4±0.12	6.73±0.1
		50**	25±0.09	19.85±0.08	13.98±0.05	10.69±0.04	3.4±0.07
		100***	20.93±0.08	13.83±0.07	8.2±0.04	5.26±0.05	1.22±0.01
Ind. (10 mg/kg)	Car.*	100 µL (%1 Car.)/paw	10.03±3.01	19.87±4.03	18.7±3.45	19.99±2.14	16.01±2.59
	*M. l. obtusa**	75 µg /100 µL/paw	12.67±6.78	12.49±5.67	8.92±5.24	8.06±4.37	7.96±3.72

Values are represented as mean ± SD. Carrageenan (Car.), Indomethacin (Ind.). *Based on the previous study (Demiroz et al., 2018) (*A. tricolor* and indomethacin **p < 0.05, ***p < 0.01 compared to *M. l. obtusa* venom) (For the bold written values, p < 0.001 compared to carrageenan control group data)

12.67% and 7.96% at 0.5 and 4 h respectively (Demiroz et al., 2018). In the current study, the volume of the paw increase (%) calculated for 25, 50 and 100 mg/kg doses of *A. tricolor* chloroform extract against venom induced-edema is given in Figure 2. The most effective dose was the 100 mg/kg extract, which inhibited the edema at 79.07% at the first half hour. On the other hand, all of the extracts which reduced the edema to 1.22-6.73% had a very strong effect at the end of 4h. While the 100 mg/kg dose of the extract inhibited the edema approximately 99% at 4 h, the anti-inflammatory effect of indomethacin was 92% (Figure 2).

DISCUSSION

In vitro experiments

In the present study, the model cells were used for each method. While HL60 cells are often preferred for ROS generation (Teufelhofer et al., 2003), in the NAG-1 activation assay the SW1353 cell line has been established as an inflammatory model (Pang et al., 2021). Using the same cell lines, Zhao's team evaluated the inhibition of ROS, iNOS and NF-κB and the activation of NAG-1 of six octulosonic acid derivatives isolated from *A. nobilis*. Although none of the molecules had significant activity against NF-κB or iNOS, all of them enhanced NAG-1 activity 2-3-fold at 50 μM and decreased ROS generation (Zhao et al., 2014). In our case, all extracts at 50 ug/mL had a stronger activity in the activation of NAG-1 than the standard drug. Also, 50 ug/mL of the chloroform extract (7.9-fold) was approximately twice that of the 5 ug/mL extract (4.1-fold), whereas the efficacy of the hexane and methanol extracts was similarly increased in a dose-dependent manner. In addition to NAG-1 activation, strong inhibition of iNOS and NF-κB of the chloroform and hexane extracts may be due to the synergistic effect of the molecules in the extracts. Likewise, the essential oil of *A. weidmanniana*, rich in terpene molecules and collected from Turkey, also exhibited a strong inhibitory effect on NO production (IC_{50}:41.2 μg/mL) (Conforti et al., 2012). There are many reports on *in vitro* antioxidant and anti-inflammatory effects of *Anthemis* species, such as *A. nobilis, A. aetnensis, A. arvensis* and *A. cotula* (Acquaviva et al., 2012; Al-Snafi, 2016; Boukhary et al., 2019; Mantle, Eddeb, & Pickering, 2000; Vučković et al., 2011; Zhao et al., 2014). However, antioxidant and anti-inflammatory effects and NAG-1 activation of *A. tricolor* extracts have been for the first time in this study.

To our knowledge, there is no report on the cytotoxic effect of *A. tricolor* and interestingly, there are also limited studies on extracts, essential oils or isolated compounds from *Anthemis* genus (Conforti et al., 2012; Radulović et al., 2013). However, it is well known, that biological activities of the plants are associated with the polarity of extraction solvent and the chemical composition of extracts. Thus, while the polar (aqueous) extract of *A. atropatana* showed negligible toxicity against HEK293 cells, dichloromethane extract of *A. mirheydari* had more toxicity against LS180 (human Caucasian colon adenocarcinoma), MCF-7 (breast cancer) and MOLT-4 (human T lymphoblast) cells than methanol extract (Jassbi et al., 2016; Khosravi, Mirzaie, Kashtali, & Noorbazargan, 2020). In our case, non-polar extracts (hexane and chloroform) were more effective than the polar (methanol) extract, concordant with the literature.

Sesquiterpene lactones are the chemical markers of the *Anthemis* genus (Staneva et al., 2008) and their cytotoxic and anti-inflammatory activities have been well demonstrated like many terpenoids (Rüngeler et al., 1999; Wong & Menendez, 1999). Non-polar fractions are rich in these compounds. Thus, the non-polar (chloroform) extract of *A. ruthenica* and isolated sesquiterpenes and flavonoids from this extract had strong inhibition of HeLa, MCF7 and A431 (human squamous carcinoma) cells proliferation (Hajdú et al., 2010; Réthy et al., 2007). Additionally, NF-κB DNA binding activity of sesquiterpene lactones from *A. arvensis* and *A. cotula* have been reported (Vučković et al., 2011). In the present study, biological activities of *A. tricolor* extracts in different polarities were tested against seven different cell lines (HL-60, SW1353, RAW 264-7, U205, A549, HeLa, HEK293) and the highest effects were observed in chloroform and hexane extracts in general. From this point of view, the effects of apolar fractions of *A. tricolor* (hexane and chloroform extracts) on cytotoxicity, NO generation and NF-κB may be attributed to sesquiterpenic compounds. In a previous study, sesquiterpenes of *A. plutonia*, an endemic species to Cyprus and related to *A. tricolor* have been identified (Bruno, Maggio, Arnold, Diaz, & Herz, 1998; Oberprieler & Vogt, 1999). However, further detailed phytochemical investigations on *A. tricolor* are needed.

In vivo experiments

According to the literature survey, hexane and sesquiterpene lactone extracts (SLE) of *A. tricolor* have been more effective than methanol extract against erythema in rats (Demirkan et al., 2019). In addition, there are many studies on the anti-inflammatory activities of other *Anthemis* species. For example, 500 mg/kg of methanol extract of *A. scrobicularis* had 63.06% inhibition at the end of 2 h (Yusufoglu, Alam, Salkini, & Zaghloul, 2014). Gonenc's team reported SLE, hexane and diethyl ether extracts of *A. wiedemanniana* inhibited carrageenan-induced edema in mice. While 200 mg/kg of hexane and diethyl ether extracts shrank the swelling at 180 min (25.5 and 23.8% inhibition), the same dose of SLE was effective for three hours (26.7-30.7% inhibitions) (Gönenç et al., 2014). In another study, while 200 mg/kg of SLE of *A. aciphylla* var. *aciphylla* inhibited 78% of carrageenan-induced paw edema at 5 h, the same dose of ethanol extract was effective at 1 h (68% inhibition) (Baltaci et al., 2011). Based on these studies, non-polar extracts of *Anthemis* genus such as SLE, hexane, and diethyl ether are more effective than polar fractions against inflammation and our results were confirmed by the literature. Chloroform extract of *A. tricolor* was the most effective fraction among all of extracts in the *in vitro* studies. Similarly, the *in vivo* studies showed low doses of chloroform extract of *A. tricolor* had the strongest activity against inflammation induced by carrageenan compared to the literature.

M. l. obtusa, blunt-nosed viper, the largest snake from the Viperidae family in Turkey, causes a significant percentage of snake bite cases (Mermer, Gocmen, & Cicek, 2012). It is known that *Vipera lebetina* (*Macrovipera lebetina*) venom triggers the

inflammation mechanism via their metalloproteases, which can stimulate proinflammatory factors such as TNF-α, IL-1, IL-6, IL-10 and IFNγ (De Toni et al., 2015; Farsky et al., 2000; Gutierrez, Rucavado, Escalante, & Diaz, 2005; Moura-da-Silva, Butera, & Tanjoni, 2007; Rucavado, Nunez, & Gutierrez, 1998; Teixeira, Cury, Moreira, Picolob, & Chaves, 2009; Trummal et al., 2005). PLA2 homologs, a kind of metalloprotease, exhibit severe myotoxic activity, affect plasma membrane integrity, cause toxicities such as hyperalgesia, edema and the release of proinflammatory cytokines (Kang et al., 2011; Lomonte & Rangel, 2012; Teixeira et al., 2009). Proinflammatory cytokines are usually produced by macrophages and affect COXs expression, and increase the level of iNOS production via activating the nuclear factor (NF)-κB signaling pathway (Hanada & Yoshimura, 2002; Hung, Hsu, Chung, & Huang, 2016; Zhang & An, 2007). Active COXs are involved in the synthesis of prostaglandin E2s, vasodilatation and vascular permeability increase in the area where snake venom is injected (Harris, Padilla, Koumas, Ray, & Phipps, 2002; Hirata & Narumiya, 2012; Moreiraa, Teixeiraa, Silva, D'Império Limab, & Dos-Santos, 2016; Teixeira et al., 2009).

The inflammatory effect of snake venom can vary according to the amount of venom exposure, protein concentration and the substances which are involved in the inflammatory mechanism in the protein content (Chippaux et al., 1991). Determining the amount of protein in venom is important to understand the toxicity and inflammatory potential of the venom. The content and protein concentration of venom varies according to species and subspecies of the snake, location, gender, age, nutrition, season and even captivity (Chippaux, 1998; Chippaux et al., 1991; Creer, Chou, Malhotra, & Thorpe, 2002; Daltry, Wüster, & Thorpe, 1996; Durban et al., 2011; Gubensek, Sket, Turk, & Lebez, 1974; Igci & Demiralp, 2012; McCleary, Sridharan, Dunstan, Mirtschin, & Kini, 2016; Menezes, Furtado, Travaglia-Cardoso, Camargo, & Serrano, 2006; Sarhan, Mostafa, Elbehiry, & Saber, 2017). In our previous study, the protein concentration of *M. l. obtusa* venom was found to be quite high (1999.5 µg/mL) (Demiroz et al., 2018). According to Igci and Demiralp (2012), *M. l. obtusa* venom contains 24% metalloprotease and 34% PLA2 (Igci & Demiralp, 2012). Additionally, characterization studies show *M. l. obtusa* venom contains bradykinin-activating peptide, disintegrin fragment, C-natriuretic peptide A, dimeric disintegrin, PLA2 PIII-metalloproteinase, serine proteinase- Factor V serine proteinase, thrombin-like serine proteinase, and L-amino acid oxidase protein families (Igci & Demiralp, 2012; Sanz, Ayvazyan, & Calvete, 2008).

Because of the venom's content, edema peak times and amount aren't clearly known. However, in general, the first hour is defined as the golden hour and is considered critical for these cases (Forgey, 2008; Isbister et al., 2013). In some case procedures, it is recommended to check the patient for the first three hours, hourly, then every few hours (India Ministry of Health & Family Welfare, 2006). According to the values we found in our study, although the amount of edema at the 4th hour still has a significant difference, the critical time for *M. l. obtusa* venom seems to be the first three hours. In the previous study, it was seen that there was a similar timeline for edema caused by another viper species, *Montivipera xanthina* venom (Demiroz et al., 2018).

Chloroform extract of the plant, which showed strong anti-inflammatory effect in the *in vitro* experiments, had similar effect as the *in vivo* studies against carrageenan-induced and *M. l. obtusa* venom inflammation model in rats. Snake venom reached its peak in 0.5 h and caused a rapid inflammatory effect, while carrageenan produced inflammation for a longer time. Concentrations at 12.5, 25 and 50 mg/kg of *A. tricolor* chloroform extract were more effective on carrageenan-induced inflammation compared to venom. However, 75 µg/paw of *M. l. obtusa* venom caused a 73.5% increase in paw volume at 0.5 h, while three doses of *A. tricolor* chloroform extract (25, 50 and 100 mg/kg) reduced paw volume nearly to 20-25% at the same time. It has been shown that *A. tricolor*, which has strong anti-inflammatory effect in the *in vitro* tests performed, showed similar results in *in vivo* studies. Compared to the standard drug, indomethacin, 2.5-100 mg/kg of *A. tricolor* chloroform extracts had more effect against the inflammation caused by carrageenan and *M. l. obtusa* venom. Based on the literature survey, there is only one study on the inhibition of edema caused by *M. l. obtusa* venom. It was performed with *Centaurea calolepis* and cnicin by our team. Fifty mg/kg of the extract was chosen as the most effective dose against venom-induced inflammation (22.55% inhibition), while 2.5 mg/kg of cnicin shrank the edema to 19.10% at 4 h (Demiroz et al., 2018). Our results showed all of the doses of *A. tricolor* (25, 50 and 100 mg/kg) had stronger effect than *C. calolepis*. The anti-inflammatory effect in rats may be due to sesquiterpene molecules such as other *Anthemis* species or *Centaurea*, another member of Asteraceae (Baltaci et al., 2011; Demirkan et al., 2019; Demiroz et al., 2018; Gonenc et al., 2011).

CONCLUSION

To our knowledge, there has been no study on the antioxidant, cytotoxic and anti-inflammatory effects of endemic *A. tricolor* by *in vitro* studies up to now. Moreover, by this study, anti-inflammatory activity of *A. tricolor* against carrageenan and snake venom-induced paw edema have been evaluated for the first time.

A. tricolor has enormous potential for the treatment of inflammation and local viper bites. Further studies should focus on purification of bioactive compounds as lead compounds for anticancer and anti-inflammatory drugs.

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Original Article

Essential oil content, *in-vitro* and *in-silico* activities of *Hypericum triquetrifolium* Turra, *H. empetrifolium* subsp. *empetrifolium* Willd., and *H. pruinatum* Boiss. & Balansa species

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ABSTRACT

Background and Aims: The importance of *Hypericum* species that are used traditionally against many diseases is increasing day by day.

Methods: In this study, the essential oil contents of *Hypericum triquetrifolium*, *H. empetrifolium* subsp. *empetrifolium*, and *H. pruinatum* species were determined with GC-MS/FID. This is the first study on the antioxidant, anticholinesterase, antiurease, antityrosinase, antielastase, and anticollagenase activities of these species. Also, *in silico* and *in vitro* enzyme inhibitory activities of the major compounds in the essential oil samples of the species have been evaluated. In addition, the cytotoxic effects of the essential oils were determined by the MTT method.

Results: According to GC-MS/FID results, the major compounds were determined as caryophyllene oxide (16.76%) for *H. triquetrifolium*, α-pinene (21.67%) for *H. empetrifolium* subsp. *empetrifolium*, and germacrene D (22.47%) for *H. pruinatum*. Especially, *H. pruinatum* sample showed a high cytotoxic effect (IC_{50}: 34.78±0.22 and 29.06±0.40 µg/mL, respectively) on HT-29 and MCF-7 cell lines. It was determined that the same sample showed a promising inhibitory activity on acetyl (18.33±2.79, 36.48±2.40, and 56.97±0.94, respectively) and butyryl (71.63±2.78, 73.88±1.16, and 56.97±0.97, respectively) cholinesterase enzymes.

Conclusion: Results of the *in-vitro* activity studies indicated that *H. pruinatum* essential oil could be used in the pharmaceutical industry.

Keywords: *Hypericum triquetrifolium, H. empetrifolium, H. pruinatum*, essential oil, cytotoxicity, anti-aging, *in silico*

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INTRODUCTION

Hypericum L. genus, belonging to the Hypericaceae family; has about 500 species known in the world and 97 species in Turkey (Babacan, Aytac, & Pinar, 2017). They are called "sarı kantaron, binbirdelik otu, koyunkıran, and kılıç otu" in Anatolia (Baytop, 1984). Extracts obtained from *Hypericum* species are traditionally used against depression, stomach ailments (gastritis, ulcer), loss of appetite, jaundice, athlete's foot, gingivitis (gargling), sinusitis, intestinal inflammation, hemorrhoids, and fever, and as an inflammation dryer in external wounds (by making an ointment), expectorant, and blood production enhancer in folk medicine (Volz, 1997; Karatoprak et al., 2019). There are many studies in the literature on the essential oil contents of *Hypericum* species (Bertoli, Menichini, Mazzetti, Spinelli, & Morelli, 2003; Cirak & Bertoli, 2013; Sajjadi, S. E., Mehregan, I., & Taheri, 2015; Akdeniz et al., 2020; Grafakou et al.,2020; Silva, Taofiq, Ferreira, & Barros, 2021) Hydrocarbons such as 2-methyloctane, nonane, and undecane, monoterpenes such as *α*-pinene, limonene, *β*-myrcene, and cis-*β*-ocimene, and sesquiterpenes such as caryophyllene and caryophyllene oxide are present in essential oils of *Hypericum* species (Akdeniz et al., 2020). While there are many studies in the literature on the *in vitro* activities of various extracts of species belonging to the genus *Hypericum*, in particular, there are few studies on the *in vitro* activities of essential oils (Akdeniz et al., 2020; Tahir et al., 2019).

The essential oil contents of *Hypericum triquetrifolium* Turra., *H. empetrifolium* subsp. *empetrifolium* Willd., and *H. pruinatum* Boiss. & Balansa obtained by hydrodistillation were determined by GC-MS/FID. Antioxidant (DPPH, ABTS, and CUPRAC), acetyl- and butyryl-cholinesterase inhibitory, antiurease, antityrosinase, antielastase, and anticollagenase activities of these essential oils, which had not been studied before, were investigated. Also, the toxic effects of these essential oils on healthy cell line (PDF) and their cytotoxic effects on cancerous HT-29 (colon cancer) and MCF-7 (breast cancer) cell lines were determined by the MTT method. Both *in silico* and *in vitro* enzyme studies of the major compounds, which were detected at high percentages according to the GC-MS results, were carried out to reveal the content-activity relationship.

MATERIAL AND METHODS

Plant material

H. triquetrifolium (S1) was collected by Dr. Abdulselam Ertas from Diyarbakır in July 2014 and identified by Dr. Yeter Yeşil (Istanbul University). *H. empetrifolium* subsp. *empetrifolium* (S2) was collected from Muğla and *H. pruinatum* (S3) from Trabzon and they were identified by Dr. Yeter Yeşil in July 2015. The herbarium numbers of the samples are ISTE 98926, ISTE 113613, and ISTE 110750, respectively.

Studies of GC-MS analysis

Essential oils of studied samples (shadow dried 100g aerial parts) obtained by a hydrodistilation method using Clevenger apparatus, and the yields of essential oils (*H. triquetrifolium* (S1): 0.65%, *H. empetrifolium* subsp. *empetrifolium* (S2): 0.21% and *H. pruinatum* (S3): 0.45%) were calculated. 20 µL of pure essential oil samples were taken and diluted with hexane to a total volume of 1000 µL. The GC-MS conditions are the same as those given in the relevant references and are detailed below.

Analysis of GC-MS/FID was carried out using Agilent Tecnolocies 7890A GC-FID and 5977B MS detectors, respectively. HP-5MS UI capillary column (30 m–0.25 mm i.d. and 0.25 µm film thickness) was used. The injector temperature was adjusted to 250 °C. Split flow and split ratio were 25 mL/min and 25:1, respectively. The injection volume was 1.0 µL. Mass spectra were detected at 70 eV, and mass range was *m/z* 40–500 amu. GC oven temperature started at 50°C and was held at this temperature for 4 mins and then increased to 240°C by a rate of 3°C per minute and held at final temperature for 5 mins. The MSD and FID detectors temperature were 230°C and 300°C, respectively. Helium gas as carrier has a flow rate of 1 mL/min. The compounds were identified by comparing their retention times and mass spectra with those obtained from authentic samples and/or the NIST and Wiley spectra as well as data from the published literature (Akdeniz et al., 2020; Bakir et al., 2020).

Antioxidant and cytotoxic activities

ABTS cation radical (Re et al., 1999), CUPRAC (Copper (II) ion reducing antioxidant capacity) (Apak, Guclu, Ozyurek, & Karademir, 2004), and DPPH free radical scavenging (Blois, 1958) methods were used to determine the antioxidant activities of the samples. Additionally, on the healthy cell lines (PDF) and on the cancerous MCF-7 and HT-29 cell lines were used by the MTT method to determine the cytotoxic and toxic effects of the samples (Mojarraba, Langzian, Emamic, Asilic, & Tayarani-Najaranb, (2013). IC_{50} values were calculated using the different concentrations (250, 100, 50, 25, 10 and 1 µg/mL concentrations) of the samples (Ertas Yener, 2020).

Enzyme inhibitory activities

A spectrophotometric method based on acetyl- (AChE: from electric eel, Type-VI-S, Sigma) and butyryl-cholinesterase (BChE: from horse serum, Sigma), urease (from Canavalia ensiformis, Type III, Sigma), tyrosinase (from *mushroom*, Sigma), elastase (from *Porcine pancreas,* Type I, Sigma) and collagenase (from *Clostridium histolyticum*, Type I, Sigma) inhibitory activities developed by Ellman, Courtney, Andres, & Featherstone (1961), Hina et al. (2015), Hearing & Jimenez (1978), Kraunsoe, Claridge, & Lowe (1996) and Thring, Hili, & Naughton (2009) with slight modifications, respectively were used to determine the enzyme inhibitory activities. Ethanol (99.9%, Merck) was used to prepare the stock solutions and to dilute the solutions in all enzyme experiments (Ertas et al., 2021; Yener et al., 2018).

Molecular docking

The compatibility of *α*-pinene, caryophyllene oxide and germacrene D, which were determined as the major compounds in the essential oils, to the active site of cholinesterase, tyrosinase, elastase, and collagenase enzymes were determined using the Dock 6.5 program. The coordinates of the protein mentioned above were taken from the Protein Data Bank (4cex.pdb for urease, 5i38.pdb for tyrosinase, 4bbz.pdb for BuChE, 2x8b.pdb for AChE). Crystallographic water molecules were removed from all structures. In 4cex.pdb, 5i38.pdb, 4bbz.pdb, 2x8b.pdb, the missing coordinates were modelled using XLEAP and ff99SB force fields. (Kohno, Hochigai, Yamashita, Tsukihara,

& Kanaoka, 2006; Lang et al., 2007; Carletti et al., 2010; Carletti, Colletier, Schopfer, Santoni, & Masson, 2013; Deri et al., 2016; Nakanishi, Kinoshita, Sato, & Tada, 2020). Molecular modeling study was not performed for urease enzyme because the anti-urease activities of the samples were found to be insignificant. The details related to the current *in silico* studies were given in previous studies (Yener et al., 2020).

RESULTS AND DISCUSSION

Essential oil content and *in vitro* activities

The essential oils of the shade-dried samples of *H. triquetrifolium* (S1), *H. empetrifolium* subsp. *empetrifolium* (S2), and *H. pruinatum* (S3) species were determined by hydrodistillation with GC-MS and GC-FID (Table 1 and Figure 1). The contents of the essential oils of three samples were 90.50, 95.55, and 91.68%, respectively, and 61 compounds were determined in total (Table 1). Essential oils of the samples have been found to contain monoterpene hydrocarbons (18.50, 32.10, and 3.12%, respectively), oxygenated monoterpenes (2.45, 1.31, and 0.25%, respectively) sesquiterpene hydrocarbons (28.7, 17.95, and 66.62%), oxygenated sesquiterpenes (26.82, 10.54, and 16.08%) and other hydrocarbons (14.03, 33.65, and 5.61%) (Table 1 and Figure 1). It was determined that *H. triquetrifolium* and *H. pruinatum* are rich in sesquiterpenes and *H. empetrifolium* subsp. *empetrifolium* is rich in other hydrocarbons. The major components are determined as caryophyllene oxide (16.76%), *α*-pinene (9.92%), and 2-methyloctane (6.58%), for S1, *α*-pinene (21.67%), humulene (15.00%), and 2-methyloctane (12.10%) for S2, germacrene D (22.47%), *α*-cadinol (12.73%), and *δ*-cadinene (9.59%) for S3.

In the previous studies, germacrene-D (21.7%), *β*-caryophyllene (18.3%), and *δ*-cadinene (6.4%) (Sajjadi et al., 2015), myrcene (16%), *α*-pinene (13%), sabinene (13%), germacrene-D (10%), *β*-pinene (8%), and caryophyllene oxide (5%) (Bertoli, Menichini, Mazzetti, Spinelli, & Morelli, 2003) were determined as the main components of the *H. triquetrifolium* essential oil.

In the presented study, major components (caryophyllene oxide: 16.76%, oxygenated sesquiterpenes; *α*-pinene: 9.92%, monoterpene hydrocarbons; 2-methyloctane: 6.58%, others; *β*-caryophyllene: 5.39%, sesquiterpene hydrocarbons; germacrene-D: 4.80%, sesquiterpene hydrocarbons and *δ*-cadinene: 3.67%, sesquiterpene hydrocarbons) of *H. triquetrifolium* essential oil were determined in parallel with the literature (Sajjadi et al., 2015; Al-Snafi 2018). In the literature, the major constituents of the essential oil of *H. empetrifolium* subsp. *empetrifolium* were determined as *α*-pinene (19.0%), *β*-pinene (8.7%), and germacrene D (12.5%) (Grafakou et al., 2020), and *β*-selinene (15%), caryophyllene oxide (9%), *β*-caryophyllene (8%), and *γ*-muurolene (7%) were determined from *H. pruinatum* essential oil (Cirak & Bertoli, 2013). In another study on essential oil of *H. empetrifolium* subsp. *empetrifolium*, *α*-pinene (35.60%) and γ-gurjunene (10.50%) were determined as the major components (Petrakis et al., 2005).

The antioxidant activity results of the essential oils of the samples are given in Table 2. In general, the DPPH free radical scavenging activity of all samples was determined to be low (Table 2). ABTS radical scavenging activities of the S1 and S3 were significant (IC_{50}: 36.65±0.78 and 77.58±1.15 µg/mL, respectively), and the S2 sample was low (IC_{50}: 858.77±1.45 µg/mL). In the CUPRAC method, the results of three samples were found to be close to each other with moderate antioxidant activity. The toxic effects of the samples on the healthy cell–lines (PDF) and the cytotoxic effects on the cancerous MCF-7 and HT-29 cell–lines were determined by the MTT method (Table 2). All samples appear to have toxic effects on the healthy cell–lines (PDF) at high concentrations. In particular, it was determined that the toxic effect of the S3 sample was lower than the others (IC_{50}: 76.09±0.34 µg/mL) on PDF cell-lines and highly cytotoxic (IC_{50}: 34.78±0.22 and 29.06±0.40 µg/mL, respectively) on HT-29 and MCF-7 cell-lines. It was determined that the BChE inhibitory activity of all samples was promising (inhibition%: 71.63±2.78, 73.88±1.16 and 82.08±1.99, respectively), but only the S3 sample had moderate AChE inhibitory activity (inhibition%: 56.97±0.94). Urease, tyrosinase, elastase and collagenase enzyme inhibitory activities potentials of all samples were determined to be low (Table 2).

In silico and *in vitro* studies of the major components

When the *in vitro* enzyme activity of *α*-pinene, caryophyllene oxide and germacrene D at 100 µg/mL concentration were examined, it was determined that they showed low-moderate activity. *α*-Pinene, caryophyllene oxide and germacrene D, which are the main components of the samples, showed moderate AChE (inhibition%: 47.89±0.91, 52.34±0.41 and 49.58±0.21, respectively), BChE (inhibition%: 11.43±0.06, 34.39±0.17 and 43.72±0.26), elastase (inhibition%: 16.31±0.11, 28.30±0.27 and 35.80±0.42) and collagenase (inhibition%: 12.86±0.10, 18.03±0.20 and 32.93±0.52) enzyme inhibitory activities. In the AChE and BChE methods, galanthamine were used (inhibition%: 91.45±0.84 and 78.92±0.65) as a standard reference. The thiourea (inhibition%: 96.75±0.42), kojic acid (93.47±0.48), oleanolic acid (43.25±0.68), epicatechin gallate (84.52±1.98) were used in urease, tyrosinase, elastase and collagenase enzyme activities as standard references, respectively.

According to the results of *in silico* studies, enzyme-inhibitor interactions were assessed with help of docking calculations where binding free energy was recorded at each possible position. The molecular docking result of AChE complexation energy was observed in range from -16.86 kcal/mol to -21.69 kcal/mol, the result of BChE complexation energy was observed in range from -24.18 kcal/mol to -32.36 kcal/mol, the result of tyrosinase complexation energy was observed in range from -20.75 kcal/mol to -25.34 kcal/mol, the result of elastase complexation energy was observed in range from -17.07 kcal/mol to -28.93 kcal/mol, the result of collagenase complexation energy was observed in range from -22.35 kcal/mol to -28.87 kcal/mol, respectively (Table 3). Since the major components have similar chemical structures, it was determined that they showed activity with similar interactions in all the enzymes studied, with Van der Waals and pi alkyl interactions being dominant (Figure 2).

AChE and BChE enzymes bind to ligands in their active sites with the help of similar amino acid residues, especially Trp, Hie,

Table 1. Essential oil composition of aerial parts of *Hypericum* species.

No	RI[a]	Constituents[b]	S1[c]	S2[c]	S3[c]	95% RI range in literatured	No	RI[a]	Constituents[b]	S1[c]	S2[c]	S3[c]	95% RI range in literatured
1	848	2-Hexenal	0.43	0.12	0.11	817–853	34	1457	α-Himachalene	0.29	-	-	1445- 1664
2	859	2-methyloctane	6.58	12.10	0.10	-	35	1462	Humulene	0.53	15.00	2.20	1425–1472
3	899	Nonane	2.65	1.06	0.12	-	36	1468	Alloaromadendrene	-	-	1.19	-
4	935	α-Pinene	9.92	21.67	1.98	912–948	37	1473	Cyclodecane	0.17	1.44	-	-
5	950	Camphene	0.25	0.37	0.12	928–964	38	1483	γ-Muurolene	2.93	0.23	5.47	1450–1501
6	967	3-Methylnonane	3.92	2.46	0.21	-	39	1490	Germacrene D	4.80	0.11	22.47	1463–1499
7	981	β-Pinene	1.75	2.93	0.10	962–989	40	1495	β-Selinene	1.26	-	1.38	1458–1502
8	991	β-Myrcene	0.60	0.11	0.13	975–998	41	1501	Valencene	4.17	-	-	1492- 1728
9	999	Decane	-	0.14	-	-	42	1503	α-Selinene	-	-	2.04	1467–1512
10	1026	*p*-Cymene	0.25	0.72	0.10	1010–1034	43	1506	α-Muurolene	0.61	-	4.47	1473–1506
11	1030	Limonene	0.28	1.38	0.14	1014–1040	44	1509	α-Farnesene	-	-	3.15	1479–1518
12	1033	Eucalyptol	-	0.27	-		45	1521	γ-Cadinene	2.90	0.12	3.52	1480–1526
13	1036	*trans*-β-Ocimene	0.12	0.11	0.06	1022–1049	46	1530	δ-Cadinene	3.67	0.44	9.59	1497–1529
14	1047	*cis*-β-Ocimene	0.11	0.12	0.15	1035–1058	47	1540	Cubenene	-	-	0.40	-
15	1059	γ-Terpinene	0.1	0.11	0.13	1043–1073	48	1551	α-Calacorene	0.85	-	0.28	1514–1543
16	1062	2-Methyldecane	2.07	5.72	tr	-	49	1566	Nerolidol	-	0.47	-	1536–1565
17	1099	Undecane	2.08	3.03	5.22	-	50	1587	Spathulenol	2.86	1.68	0.81	1545–1581
18	1104	Nonanal	0.22	0.51	-	-	51	1594	Caryophyllene oxide	16.76	6.69	0.12	1560–1596
19	1169	*endo*-Borneol	1.03	0.54	-	1134–1180	52	1602	Viridiflorol	-	-	0.49	1569–1604
20	1193	α-Terpineol	0.22	0.78	0.25	1163–1207	53	1613	Globulol	3.20	-	0.98	1559–1595
21	1200	Myrtenol	-	0.26	-	1168–1200	54	1620	Bisabolene epoxide	3.26	1.70	-	-
22	1263	2-Methyldodecane	-	1.05	-	-	55	1630	Junenol	-	-	0.95	-
23	1299	Tridecane	-	0.28	0.06	-	56	1640	1-Dodecanol	-	5.32	-	1473-1959
24	1301	Thymol	2.23	-	-	1272–1304	57	1649	*tau*-Cadinol	0.74	tr	tr	1618–1669
25	1354	α-Cubebene	-	-	0.39	1332–1381	58	1663	α-Cadinol	-	tr	12.73	1618–1669
26	1370	β-Cubebene	0.34	0.92	-	1364–1395	59	1676	1-Tetradecene	-	2.11	-	-

Table 1. Continue.

No	RI[a]	Constituents[b]	S1[c]	S2[c]	S3[c]	95% RI range in literatured	No	RI[a]	Constituents[b]	S1[c]	S2[c]	S3[c]	95% RI range in literatured
27	1376	α-Ylangene	-	-	0.31	1370-1484	60	1690	α-Bisabolol	-	-	0.53	1682- 2213
28	1382	α-Copaene	0.51	0.10	1.38	1355–1395	61	1846	Hexahydrofarnesyl acetone	-	2.35	-	1817–1850
29	1391	β-Bourbonene	0.12	0.18	0.12	1362–1405			Total identified (%)	90.50	95.55	91.68	
30	1396	β-Elemene	0.33	-	3.29	1370–1404			Monoterpene hydrocarbons	18.50	32.10	3.12	
31	1422	α-Cedrene	-	0.42	-	1412-1583			Oxygenated monoterpenes	2.45	1.31	0.25	
32	1427	Caryophyllene	5.39	0.43	4.73	1397–1449			Sesquiterpene hydrocarbons	28.70	17.95	66.62	
33	1446	Aromadendrene	-	-	0.24	1419–1464			Oxygenated sesquiterpenes	26.82	10.54	16.08	
									Others	14.03	33.65	5.61	

[a]Retention index on HP–5MS fused silica column, [b]A nonpolar Agilent HP-5MS fused silica column, [c]S1: *H. triquetrifolium* collected in Diyarbakır, S2: *H. empetrifolium* subsp. *empetrifolium* collected in Muğla, S3: *H. pruinatum* collected in Trabzon, [d]Retention indices for most frequently (95%) reported essential oil compounds in literature, [tr] trace

Table 2. *In vitro* activities of essential oil of aerial parts of *Hypericum* species

	Antioxidant activity[1]			Cytotoxic activity[1]			Enzyme activity (100 µg/mL)[1]					
	IC_{50} (µg/mL)		$A_{0.5}$ (µg/mL)	IC_{50} (µg/mL)			Inhibition (%)					
Samples[2]	DPPH	ABTS	CUPRAC	HT-29	MCF-7	PDF	AChE	BChE	Urease	Tyrosinase	Elastase	Collagenase
S1	≥250[a]	36.65±0.78[a]	102.30±3.32[a]	112.19±1.20[a]	109.42±1.17[a]	39.03±0.50[a]	18.33±2.79[a]	71.63±2.78[a]	NA[a]	2.35±0.31[a]	NA[a]	8.88±0.03[a]
S2	≥250[a]	≥250[b]	197.59±2.53[b]	86.36±2.21[b]	110.82±0.89[a]	48.89±1.52[b]	36.48±2.40[b]	73.88±1.16[a]	NA[a]	2.56±0.82[a]	NA[a]	11.07±0.08[b]
S3	≥250[a]	77.58±1.15[c]	164.22±3.54[c]	34.78±0.22[c]	29.06±0.40[b]	76.09±0.34[c]	56.97±0.94[c]	82.08±1.99[b]	NA[a]	2.16±0.17[a]	NA[a]	14.92±0.01[c]
α-Pinene	-	-	-	-	-	-	47.89±0.91[d]	11.43±0.06[c]	38.62±0.31[b]	NA[b]	16.31±0.11[b]	12.86±0.10[d]
Caryophyllene oxide	-	-	-	-	-	-	52.34±0.41[e]	34.39±0.17[d]	14.70±0.23[c]	NA[b]	28.30±0.27[c]	18.03±0.20[e]
Germacrene D	-	-	-	-	-	-	49.58±0.21[f]	43.72±0.26[e]	27.58±0.17[d]	NA[b]	35.80±0.42[d]	32.93±0.52[f]
BHT[3]	54.68±0.47[b]	15.24±0.63[d]	8.42±0.25[d]	-	-	-	-	-	-	-	-	-
α-TOC[3]	14.55±0.26[c]	9.52±0.36[e]	19.53±0.34[e]	-	-	-	-	-	-	-	-	-
Galanthamine[3]	-	-	-	-	-	-	91.45±0.84[g]	78.92±0.65[f]		-	-	-
Thiourea[3]	-	-	-	-	-	-	-	-	96.75±0.42[e]	-	-	-
Kojic acid[3]	-	-	-	-	-	-	-	-	-	93.47±0.48[c]	-	-
Oleanolic acid[3]	-	-	-	-	-	-	-	-	-	-	43.25±0.68[e]	-
Epicatechin gallate[3]	-	-	-	-	-	-	-	-	-	-	-	84.52±1.98[g]

[1]Values expressed are means ± S.D. of three parallel measurements and values were calculated according to negative control Values with different letters in the same column were significantly different ($p < 0.05$), [2]S1: *H. triquetrifolium* collected in Diyarbakır, S2: *H. empetrifolium* subsp. *empetrifolium* collected in Muğla, S3: *H. pruinatum* collected in Trabzon, [3]Standard compound, NA: Not active

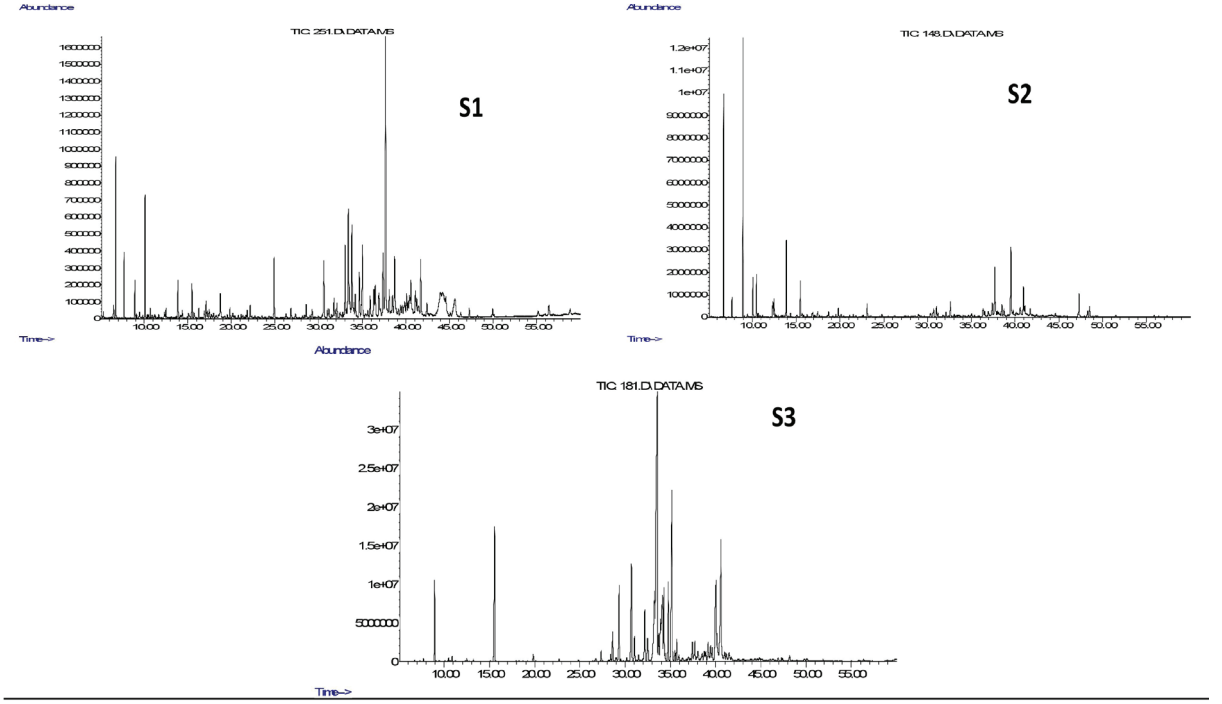

Figure 1. TIC chromatograms of essential oil of studied Hypericum species by GC-MS.
S1: H. triquetrifolium collected in Diyarbakır, S2: H. empetrifolium subsp. empetrifolium collected in Muğla, S3: H. pruinatum collected in Trabzon

Phe, Tyr. It is predicted that π-π and alkyl-π interactions between ligands and aromatic rings in the catalytic region and van der Waals interactions between alkyl groups play an important role. It is thought that the presence of donor oxygen and nitrogen atoms of the galantamine molecule, which is used as a reference, differs from the natural components, making a difference in its effectiveness. The π bonds of the germacren D molecule are thought to play an important role in its effectiveness.

Interactions with Phe, Asn, Val and Ser amino acids are observed in the active binding site of the tyrosinase enzyme. In addition to the -OH groups Vdw and π-π interactions of the reference kojic acid molecule, hydrogen bonds with Met amino acid residues in the active region in particular. It is remarkable that natural components with relatively high activity have a strong effect on the enzyme with non-covalent interactions.

Electrostatic interactions and hydrogen bonds with ligands of Ser, Asn, Gly, His and Cys amino acids in the active site of the elastase enzyme appear to play a role in the activity. The reference compound olenaoic acid has a high affinity with its carboxylic acid functional group, which differs from natural components. It has been determined that it shows activity by forming a large number of hydrogen bonds, especially to Ser and Hie active amino acids.

Collagenase enzyme shows chemical activity with Gly, Leu, Ala, Glu, Tyr and Pro amino acid residues in its active site with natural components and reference molecule. H-bond interactions between the ligands and the enzyme were generally observed with Leu, Ala, Glu, His. The epicatechin gallate used as a reference in the study has a large number of –OH groups and oxygen atoms, unlike the natural components that show activity. It is clearly seen in the results that both the non-covalent interactions of the methyl groups in the *α*-pinene molecule and the effect of the π electrons in the germacren and caryoxyphlene oxide compounds are not that strong.

The *in vitro* test results of the compounds identified as major ones in the essential oils of the species show parallelism with the *in silico* results. In particular, it is observed that the BChE activity of all studied samples is high, but the major compounds are moderately active. However, it is seen that the BChE activity of the essential oil samples, in which these major compounds are mixed in certain proportions, is quite high. It can be suggested that the high BChE enzyme activity of essential oils is due to the synergistic effect of the components. It was determined that the major component germacrene D in the S3 sample was moderately effective in elastase and collagenase (Inhibition%: 35.80±0.42 and 32.93±0.52, respectively) enzyme inhibitory activities, while the relevant sample showed low activity. Therefore, the major component of the S3 sample might have an antagonist effect against these enzymes with other components.

CONCLUSION

In the last decade, more than three thousand studies have been published on this genus, mainly *H. perforatum* L. (Silva, Taofiq, Ferreira, & Barros, 2021). In particular, many *in vivo* and *in vitro* activities related to the cosmetic field of different extracts (methanol, ethanol and water etc.) of the genus were investigated (Silva, Taofiq, Ferreira, & Barros, 2021). However, there are few studies on the *in vitro* activities of the essential oil of *Hypericum* species. The aerial parts of *H. triquetrifolium* (S1), *H.*

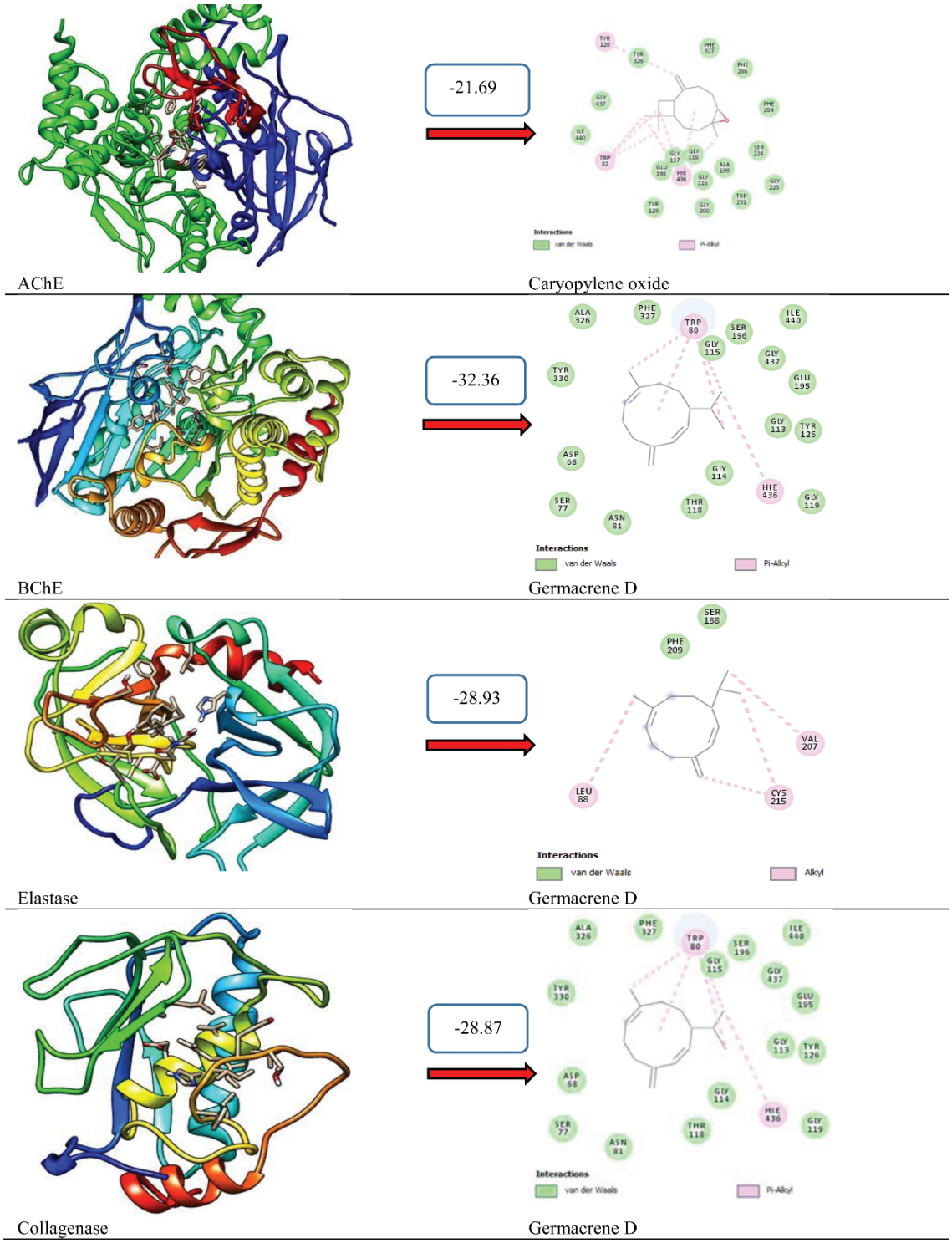

Figure 2. Ribbon representation of the active site pocket enzymes with the bound ligands. The wide opening of the binding site pocket allows the compounds to adopt flexible conformation in this area.

empetrifolium subsp. *empetrifolium* (S2) and *H. pruinatum* (S3) samples collected in this framework were dried in the shade, and the content of their essential oils obtained by hydrodistilation method was indicated by GC-MS/FID. At the same time, cytotoxic, antioxidant, cholinesterase, urease, tyrosinase, elastase and collagenase enzyme inhibitory activities of essential oils were evaluated. It was determined that *H. triquetrifolium* and *H. pruinatum* species are rich in sesquiterpene, and *H. empetrifolium* subsp. *empetrifolium* species are rich in other hydrocarbons. The major components are determined as caryophyllene oxide (16.76%) for S1, *α*-pinene (21.67%) for S2, and germacrene D (22.47%) for S3. This is the first study on the *in*

Table 3. Calculated thermodynamic parameters for complexation of ligands by docking method.

Compounds	AChE			BChE			Tyrosinase			Elastase			Collagenase		
	VdW	es	DockS	VdW	es	DockS	VdW	es	DockS	VdW	es	DockS	VdW	es	DockS
α- pinene	-16.77	-0.09	-16.86	-23.99	-0.18	-24.18	-21.85	-0.03	-21.88	-16.95	-0.12	-17.07	-22.16	-0.18	-22.35
Caryophylene oxide	20.96	-0.65	-21.69	-31.63	-0.05	-31.63	-20.29	-0.46	-20.75	-22.33	-0.07	-22.40	-25.36	-0.46	-25.82
Germacrene D	-17.23	-0.16	-17.40	-32.28	-0.08	-32.36	-25.21	-0.12	-25.34	-28.80	-0.13	-28.93	-28.73	-0.14	-28.87
Galantamine	-66.53	-11.68	-78.21	-63.05	-7.14	-71.19	-	-	-	-	-	-	-	-	-
Kojic acid	-	-	-	-	-	-	-69.19	-10.97	-80.16	-	-	-	-	-	-
Oleanolic acid	-	-	-	-	-	-	-	-	-	-31.39	-0.86	-32.25	-	-	-
Epicatechin gallate	-	-	-	-	-	-	-	-	-	-	-	-	-7.20	-53.90	-61.11

vitro activities of essential oils of *H. empetrifolium* subsp. *empetrifolium, H. pruinatum,* and *H. triquetrifolium*.

It has been determined that the essential oil of *H. pruinatum* has a high cytotoxic effect on HT-29 and MCF-7 and a high inhibitory effect on both AChE and BChE enzymes. The essential oil of *H. pruinatum* might be used in the pharmaceutical industry in treatment of diseases such as memory loss, colon cancer, and breast cancer.

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Original Article

The cytotoxic and apoptotic effects of *Thymus vulgaris* extracts on human breast cancer cell lines

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ABSTRACT

Background and Aims: Plant extracts are an important source of cytotoxic compounds and have consistently been an interesting field of research. The aim of this study is to investigate the cytotoxic and apoptotic effects of *Thymus vulgaris (T. vulgaris)* extracts on human breast cancer cell lines.

Methods: This study was carried out using human breast cancer cell lines (MCF-7 and MDA-MB-231) as experimental groups and the healthy human fibroblast cell line (PCS-201-012) as the control group. Petroleum ether and ethanol extracts were obtained from *T. vulgaris*. The extracts were applied to MCF-7 and MDA-MB-231 human breast cancer cell lines and human breast cancer stem cells. Cytotoxicity studies were performed using the RTCA iCELLigence system (Agilent Technologies), and apoptosis studies were performed using terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) and 4',6-diamidino-2 phenylindole (DAPI) methods.

Results: The *T. vulgaris* extracts were found to have concentration-dependent cytotoxic effects on human breast cancer cells. The growth of breast cancer stem cells was also determined to be inhibited when an effective concentration (45 µg/mL) of the extracts was applied. Lastly, specific morphological changes related to apoptosis were detected in the cells that had been treated with the effective concentration.

Conclusion: The *T. vulgaris* extracts were found to inhibit the proliferation of human breast cancer cells and human breast cancer stem cells selectively and concentration-dependently via an apoptosis-dependent pathway. The results suggest that the extracts may make promising sources for developing drugs for breast cancer therapy.

Keywords: DAPI, iCELLigence, MCF-7, MDA-MB-231, *Thymus vulgaris*, TUNEL

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INTRODUCTION

Cancer involves genetic changes that lead to the transformation of a normal cell into a malignant cell by escaping cell death (Hanahan & Weinberg, 2000). The following are some of the pathways for eliminating apoptosis or the apoptosis resistance of malignant cells: (i) degradation of apoptosis precursors and anti-apoptosis proteins, (ii) suppression of caspase functions, and (iii) disruption of cell death receptors. Therefore, one of the strategies for treating cancer is to induce apoptosis (Wong, 2011).

Anticancer drugs today are derived from natural products or derivatives of natural products (Cragg & Pezzuto, 2016; Thomford et al., 2018). The *Thymus* (thyme) plant is one of the most important genera in the Lamiaceae family and belongs to the Angiosperm phyla (Kuete, 2017). *Thymus vulgaris* (*T. vulgaris*), also known as thyme, is an ornamental plant that grows in the ground and is endemic to the Western Mediterranean coastline (Bone & Simon, 2012; Hosseinzadeh, Jafarikukhdan, Hosseini, & Armand, 2015). Various studies have shown thyme to have antimicrobial (Nikolić et al., 2014; Hosseinzadeh et al., 2015), antioxidant (Roby, Sarhan, Selim, & Khalel, 2013; Nikolić et al., 2014), and anticancer (Berrington & Lall, 2012; Nikolić et al., 2014; Hosseinzadeh et al., 2015) properties. Natural phenolic compounds (e.g., carvacrol) found in *T. vulgaris* are known to have anti-proliferative and apoptotic effects on cancer cells (Yin et al., 2012).

Cancer stem cells occur as a hidden group within cancer cells and are also referred to as the group from which cancer arises (Zheng, Xin, Liang, & Fu, 2013; Batlle & Clevers, 2017). In samples obtained from breast cancer tissues, the cells with the phenotype cluster of differentiation (CD)24^-/$CD44^+$ have been identified as breast cancer stem cells (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003; Albeniz & Alkanlı, 2020). Breast cancer treatment failure and treatment resistance are well known for being associated with breast cancer stem cells (Bozorgi, Khazaei, & Khazaei, 2015).

This study hypothesizes *T. vulgaris* extracts to be able to possess anticancer and antiapoptotic properties regarding human breast cancer cell lines due to *T. vulgaris* being rich in active compounds such as thymol and carvacrol. Therefore, the study evaluates *T. vulgaris* extracts for their cytotoxic activity against human breast cancer cell lines (MCF-7 and MDA-MB-231) and human breast cancer stem cells. The study also identified the apoptotic induction of the extracts using the terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay and 4',6-diamidino-2 phenylindole (DAPI) staining methods.

MATERIALS AND METHODS

Chemicals

DAPI, dimethyl sulfoxide (DMSO), ethanol (EtOH), fetal bovine serum (FBS), phosphate buffered saline (PBS), and TUNEL kit were acquired from Sigma (St. Louis, MO, USA). Dulbecco's modified eagle medium-12 (DMEM-F12) was acquired from Lonza (Basel, Switzerland). Penicillin-streptomycin and $CD24^-$/$CD44^+$ stem cell markers were purchased from Thermo Fisher Scientific Life Sciences (Rockford, IL, USA). Petroleum ether (PE) was purchased from Honeywell (Charlotte, NC, USA). All other chemicals were of analytical grade.

Plant material

The aerial parts of *T. vulgaris* were collected during the flowering stage of growth from Nezahat Gökyiğit Botanical Garden (NGGB) in Istanbul in the northwestern part of Turkey at an altitude of 81 m and registered with the NGBB Herbarium as 20070071. Botanist MSc. Burçin Çıngay was responsible for NGBB Herbarium and performed the species identification.

Extract preparations

The aerial parts of *T. vulgaris* (90 g) were dried in the shade and then powdered in a mill. The powdered plant was first macerated in PE once for 3 days, and then extracted by acetone twice for 3 days, before finally being extracted by EtOH twice for 3 days. The obtained mixtures were then filtered. The PE and EtOH extracts were obtained by evaporating the solvents in a rotary evaporator then stored at -20°C for use in the experiments.

The extracts were prepared at different concentrations (360 µg/mL, 180 µg/mL, 90 µg/mL, 45 µg/mL, 20 µg/mL, and 10 µg/mL) by dissolving with DMSO (Esmaeili-Mahani, Falahi, & Yaghoobi, 2014; Nikolić et al., 2014).

Cell culture conditions

MCF-7 and MDA-MB-231 human breast cancer cell lines, as well as the PCS-201-012 healthy human fibroblast cells, were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in a culture medium containing DMEM-F12, FBS (10%, v/v), and penicillin-streptomycin (1%, v/v). The cells were maintained in 25 cm^2 and 75 cm^2 cell culture dishes in an incubator (SANYO, Osaka, Japan) with 5% CO_2 at 37°C. When the cells reached a density of 75% in the culture dishes, they were sub-cultured.

Real-time cytotoxicity assay (RTCA)

The RTCA was performed using the iCELLegince system (ACEA Biosciences Inc., CA, USA). The iCELLigence system offers several significant benefits, including the elimination of the need to label cells, real-time monitoring capabilities, reduced potential for human error, and the generation of more accurate results (Düzgün et al., 2017; Türker Şener, Albeniz, Dinç, & Albeniz, 2017).

The cells ($1.5x10^4$ cells/well) were seeded in each well of the E-plates (ACEA Biosciences Inc., CA, USA), and the cell index (i.e., cell-electrode impedance of the E-plate well) was checked using an iPad device (Apple, Cupertino, CA, USA) containing the RTCA iCELLigence software. The extracts were then applied to the cells. The cell index values were measured every 15 min using the RTCA iCELLigence system for 96 h once the extracts were applied. Results are expressed as IC_{50} values (the concentration required to inhibit 50% of cell growth), with IC_{50} values being calculated using the cell index values obtained from the RTCA iCELLigence software.

In order to determine the cytotoxic effects of extracts on breast cancer stem cells and apoptosis studies, the effective concentrations of the extracts were determined according to the results from the cytotoxicity analysis using the RTCA iCELLigence system. The effective concentration was applied to breast cancer stem cells and breast cancer cells for the TUNEL assay and DAPI staining.

Identifying breast cancer stem cells using flow cytometry

MDA-MB-231 human breast cancer cells were used as the primary culture for sorting the cancer stem cells. The cancer stem cells were identified and isolated using a flow cytometer (Beckman Coulter, CA, USA) based on the CD24 and CD44 markers. The cell concentrations in the test tube and control tube were adjusted to $1.5x10^4$ cells. Anti-human CD24 and anti-human CD44 antibodies were added to the test tube, mixed, and incubated at room temperature in the dark for 30 min. The population of $CD24^-/CD44^+$ cells was sorted by flow cytometry (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003).

Determination of apoptotic activity

$15x10^4$ cells/well and $2x10^4$ cells/well were seeded in 24-well plates for the TUNEL assay and DAPI staining, respectively. The cells were then treated with an effective concentration of extracts and incubated for 72 h to determine apoptosis.

DAPI staining was determined by fluorescence microscopy (Leica, Wetzlar, Germany), and the results from the TUNEL assay were obtained using a light microscope (Leica, Wetzlar, Germany). Information about the morphological structure of apoptosis was observed by DAPI staining, and the morphological changes and quantification were determined by the TUNEL assay.

An apoptotic index is a numerical number that counts both the morphological changes of apoptotic nuclei in the cell, which can be referred to as TUNEL (+) cells, and the healthy non-apoptotic nucleating cells, which can be referred to as TUNEL (–) cells. The mathematical calculation of the apoptotic index is as follows:

$$\text{Apoptotic index (\%)} = \left(\frac{\text{Number of apoptotic nuclei}}{\text{Total number of cells}}\right) \times 100$$

Statistical analysis

Statistical calculations were performed using the software IBM SPSS Statistics 22 (IBM, NY, USA). When analyzing the data, the suitability of the parameters for normal distribution was tested using the Shapiro-Wilks test. The data were found to not conform to a normal distribution. The Mann Whitney U test was used to compare the quantitative data between the two groups. The Kruskall Wallis test was used for comparisons between more than two groups, while the Mann Whitney U test was used to determine the group that caused the difference. The Wilcoxon signed-rank test was used to evaluate the cell indexes at the 48th, 72nd, and 96th h according to 24th h. Significance was evaluated at the $p < 0.05$ and $p < 0.0001$ levels.

RESULTS

Real-time cytotoxicity assay (RTCA)

The concentrations of the 360 µg/mL, 180 µg/mL, 90 µg/mL and 45 µg/mL of the PE extract had cytotoxic effects on MCF-7 and MDA-MB-231 human breast cancer cell lines, while the concentrations of 20 µg/mL and 10 µg/mL showed no cytotoxicity on the cells ($p < 0.05$) (Figure 1). The PE extract was cytotoxic only at a concentration of 360 µg/mL against the PCS-201-012 cell line, which was used as the control group ($p < 0.05$; Figure 1).

The EtOH extract of *T. vulgaris* had a concentration-dependent cytotoxic effect at all concentrations (360-10 µg/mL) on MCF-7 and MDA-MB-231 human breast cancer cell lines ($p < 0.01$; Figure 2). In contrast, the EtOH extract of *T. vulgaris* was not cytotoxic against the PCS 201-012 cell line used as a control group ($p < 0.05$; Figure 2).

The RTCA software analyzed the results obtained 72 h after the application of the PE and EtOH extracts. IC_{50} was determined using time-dependent impedance values for MCF-7 and MDA-MB-231 human breast cancer cells and for the PCS 201-012 healthy fibroblast cells at 24, 48, and 72 h (Table 1).

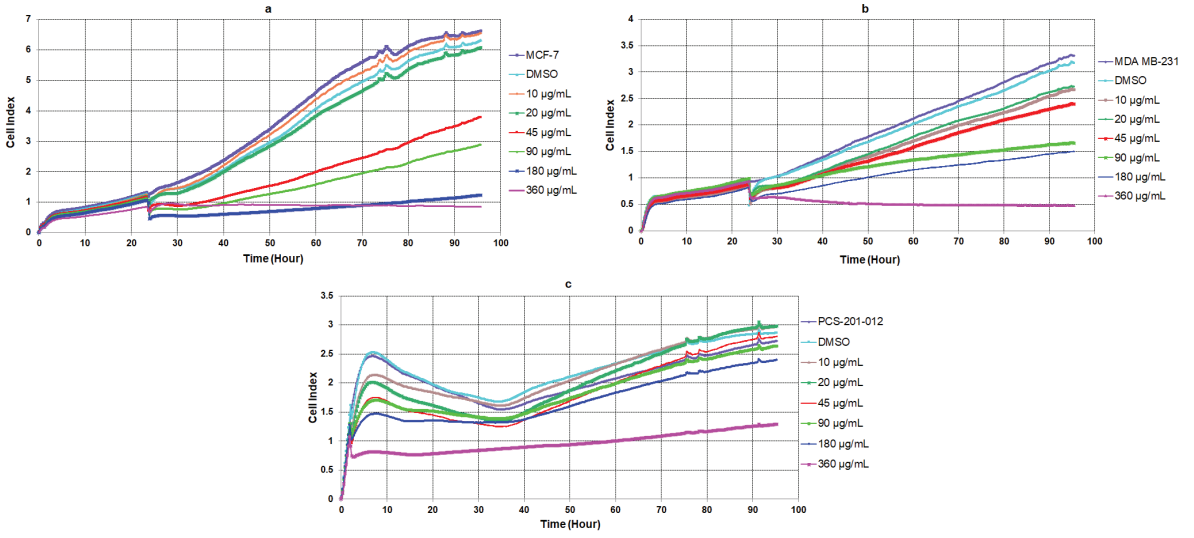

Figure 1. The cytotoxic effects of the petroleum ether extract from *T. vulgaris* on the (a) MCF-7 and (b) MDA-MB-231 human breast cancer cells and on the (c) PCS-201-012 healthy fibroblast cells.

Based on the cytotoxicity results (at 24th, 48th, and 72nd h) for both extracts on both breast cancer cell lines, the 10 and 20 µg/mL concentrations exhibited very low cytotoxic effects, while the 90, 180, and 360 µg/mL concentrations showed very high cytotoxic effects (Figures 1 & 2). Since the 45 µg/mL concentration showed a moderate cytotoxic effect on the cells among the concentrations applied in the RTCA (10-360 µg/mL), 45 µg/mL was determined as the effective extract concentration. The effective concentration was then used for determining the cytotoxic effect of extracts on human breast cancer stem cells.

The effective concentration of *T. vulgaris* PE and EtOH extracts was applied by flow cytometry to the $CD24^{-}/CD44^{+}$ cell-specific antigen profile of MDA-MB-231 human breast cancer cell line. A cytotoxic effect was observed at the administered effective concentration (Figure 3).

Determination of apoptotic activity

The effective concentration was also used for determining apoptotic activity. After incubation with 45 µg/ml of both extracts, morphological alterations in breast cancer cells showed comparisons with the control cells.

TUNEL assay

Brown-stained cells were noted as a result of the TUNEL assay for detecting cells with apoptotically labeled nuclei. Figure 4 shows the changes in the apoptotic cell index of the PE and EtOH extracts applied to MCF-7 cells compared with the control cells. The PE extract applied to MCF-7 cells was found to have a higher apoptotic cell index than the EtOH extract. The apoptotic cell index was significantly different from that of the control ($p < 0.0001$).

The changes in the apoptotic cell index of the PE and EtOH extracts applied to MDA-MB-231 cells compared with the control cells are shown in Figure 5. The PE and EtOH extracts show MDA-MB-231 cells to have been killed by very high rates of apoptosis. The index of apoptotic cells is significantly different from that of the control cells ($p < 0.0001$).

Table 2 shows the apoptotic index values obtained by applying the *T. vulgaris* extracts to two different cell lines. MCF-7 cells were more resistant to apoptosis than MDA-MB-231 cells and had a slightly lower apoptotic index ($p < 0.0001$).

DAPI staining

For the nuclear morphological analysis, the effective concentration (45 µg/mL) of the *T. vulgaris* PE and EtOH extracts was applied to MCF-7 and MDA-MB-231 human breast cancer

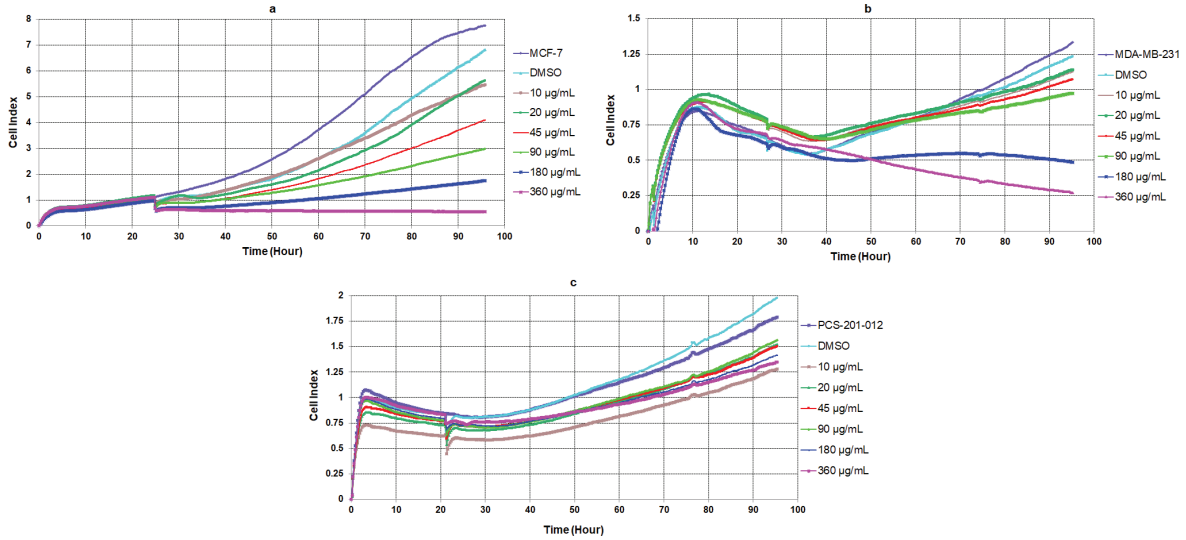

Figure 2. The cytotoxic effects of the ethanol extract from *T. vulgaris* on (a) MCF-7 and (b) MDA-MB-231 human breast cancer cells and on the (c) PCS-201-012 healthy fibroblast cells.

Table 1. IC_{50}* values for the *T. vulgaris* extracts on breast cancer cells and healthy cells.

	IC_{50} Values (µg/mL)					
	Petroleum ether extract			Ethanol extract		
	24th h	48th h	72nd h	24th h	48th h	72nd h
MCF-7	30.155	26.622	32.549	106.32	59.915	78.043
MDA-MB-231	100.9	50.618	49.888	42.457	84.698	74.826
PCS-201-012	$5.48x10^8$	$33.3x10^8$	$26.04x10^8$	$8.91x10^8$	$34.75x10^8$	$15.84x10^8$

* IC_{50} is the concentration required to inhibit 50% of cell growth.

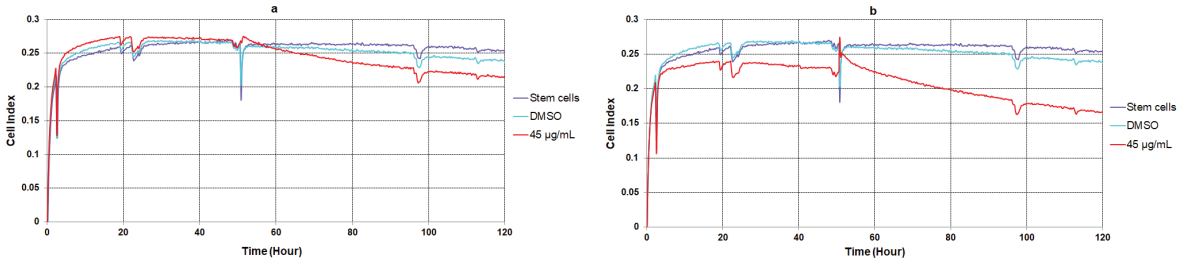

Figure 3. The cytotoxic effects of the (a) PE and (b) EtOH extracts from *T. vulgaris* on human breast cancer stem cells.

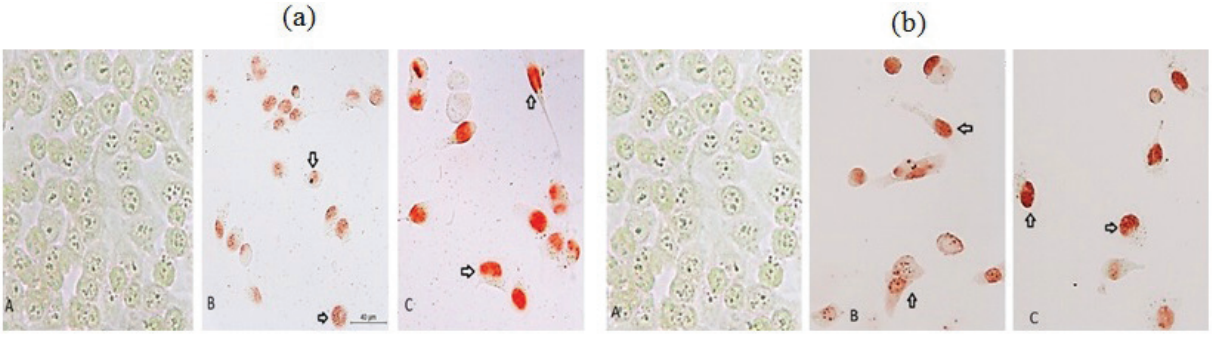

Figure 4. The apoptotic effects of the (a) PE and (b) EtOH extracts from *T. vulgaris* on MCF-7 human breast cancer cells. (A) shows non-treated cells; (B) and (C) show cells treated with effective concentration of the extracts (45 µg/mL) after 24 h, and the TUNEL-positive apoptotic cells, respectively (Bar; A = 80 µm, B and C = 40 µm).

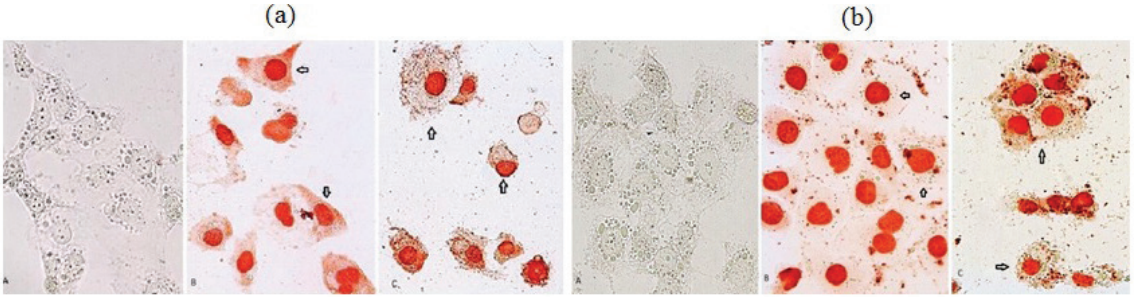

Figure 5. The apoptotic effects of (a) PE and (b) EtOH extracts from *T. vulgaris* on MDA-MB-231 human breast cancer cells. (A) shows non-treated cells; (B) and (C) show cells treated with effective concentration of the extracts (45 µg/mL) after 24 h, and TUNEL-positive apoptotic cells, respectively (Bar; A = 80 µm, B and C = 40 µm).

Table 2. Apoptotic index values (%) of breast cancer cells treated with *T. vulgaris* extracts.

	Apoptotic index values (%)		
	Control	**PE**	**EtOH**
MCF-7	2.38 ± 0.36	76.27 ± 6.33	67.18 ± 23.42
MDA-MB-231	1.52 ± 0.23	96.88 ± 1.68	93.87 ± 1.84

EtOH = Ethanol extract; PE = Petroleum ether extract

cells. After 24 h, the cells were stained with DAPI and visualized under fluorescence microscopy. The images of the apoptotic cell nuclei morphology as a result of applying the PE and EtOH extracts to MCF-7 and MDA-MB-231 cells are shown in Figures 6 and 7. The changes in the nuclei of the cells were observed after treatment with the extracts. The treated cancer cells showed condensed chromatin and nuclear fragmentation, which are characteristics of apoptosis compared to the non-treated cells, which showed clear round nuclei. Also, the number of apoptotic cells was observed to have increase in the treated cells compared to the non-treated cells.

DISCUSSION

The present study has shown the effects of the PE and EtOH extracts of *T. vulgaris* on MCF-7 human breast cancer cell line. The results show a dose-dependent cytotoxic effect, with the IC_{50} values of the PE and EtOH extracts after 24 h incubation be-

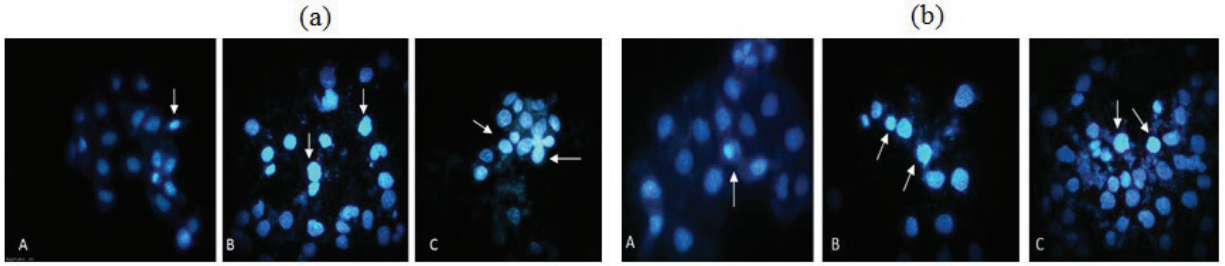

Figure 6. Image of nucleus morphologies of apoptotic cells in fluorescent microscopy by DAPI staining of MCF-7 human breast cancer cells treated with the (a) PE and (b) EtOH extracts from *T. vulgaris*. Normal and mitotic nuclei in the (A) non-treated cells and (B & C) apoptotic cells were observed at the end of 24 h in the treated cells. The cells were treated with the effective concentration of the extracts (45 µg/mL; magnification: x40).

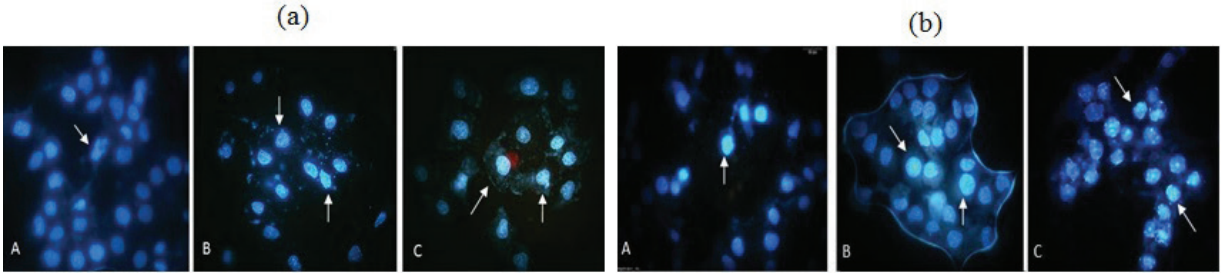

Figure 7. Image of nucleus morphologies of apoptotic cells in fluorescent microscopy by DAPI staining of MDA-MB-231 human breast cancer cells treated with the (a) PE and (b) EtOH extracts from *T. vulgaris*. Normal and mitotic nuclei in the (A) non-treated cells and (B & C) apoptotic cells were observed at the end of 24 h in the treated cells. The cells were treated with the effective concentration of the extracts (45 µg/mL; magnification: x40).

ing 30.155 µg/mL and 106.32 µg/mL, respectively. Some studies have focused on the biological activity of the *Thymus* species. One of these studies indicated the essential oils of *T. serpyllum* (52.69 µg/mL), *T. algeriensis*, (62.53 µg/mL), and *T. vulgaris* (180.40 µg/mL) to exhibit cytotoxic effects in terms of IC_{50} values on MCF-7 cells (Nikolić et al., 2014). Esmaeili-Mahani et al. (2014) reported the *T. caramanicus* extract to be effective against MCF-7 cells at a concentration of 80 µg/mL and ineffective at a concentration of 40 µg/mL. Another study applied the methanol extracts from *T. serpyllum* and *T. vulgaris* to MCF-7 cells and determined the effective concentrations to be 399.407 µg/mL and 407 µg/mL, respectively (Berdowska et al., 2013). Compared to previous studies on the effects of extracts/essential oils from *T. vulgaris* on MCF-7 cells, the current study shows the cytotoxic effects to have occurred at lower concentrations. The better results obtained in the present study may be due to the different plant extract preparation techniques and the solvents used to prepare the extracts from *T. vulgaris*. In addition, all other previous studies had been performed with classical colorimetric cytotoxicity methods. More sensitive and accurate results may have been obtained compared to the classical methods as a result of the present study's use of the RTCA iCELLigence.

In this study, MDA-MB-231 cells were also treated with both the PE and EtOH extracts of *T. vulgaris*, and the findings show a concentration-dependent cytotoxic effect. After a 24 h incubation, the IC_{50} values of the PE and EtOH extracts were 100.9 µg/mL and 42.457 µg/mL, respectively. These values show *T. vulgaris* to have a cytotoxic effect on MDA-MB-231 cells. A previous study showed the IC_{50} values of the essential oils from *T. vulgaris* on MDA-MB-231 cells to have been 108.71 µg/mL and 71 µg/mL after respective incubations of 24 h and 48 h (Al-Shahrani, Mahfoud, Anvarbatcha, Athar, & Al Asmari, 2017). The literature has had no study on the cytotoxic effects of *T. vulgaris* extracts on MDA-MB-231 cells. In this sense, this is the first study to have investigated the effects of *T. vulgaris* extracts on MDA-MB-231 cells. The study hypothesizes that the *T. vulgaris* extracts could be useful in treating MDA-MB-231 cells, which has a different phenotype than MCF-7 cells.

Stem cells with the $CD24^{-}/CD44^{+}$ antigen profile derived from MDA-MB-231 cells were also treated with the effective concentration of *T. vulgaris*. The cytotoxic effect of the extracts was then observed using the RTCA iCELLigence system. With respect to both the cytotoxic effects of *T. vulgaris* extracts on breast cancer stem cells and using the RTCA iCELLigence system for the determination of the cytotoxicity, no such study is noted to have existed in the literature, and this is the first study to have identified the effect of *T. vulgaris.* Therefore, *T. vulgaris* is thought to be a natural source for preventing the spread of cancer in terms of inhibiting cancer stem cells.

The cytotoxic effective concentration in the human breast cancer cell lines (MCF-7 and MDA-MB-231) induced apoptotic cell death. As a result, the present study has found the PE and EtOH extracts of *T. vulgaris* to have an apoptotic effect on both MCF-7 and MDA-MB-231 cells and to have determined morphological changes specific to apoptosis such as rounding, membrane budding, chromatin condensation, and apoptotic bodies. Compared to the control cells, the nucleus condensation in some TUNEL-positive cells was found to be significantly located along the periphery of the nucleus. Based on these results, *T. vulgaris* extracts are thought to be useful as a natural compound for treating human breast cancer.

CONCLUSION

This study has revealed the extracts of *T. vulgaris* to show a significant cytotoxic effect on breast cancer cells and breast cancer stem cells compared to healthy cells. In addition, the extracts induced high levels of apoptosis in the breast cancer cells. These extracts may be a new source for breast cancer therapy due to their selective cytotoxic and high apoptosis induction effects. Further studies are needed to determine the molecular effects of the active compounds from *T. vulgaris* on the cytotoxic, anti-proliferative, and apoptotic pathways.

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

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Original Article

Antioxidant, tyrosinase inhibitor, and cytotoxic effects of *Anthemis aciphylla* Boiss. var. *aciphylla* and *Cota dipsacea* (Bornm.) Oberpr. & Greuter

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ABSTRACT

Background and Aims: The study aims to examine the antioxidant , tyrosinase inhibitory, and cytotoxic activities of the methanol and chloroform extracts of *Anthemis aciphylla* Boiss. var. *aciphylla* and *Cota dipsaceae* (Bornm.) Oberpr. & Greuter in order to evaluate the results regarding different polarities.

Methods: The study evaluates the total phenolic and total flavonoid content of the extract sand the *in vitro* antioxidant activities using the DPPH, ABTS, superoxide radical, and nitric oxide scavenging activities. Tyrosinase inhibitor activity was identified by Masuda's method. Cytotoxicity was investigated using the MTT assay.

Results: The *A. aciphylla* var. aciphylla methanol extract (*AAM*) was the most active extract in term of overall antioxidant activity. The IC_{50} values for DPPH, ABTS, and superoxide anion radical activity were found to be 19.57, 46.82, and 5.610 µg/mL, respectively. Nitric oxide scavenging activity percent inhibition of the *AAM* was 46.47%. The tyrosinase inhibitory activity of *AAM*, was also the most effective, thus confirming the study's hypothesis. Moreover, no cytotoxic activity was observed to be present in the extracts with biological activity.

Conclusion: According to the hypothesis, the tyrosinase inhibitor effect should be observed mostly in extracts and compounds that show the high antioxidant activity mostly originating from phenolic substances. *AAM* confirmed this hypothesis as the most active extract regarding both the tyrosinase inhibitor effect as well as antioxidant activities. Furthermore, the MTT assay was used to find *AAM* to be non-cytotoxic at the tested concentrationsThis indicates *AAM* to be able to be used more reliably in medicine and as a food preservative.

Keywords: *Anthemis, Cota*, antioxidant activity, cytotoxic activity, tyrosinase inhibitor activity

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INTRODUCTION

The use of natural resources, including herbal medicines, has become quite common in the therapeutic field. The secondary metabolites they contain involve important biological activities. Phenolic compounds found in plant extracts are particularly known for being the most important group responsible for biological activities involving antioxidant activity. Oxidative stress plays an essential role in chronic diseases such as cardiovascular disease, diabetes, and cancer. Antioxidants protect the body from oxidative stress by scavenging free radicals (Zengin, Aktümsek, Güler, Çakmak & Yıldıztugay, 2011; Albayrak, Atagün & Aksoy, 2017). Several reports are found describing the involvement of free radicals in carcinogenesis, with a general relationship found between antioxidant defense systems and melanogenesis. The most important point of the relationship is the result of the synergistic effect of tyrosinase inhibitors by increasing their antioxidant effectiveness in scavenging free radicals while working (Wang et al., 2018). UV radiation stimulates reactive oxygen species (ROS) production in the skin, increasing skin pigmentation. ROS are formed as a result of DNA damage, UV radiation, and immune responses to cancerous cells. As mentioned, increased ROS production has long been observed in many types of cancers and different diseases (Witgen and Kempen 2007; Fuchs-Tarlovsky, 2013; Venza et al., 2021).

Skin rejuvenation and hyperpigmentation disorders have become very important in recent years. Nowadays, the focus is on tyrosinase enzyme, which is an essential and rate-limiting enzyme involved in the biosynthesis of melanin, the group of pigments responsible for pigmentation in mammals. Melanin is a copper-containing multifunctional oxidase (Masuda, Yamashita, Takeda & Yonemon, 2005). In the course of melanogenesis, the process for synthesizing melanin, tyrosinase catalyzes the hydroxylation of L-tyrosine to DOPA (β-3,4-dihydroxy phenylalanine), then allowing Dopaquinone to be oxidized. Overproduction of melanin causes hyperpigmentation disorders such as melasma and age spots. Tyrosinase inhibitors show activity through hydroxyl groups that bind copper at the active site of tyrosine. Polyphenols, especially flavonoids, long-chain lipids, and steroids, are the most common enzyme inhibitors. A complex is thought to bind mechanically, the oxygen of phenol to copper, followed by electrophilic monooxygenation of the ring to both copper ions (Zolghadri et al., 2011; Sarıkürkçü, 2020).

The genus *Anthemis* is in the Asteracea family, with 81 taxa and 51 species registered in the flora of Türkiye (Davis, 1984; Güner, Ozhatay, Ekim & Baser 2000). Ethnobotanically, *Anthemis* is used internally for gastrointestinal disorders, abdominal pain, stomach pain, hepatic diseases, coughs, carminative, and kidney stone reduction and externally for burns and skin irritation (Baytop, 1984; Gürhan & Ezer, 2004). Its major secondary metabolites are sesquiterpene lactones, flavonoids, phenolics, acetylene, and essential oils. In addition, the genus is known to have antioxidant, antimicrobial, anticholinesterase, antiproliferative, antispasmodic, and anti-Helicobacter pylori effects (Honda, Yesilada & Tabata, 1996; Kultur, 2007; Uğurlu and Seçmen, 2008).

The plant *Anthemis dipsacea* was included in the *Cota* genus in 2012 with the study of Güner et al. . *Anthemis dipsacea* Boiss. is known as the synonym for the plant *Cota dipsacea* (Bornm.) Oberpr. & Greuter (Güner et al., 2012). While the genus *Cota* had previously been considered a species in the genus *Anthemis*, it is now considered a separate genus consisting of 63 taxa worldwide and being distributed throughout Western Europe, North Africa, and Asia. There are 22 taxa in Türkiye, 9 of which are endemic. The ethnobotanical uses of the *Cota* species indicate that the infusion prepared from their capitulum is beneficial for stomachaches, sore throats, and coughs. Fresh capitulum are crushed and used for gingivitis. In addition, the aerial parts are known to be used for cancer treatment among people. Although not many detailed studies are found on the genus, it is known to have many common points in terms of biological activity and secondary metabolites due to its similarities to the genus *Anthemis*. The *Cota* genus contains polyphenols and sesquiterpene lactones as major secondary metabolites. In addition, anti-inflammatory, antioxidant, and various enzyme inhibitory activities (e.g., cholinesterase, tyrosinase) have been detected in the genus. *Anthemis* and *Cota* are both similar genera from the *Asteracea* family, which contains many phenolic components. Therefore, both can be considered as potential inhibitors due to their rich phenolic content (Karadeniz, Cinbilgel, Gün & Çetin, 2015; Talhouk et al., 2015; Ozek et al., 2019).

This study aims to reveal the antioxidant and tyrosinase inhibitory activities in accordance with the overall phenolic and flavonoid content and assumes that active extracts will show non-cytotoxic properties suitable for consumption. Hence, this study uses the methanol and chloroform extracts of different polarities were used.

MATERIALS AND METHODS

Plant material

The aerial parts of *Anthemis aciphylla* var. *aciphylla* and *Cota dipsaceae* were collected from Bozdağ, İzmir in May 2022 and identified by Prof. Dr. Tuğçe Fafal. Voucher specimens were deposited in the herbarium of Ege University Faculty of Pharmacy, Department of Pharmacognosy and registered under Nos. 1654 and 1655, respectively.

Extraction

After the *Anthemis* and *Cota* species were air-dried and powdered by mill, 10 g of the plants were macerated with 200 mL of methanol at room temperature for 24 hours to prepare the extracts. Plants were extracted three times. After filtering, the extract was evaporated to dryness in a rotavapor. The same procedures were repeated with chloroform and then stored at 4°C for use.

Determining the total phenolic content of the extracts

Total phenolic substance analysis was performed according to the common Folin-Ciocalteu method. The total amounts of the phenolic substances in the extract were calculated with the graph formula prepared from different concentrations of gallic acid. 2.8 ml of deionized water was added to 0.1 ml of the extract diluted at the appropriate rate, and then 2 ml of 2% Na_2CO_3 was added. This was lastly mixed with 0.1 ml of Folin

reagent and left for 30 minutes and measured at 750 nm. Experiments were performed three times with the results averaged (Orlando et al., 2019).

Determining the total flavonoid content of the extracts

The total flavonoid concentration was calculated colorimetrically using a UV spectrophotometer. According to this method, 1.5 ml of ethanol was added to 0.5 ml of the appropriately diluted sample, then 0.1 ml of $AlCl_3$ was added. Lastly, 2.8 ml of deionized water was added and incubated for 40 minutes. Absorbance was measured against ethanol at 415 nm. Experiments were performed three times, and the results were averaged (Orlando et al., 2019).

***In-vitro* antioxidant activities**

DPPH radical scavenging activity

Esmaeili et al.'s (2010) method has been modified to measure this. Accordingly, the DPPH solution prepared in appropriate media was added to the samples within a certain concentration range and incubated for 30 minutes in the dark. The absorbances were read against methanol at 517 nm, with α-Tocopherol being used as normal (Esmaeili and Sonboli, 2010).

ABTS radical scavenging activity

Rer et al.'s1999 modified method was used for checking the ABTS radical scavenging activity. The ABTS radical was prepared from a stock ABTS solution with potassium persulfate, then diluted with ethanol until obtaining an absorbance of 0.7 at 734 nm. After adding 0.1 mL of extract/standard to the 1 mL of diluted ABTS solution, the change in absorbance at 734 nm was incubated for 6 min. at room temperature, with α-Tocopherol being used as normal.

Superoxide anion radical scavenging activity

Checking for this was carried out according to Patel et al.'s research. 10 µL of standard extracts at different concentrations, 15 µL of 12 mM EDTA, 10 µl od 0.1 mg/mL NBT (nitro blue tetrazolium), 5 µL of 0.2 mg/mL riboflavin, and 160 µL of 0.067mM potassium phosphate buffer (pH 7.4) were placed in microplate reader and incubated under fluorescent light for 5 minutes. Ascorbic acid was used as normal. The absorbance at 560 nm was then measured, with the IC_{50} being calculated using GraphPad Prism 5(Patel et al., 2010).

Nitric oxide scavenging activity

The nitric oxide scavenging activity was carried out using the Griess reagent. 50 µl of standard extract at various concentrations were mixed with 100 µL of methanol. Next, 2.0 mL of sodium nitroprusside (10 mM) in a phosphate buffer saline at 7.2 pH was added to each tube. The solutions were incubated at room temperature for 150 minutes to produce nitrite ions. At the end of the incubation, 5.0 mL of the Griess reagent were added to each tube. The absorbance of the chromophore was measured at 546 nm. Ascorbic acid was used as normal (Patel et al., 2010).

***In-vitro* tyrosinase inhibitory activity**

Masuda et al.'s method was modified for this. 110 µL of the phosphate buffer (0.01 M, 6.8 pH), 10 µL of plant extract at different concentrations, and 20 µL of tyrosinase solution (200 unit/mL) were mixed. After 10 minutes of incubation at 37°C, the reaction was initiated by adding 20 µL of L-Dopa and allowed to incubate at 37°C for another 10 min. The absorbance was measured at 475 nm, with Kojic acid being used as normal (Masuda et al., 2005).

Cell culture

B16-F10 melanoma cells were cultured in a Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum (FBS) in a humidified incubator at 37°C with 5% CO_2. Cells were seeded into a 96-well cell culture plate at a density of 8,000 cells. After establishing the morphology of the cells, *AAC* extracts were prepared at doses ranging from 2.5-100 µg/mL and applied to the cells for 48 hours. Dimethyl sulfoxide (DMSO) was used as negative control. Activity at concentrations above 200 was not taken into account.

MTT assay

The MTT assay was carried out after a 48-hour incubation. The MTT-medium mixture was added to 96-well cell culture dishes at a final concentration of 0.5 mg/mL and incubated for 4 hours. After the medium was removed, 200 µL of DMSO was added to dissolve the formazan salts, and the measurement was taken at a wavelength of 570/690 nm with a plate reader (Varioskan, Thermo Fisher Scientific, USA).

Statistical analysis

All results are expressed as mean ± *SD*. Analysis of variance was also performed, and significant differences between means were determined using Duncan's multiple range tests at a level of $p < 0.05$ in IBM SPSS (ver. 25).(Table 3)

RESULTS AND DISCUSSION

Pharmaceutical research has recently focused on plants containing free radical scavengers due to their importance in preventing and treating several disorders. The current study evaluates the antioxidant activities of methanol and chloroform extracts from *A. aciphylla* (*AAM*, *AAC*) and *C. dipsacea* (*ADM*, *ADC*). Total phenolic and flavonoid content and antioxidant assays have been summarized in Table 1. The results for *AAM* indicate it has strong DPPH scavenging activity as well as significant amounts of total phenol and total flavonoid. *AAC* also has moderate DPPH scavenging activity; however, *ADM* and *ADC* were less effective. After searching the literature searching, medicinal plants containing high amounts of total phenol and flavonoid were seen to possess strong antioxidant activity. Given that, the results from this study are seen to be in line with previous studies regarding *A. cretica* L., *A. tinctoria* L., *A. desertii* L., and *A. palestina* L. Belhaoues et al.'s study determined a correlation to be present between phenolic compounds and antioxidant activity, with flavonoids observed to have a dose-dependent relationship with antioxidant activity. The total phenol content of *A. praecox* L. was found to be 39.65 mg GAE/g in the extract, with the total flavonoid amount being 2.28 mg QE/g. The IC_{50} values for the DPPH and ABTS radical scavenging activities were observed to be quite low (i.e., to show strong activity. (Sut et al., 2019; Belhaoues et al., 2020; Bursa, Aras, Kılıç & Buldurun, 2020). The *AAM* result regarding ABTS scavenging activity is 46.82 µg/mL, whereas the positive control (Trolox) is 18.87 µg/mL, showing almost 1/3 the ef-

fect of *AAM*. The literature shows *A. cotula*, *A. praecox*, and *A. chia* to also have significant effects (Sut et al., 2019; Belhaoues et al., 2020; Bursal et al.,2020). Chemsa et al.'s research on *A. stiparum* subsp. *sabulicola* (Pomel) Oberpr. determined the DPPH value as 92.69 µg/ml (IC_{50}) (Chemsa et al., 2018). Emir and Emir's research determined the amount of total phenolic and flavonoid substances as 21.7 mg GAE/g extract and 9.7 mg QE/g extract;(Emir and Emir, 2020) they gave DPPH as mg TE/g extract and hence is not comparable here. study on *A. cotula* L. calculated an IC_{50} value for the DPPH radical scavenging activity of 165.72 µg/ml (Gür et al., 2018).with *A. chia* L.'s total phenolic substance being 42.38 mg GAE/g extract and total flavonoid amount as 28.99 mg QE/g extract. also evaluated DPPH and ABTS radical scavenging activities but provided no trolox equivalent values, thus they could not be compared exactly(Sarıkürkçü, 2020). Total phenolic and flavonoid contents of the *A. tinctoria* L. var. *pallida* methanol extract were determined as 100.09 mg GAE/g extract and 48.54 mg RE/g extract, respectively. In addition, the *A. cretica* L. subsp. *tenuiloba* total phenolic and flavonoid contents were found to be 46.73 and 45.08, respectively. *A. desertii* total flavonoid content was found to be 2.92 mg GA/g extract. However, the DPPH radical scavenging activity again cannot be compared because the percent inhibition was given. However, after performing positive control, it was found to be the second most-effective plant (Orlando et al., 2019).

Moreover, *C. altissima* showed ABTS scavenging activity similar to *ADM* and *ADC* (Göger et al., 2021). In their recent research, the superoxide anion radical scavenging activity of *AAM* was 5.610 µg/mL, which is higher than the positive control ascorbic acid value of 5.992 µg/mL. In contrast, *AAC* was determined as the extract with the lowest activity. *ADM* and *ADC* indicated average effects on superoxide anion radicals. Regarding the percent inhibition of the nitric oxide scavenging activity, ascorbic acid inhibited 60%, while what came from the *AAM* extract had 46.47%. The extract that showed the least effect was again *AAC*.

According to this study's hypothesis, the tyrosinase inhibitor effect should be observed mostly in extracts that show antioxidant activity and should also have a significant amount of total phenol and flavonoid content. The *AAM* extract confirmed the hypothesis as the most active extract regarding both the tyrosinase inhibitor effect as well as antioxidant activity (Table 2). The results regarding antioxidant and tyrosinase inhibitor activity are also seen to be statistically compatible ($p \leq 0.01$) (Table 3). Sarıkürkçü's study identified the phenolic contents of *A. chia* L. and examined their antioxidant and enzyme inhibitory (tyrosinase, α-amylase) effects (Sarıkürkçü, 2020). Sut et al.'s , study investigated antioxidant activities of plant extracts obtained by different extraction methods using the DPPH, ABTS, FRAP, CUPRAC, and metal chelation methods. The highest effect was the CUPRAC reducing power (435.32 ± 9.60 mg TE/g extract) from the extract prepared with the accelerated solvent method. Şener et al. also examined the alpha-amylase, alpha-glucosidase, cholinesterase, and tyrosinase inhibitory activities of the extracts prepared with different techniques and observed the activities (Şener et al., 2017).*Anthemis* species and investigated tyrosinase, alpha-amylase, alpha-glucosidase, and cholinesterase in terms of inhibitory enzyme activities(Orlando et al., 2019).

When examining the studies on the *Cota* genus, performed a detailed phytochemical study on the *Cota fulvida* Grierson to examine antioxidant, antidiabetic, anti-inflammatory, and antime-

Table 2. *In-vitro* tyrosinase inhibitory activity results.

	Tyrosinase inhibitory activity results (IC_{50}) (µg/mL)	Cytotoxic activity results (IC_{50}; µg/mL)
AAM[a]	110.73±0.50	na*
ADM[b]	227.8±0.06	na*
AAC[c]	na*	11.77
ADC[d]	na*	na*
Kojic acid	37.40±0.42	

* not active; [a]*Anthemis aciphylla* var. *aciphylla* methanol extract; [b]*Anthemis dipsaceae* methanol extract; [c]*Anthemis aciphylla* var. *aciphylla* chloroform extract; [d]*Anthemis dipsaceae* chloroform extract.

Table 1. *In-vitro* antioxidant activity results.

	Total phenolic content (mg GAE/g)[e]	Total flavonoid content mg QE/g)[f]	DPPH radical scavenging activity (IC_{50}) (µg/mL)	ABTS radical cation scavenging activity (IC_{50}) (µg/mL)	Superoxide radical scavenging activity (IC_{50}) (µg/mL)	Nitric oxide scavenging activity (% inhibition) (at 324 µg/mL concentration)
AAM[a]	214.81±0.12	36.48±0.22	19.57±0.62	46.82±0.22	5.610±0.26	46.47±0.54
ADM[b]	104.66 ±0 .01	12.055±0.112	110.88±0.28	360.9±0.12	80.15±0.41	21.52±0.46
AAC[c]	1.256 ±0 .3	3.127±0.61	66.48±0.02	465.9±0.22	482.1±0.08	8.72±0.01
ADC[d]	3.425 ±0 .1	6.251±0.61	166.6±0.05	163.4±0.11	332.6±0.05	29.23±1.63
Ascorbic acid			4.474±0.31		5.992±0.01	60.0±0.05
Trolox				18.87±0.01		-

[a]*Anthemis aciphylla* var. *aciphylla* methanol extract; [b]*Anthemis dipsaceae* methanol extract; [c]*Anthemis aciphylla* var. *aciphylla* chloroform extract; [d]*Anthemis dipsaceae* chloroform extract; [e]Gallic acid equivalent; [f]Quercetin equivalent.

Table 3. Statistical analysis.

	Sum of Squares	df	Mean Square	F
Between Groups	1540542.434	24	64189.268	642178.750
Within Groups	4.998	50	.100	
Total	1540547.432	74		

Groups: Biological acitivities.

lanogenesis activities and used LC-MS/MS to identify phenolic acids, phenylpropanoid, and flavonoid-containing substances. They also determined the essential oil content using GC-MS. The methanol extract of the plant showed the highest DPPH scavenging activity (IC_{50}: 0.131 mg/mL)(Ozek et al., 2019). study examined many Turkish plants to investigate the DPPH scavenging activity of *Cota pestalozzae* Boiss and also provided the mean IC_{50} range for all plants, reporting IC_{50} values between 18.67-425.19 µg/mL(Karadeniz et al., 2015) Apart from these, no other study was found regarding the *Cota* genus in terms of genera. When comparing all the results from the current study with the literature, they are seen to be compatible.

In conclusion, this research has examined whether certain plants have a cytotoxic effect on cells and observed those with tyrosinase inhibitory activity to be non-cytotoxic. Only chloroform extract *(AAC)* gave a very low IC_{50} value in contrast to the antioxidant and tyrosinase inhibitor activities. Comparable results are also seen in similar studies on tyrosinase inhibitor activity, antioxidants, and inhibition of melanoma cells (Gomez et al., 2001; Wang et al., 2011; Sun et al., 2017; Wang et al., 2019). The fact that the plant inhibits tyrosinase activity without a cytotoxic effect (Table 2) makes it more suitable for the food and pharmaceutical industry. Further studies can examine its effects on melanogenesis in cells and accordingly provide more detailed information about their use in hyperpigmentation. These results indicate the *AAM* extract to potentially contain bioactive substances and to maybe have the potential for use as a depigmentation agent in skin disorders and as an anti-darkening agent in the food industry.

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Original Article

The combined ameliorative effects of α-lipoic acid, selenium, and vitamin E on the livers of STZ-diabetic mice

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ABSTRACT

Background and Aims: The aim of this study was to investigate the histological and biochemical effects of the antioxidant combination on liver tissue of streptozotocin (STZ)-induced diabetic mice.

Methods: Five groups of mice were given a citrate buffer (CB), the antioxidant solvents (AS), the antioxidant combination (A) (α-lipoic acid, selenium, and vitamin E), STZ (D), the antioxidant combination and STZ (A+D). The mice were sacrificed, and their liver tissues were taken out. The liver tissues were examined histologically and immune+ cell numbers of cannabinoid receptors (CB1R and CB2R) were detected. Xanthine oxidase (XO) activity, glutathione (GSH) and lipid peroxidation (LPO) levels, superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione-S-transferase (GST), γ-glutamyl transferase (GGT), paraoxonase (PON), glucose-6-phosphate dehydrogenase (G6PD) activities, protein carbonyl content (PCC) and advanced oxidation protein product (AOPP), sialic acid, fucose, hexose and hydroxyproline (OH-proline) levels were biochemically determined.

Results: Certain degenerative changes were reduced in the A+D group compared histologically to the D group. There were no significant changes in the number of CB1R immune+ cells. The number of CB2R immune+ cells was significantly reduced in the D group compared to the CB group. The GSH level, CAT, SOD, GR, GPx, GST, PON, and G6PD activities were increased while XO and GGT activity, LPO, PCC, AOPP, hexose, fucose, sialic acid, and OH-proline level were biochemically decreased in the A+D group compared to the D group.

Conclusion: The use of the antioxidant combination had a positive effect on the livers of diabetic mice with histochemical and biochemical changes, while there was no effect on the regulation of cannabinoid receptors expressions.

Keywords: Diabetes, α-Lipoic Acid, Vitamin E, Selenium, Liver, Mouse

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INTRODUCTION

Diabetes mellitus was identified as one of the deadliest diseases according to a statement made by the World Health Organization (WHO)**.** Considering 415 million people had diabetes in 2015, it is now predicted that 642 million people may have diabetes in 2040 (IDF 2015). Diabetes includes conditions such as hyperglycemia, hyperinsulinemia, hyperlipidemia, and inflammation. These situations cause oxidative stress due to the formation of reactive oxygen species (ROS) (Holst, Vilsboll, & Deacon, 2009; Aydin et al. 2019). Insulin resistance leads to oxidative stress and liver abnormalities (Mohamed, Nazratun, Zariyantey, & Budin, 2016).

The liver is important in the regulation of liver glucose metabolism and is the major organ sensitive to hyperglycemia. Liver damage occurs in early-stage diabetes (Adeyemi, Ukwenya, Obuotor, & Adewole, 2014). Type 2 diabetes and liver dysfunction are related to each other (Wannamethee, Shaper, Lennon, & Whincup, 2005; Kunutsor, Apekey, & Walley, 2013; Ballestri et al. 2016). It is assumed that liver dysfunction can increase type 2 diabetes by increasing insulin resistance. It is also thought that type 2 diabetes may impair liver function by causing lipogenesis (Smith & Adams 2011). However, it is not known exactly whether these two different relationships exist (Taylor, 2008; Loria, Lonardo, & Anania, 2013; Taylor et al. 2018).

Pathological changes increase oxidative stress and decrease antioxidant activity in internal organs, such as the liver, in diabetes (Springer et al. 2013; Zimmet, Shi, El-Osta, & Ji, 2018). Researchers have shown that ROS are overproduced by decreasing antioxidant enzymes and increasing lipid peroxidation (LPO) in liver damage caused by diabetes (Lozano et al. 2016). Alpha lipoic acid (ALA) is reduced to dihydrolipoic acid (DHLA) and regenerates glutathione (GSH), which is a potent antioxidant. It has been shown that ALA directly scavenges ROS and protects against oxidative stress (Rochette, Ghibu, Muresan, & Vergely, 2015). ALA has been shown to affect glucose uptake via the insulin signal pathway (Konrad et al. 2001). It has also been noted that in type 2 diabetes, ALA is important to improve insulin resistance as well as impaired glucose metabolism (Lee et al. 2005).

The glutathione peroxidases (GPx), iodothyronine deiodinases, and thioredoxin reductases that catalyze metabolic reactions are proteins containing selenium (Se), which is an essential nutrient (Lu & Holmgren 2009). The decreased Se level is associated with a weakened immune system and increased mortality (Rayman 2012). Conversely, additional Se intake when there is already sufficient Se may impair the function of the liver, heart, and related organs. (Zeng et al. 2012; Jablonska et al. 2016). The dose of Se intake is important. A study revealed that those who took Se supplements had a higher risk of diabetes (Stranges et al. 2007). In another study, it was shown that Se is effective in the regulation of glucose metabolism in the liver when given to diabetic rats (Vinceti, Filippini, & Rothman, 2018).

Vitamin E (Vit E), a lipid-soluble, is the most powerful micronutrient antioxidant (Peh, Tan, Liao, & Wong, 2016). It has promising positive results in the treatment of nonalcoholic fatty liver disease. Vit E ameliorated hepatocellular ballooning is a finding of nonalcoholic steatohepatitis in children (Lavine et al. 2011). Furthermore, it is suggested that inflammation and hepatic steatosis decreases in the treatment of nondiabetic adult patients with nonalcoholic steatohepatitis (Sanyal et al. 2010). Also, the risk of occurrence of nonalcoholic fatty liver disease, cirrhosis, and liver cancer in patients with type 2 diabetes is high (Younossi et al. 2018).

The endocannabinoid system contains two specific coupled G-protein receptors (CB1R and CB2R) and their ligands are called endocannabinoids. Bazwinsky-Wutschke, Zipprich, & Dehghani, (2019) asserted that the endocannabinoid system partially controls glucose metabolism in the liver. The system controls lipid and energy metabolism as well as glucose metabolism (Di Marzo 2008). Studies have shown that the endocannabinoid system is associated with diabetes (Gruden, Barutta, Kunos, & Pacher, 2016; Jourdan, Godlewski, & Kunos, 2016). The cannabinoid receptor agonists/antagonists may have potential roles in the treatment of many metabolic diseases, such as diabetes, by attenuating the autoimmune response and regulating insulin secretion, glucose, and energy metabolisms (Zhang et al., 2016; Heppenstall, Bunce, & Smith, 2012; Li, Kaminski, & Fischer, 2001).

In the present study, we investigated the effects of the ALA + Se + Vit E combination on the liver of STZ-diabetic mice and in addition, the role of the antioxidant combination on the endocannabinoid system of streptozotocin (STZ), both morphologically and biochemically.

MATERIALS AND METHODS

Animals and antioxidant treatments

This experimental study was carried out at the Institute's Animal Care and Use Committee of Istanbul University, in 2008-2009. The project was approved by the local ethics committee for animal experiments, at Istanbul University. Balb/c mice (2-2.5 months) provided by the Institute's Animal Care and Use Committee of Istanbul University, were used in the study. They were maintained under standard animal housing conditions in a controlled temperature clean room on a 12 h light/dark cycle and were given water and standard chow *ad libitum*. Five groups were randomly created for the experimental setup. The first group, a control group, was given 0.01 M citrate buffer (CB) group (pH 4.5) by intraperitoneal injections for five consecutive days (n=8), the second group, a control group, was given the antioxidant solvent (AS group) in distilled water including NaOH for ALA, distilled water for Se and flower oil for Vit E using the gavage technique (n=8), the third group, a control group, was given antioxidant combination (A) ALA (50 mg/kg), Se (0.25 mg/kg), and Vit E (100 mg/kg) for five consecutive days using the gavage technique (n=8), the fourth group, the experimental group, was given STZ (40 mg/kg) (D) group for five consecutive days by intraperitoneal injections (n=10), and the fifth group, the antioxidants+diabetes (A+D) group was given the antioxidant combination before diabetes (n=10). After 30 days, the mice were sacrificed by cervical dislocation. The liver tissues were quickly removed for histological and biochemical analyses.

Histological assay

The liver tissues were fixed for histological investigation in Bouin's solution and cleared in xylene. It was embedded in paraffin, after dehydration in ethanol series. The tissues embedded in paraffin were cut into 5 µm sections. these were then adhered to the microscope slide. Hematoxylin-Eosine and Masson's trichrome were used for staining sections.

Immunohistochemical assays

A 10% neutral buffered formalin solution was used to fix the tissue samples. 4 µm thick sections were taken. The deparaffinized sections were heated in a 0.01 mol/L CB (pH 6.0) in a microwave oven for antigen retrieval for 15 min. The immunoreactivities of antibodies were analyzed with a Histostain Plus Broad-Spectrum Kit (Invitrogen, Carlsbad, CA, USA). Endogenous peroxidase activity was blocked for 15 min with 3% hydrogen peroxide in methanol followed by the incubation of the sections with a 1:100 dilution of CB1R (rabbit polyclonal, Cayman Chemicals, Ann Arbor, MC, USA) and 1:200 dilution of CB2R (rabbit polyclonal, Santa Cruz Biotechnology, CA, USA) primary antibodies overnight at 4 °C. After incubation, the sections were washed off with phosphate-buffered saline, and were then incubated with a biotinylated secondary antibody at room temperature. They were incubated with horseradish peroxidase (HRP)-labeled streptavidin. Visualization of the reaction was carried out using 3-amino-9-ethylcarbazole (AEC, Invitrogen, Carlsbad, CA, USA) as the chromogen. Mayer's hematoxylin was used for counterstaining for 40 seconds at room temperature. The slides were incubated in the absence of CB1R and CB2R primary antibodies for negative control. The liver tissue slides were examined with a Nikon Eclipse 80i light microscope equipped with a digital camera. This was analyzed with the NIS-Elements-D 3.1 microscope imaging software program. For the expressions of CB1R and CB2R, ten randomly selected areas in each slide (n=5) were examined.

Biochemical assays

In the previous study by Karatug & Bolkent (2013), fasting blood sugar levels of mice were presented (Karatug & Bolkent 2013). The liver tissue samples were washed off with saline and kept frozen until analyzed for biochemical analyses. The liver tissue samples were homogenized with 0.9% NaCl cold physiological saline solution by means of a glass homogenizer and made up to 10% (w/v) homogenate. After that, the homogenates were centrifuged at 10000 x g for 10 minutes. Liver tissue homogenates were used for enzyme analyses and protein carbonyl content (PCC), advanced oxidation protein product (AOPP), sialic acid, fucose, hexose, and hydroxyl proline levels.

Xanthine oxidase activity

Xanthine oxidase (XO) activities of liver tissues were performed according to Corte, & Stirpe (1968), with the help of uric acid at 240 nm. The results were given as U/mg protein.

GSH levels

GSH levels of liver tissues were evaluated by the method described by Beutler (1975). Metaphosphoric acid was applied for the precipitation of protein, and the development of product color was due to 5,5′-dithiobis-2-nitrobenzoic acid. The measurements were made spectrophotometrically at 405 nm. Results were expressed as nmol GSH/mg protein.

LPO levels

The lipid peroxidation (LPO) levels of the liver tissues were measured in accordance with the Ledwozyw et al. method (Ledwozyw, Michalak, Stepien, & Kadziołka, 1986). Malondialdehyde (MDA) is among the byproducts of LPO. The measurements were made spectrophotometrically at 532 nm. Results were given as nanomoles of MDA per milligram of protein.

Superoxide dismutase activity

Superoxide dismutase (SOD) was assessed by the method described by Mylorie et al. (Mylorie, Colins, Umbles, & Kyle 1986). Phosphate buffer (pH 7.8), o-dianisidinedihydrochloride, and riboflavin were used for the reaction protocol. The reaction was performed using a fluorescent lamp and was initiated with the addition of riboflavin. The difference in absorbance between 0 and 8 minutes was recorded at 460 nm. The results were expressed as U/g protein.

Catalase activity

Catalase (CAT) activity was performed according to the Aebi (1984) method. The reduction of H_2O_2 to H_2O in the presence of CAT was assayed as the decrease of absorbance at 240 nm. The results were defined as U/mg protein.

Glutathione reductase activity

Glutathione reductase (GR) activity was determined according to the GR activity determined by Beutler (1971). GR activity was determined by calculating the amount of NADH oxidized during the reduction of oxidized glutathione with GR. GR activity was expressed as U/g protein.

Glutathione peroxidase activity

GPx activity was described by Paglia, & Valentine (1967). According to this method, GPx activity was measured by the conversion of H_2O_2 to H_2O in the presence of GSH, NADPH, and glutathione reductase (GR). The oxidation of NADPH to $NADP^+$ was considered in reaction media at 366 nm for the determination of GPx activity. GPx activity was expressed as U/g protein.

Glutathione-S-transferase activity

Glutathione-S-transferase (GST) activity was performed according to the Habig, & Jakoby (1981) method. GST catalyzes the reaction between GSH and 1-chloro-2,4-dinitrobenzene. The resultant products formed were monitored spectrophotometrically at 340 nm. GST activity was expressed as U/mg protein.

Gamma glutamyl transferase activity

Gamma glutamyl transferase (GGT) activities were determined by the method described by Szasz (1969). This method is based on the determination of the amount of p-nitroaniline formed as a result of the reaction and the absorbance value at 405 nm in the spectrophotometer. GGT activity was expressed as U/g protein.

Paraoxonase activity

Paraoxonase activity (PON) of liver samples was assayed according to Furlong et al. (Furlong, Rchter, & Seidel 1988). The enzyme activity was determined using paraoxon ethyl as a substrate and the absorbance of yellowish-colored product p-nitrophenol was assayed at 405 nm. The results were expressed as U/g protein.

Glucose-6-phosphate dehydrogenase activity
Glucose-6-phosphate dehydrogenase (G6PD) activity in liver tissues was determined according to the Beutler (1984) method, by spectrophotometric measurement of NADPH formed during the reaction in the presence of Mg^{2+} ions at 340 nm (Beutler, 1984). The results were expressed as U/g protein.

Protein carbonyl content
Liver tissue protein carbonyl (PCC) contents were determined spectrophotometrically by the method of Levine et al. (1990). The results were expressed as nmol carbonyl/mg protein.

Advanced oxidation protein product levels
Advanced oxidation protein product (AOPP) level was determined according to the Witko-Sarsat et al. (1996) method. The liver tissue samples were treated with phosphate buffer at pH 7.4 and potassium iodide. Finally, glacial acetic acid was added, and the absorbance of the product was recorded at 340 nm. The results were expressed as nmol/mg protein.

Sialic acid levels
Sialic acid was estimated by the method of Lorentz et al. (Lorentz, Weiss, & Kraas, 1986). Sialic acid, which undergoes periodic acid oxidation, reacts with ß-formyl pyruvic acid, and thiobarbituric acid. It enters a colored compound with maximum absorbance at 546 nm. This compound formed is unstable, so it is attracted to the cyclohexanone phase. Results were expressed as µmol sialic acid/g protein.

Fucose levels
The amount of fucose in the liver tissue was determined according to the method of Dische, & Shettles (1948). The method is based on the color reaction of carbohydrates with thiol groups in the presence of sulfuric acid. Results were expressed as µg fucose/mg protein.

Hexose levels
Quantification of hexose in liver tissue was determined according to the method of Winzler (1955). The method is based on the color reaction of carbohydrates with orcinol in the presence of concentrated sulfuric acid, spectrophotometrically, in order to determine hexose compounds in tissues. Results were expressed as µg glucose/mg protein.

Hydroxyproline levels
Liver hydroxyproline (OH-proline) levels were assayed by the method of Reedy, & Enwemeka (1996). Results were expressed as µg OH-proline/g protein.

Protein levels
The Lowry et al. method was used to determine the total protein levels of liver tissue samples (Lowry, Rosebrough, Farr, & Randall, 1951). The protein content of the tissue was applied with copper ions in an alkaline media and then Folin reactive was added. Finally, the blue-colored product absorbance was measured at 500 nm.

Statistical analysis
For histological analysis, the data were analyzed with one-way analysis of variance (ANOVA) followed by post hoc tests (Tukey's test) for differences among groups using a computer package (GraphPad Prism, version 6.0), and for immunohistochemical analysis, using SPSS software (version 21.0, SPSS, USA). Biochemical results were evaluated using an unpaired t-test and variance analysis (ANOVA). Analysis was performed with the NCSS statistical computer package. Findings were expressed as the mean ± standard error of the mean (SEM) for each group in the histological and immunohistochemical assays and as the mean ± standard deviation of the mean (SD) for each group in the biochemical assays. The differences were considered significant when the P value was <0.05.

RESULTS

Histological and immunohistochemical assays
The histological results are presented in Figure 1. Degenerative changes such as pyknotic nuclei, necrotic cell, hyperemia, and sinusoidal dilation increased in the D group compared to the CB group ($p<0.01$), but all of them decreased in the A+D group compared to the D group ($p<0.001$).

The immunohistological results are presented in Figures 2 and 3. There was no significant change in the number of CB1R immune$^+$ cells among all groups ($p>0.05$). A non-significant reduction in the number of CB1R immune$^+$ cells was observed in the D group as compared to the CB group ($p>0.05$) (Figure 2). There was a significant change in the number of CB2R immune+ cells among all groups ($p<0.05$) (Figure 3). The number of CB2R immune$^+$ cells was significantly reduced in the AS, A, D, and A+D groups as compared to the CB group ($p<0.05$ for all). CB1R and CB2R peptides were observed both in the nucleus and cytoplasm.

Biochemical assays
Fasting blood glucose levels were shown in our previous study. According to these results, fasting blood glucose levels in the diabetic group increased significantly compared to the control group ($p<0.001$) (Karatug & Bolkent, 2013).

XO activity, GSH, and LPO levels of liver tissue are presented in Table 1. XO activity and LPO level were increased in the D group as compared to the CB group ($p<0.05$; $p<0.01$ respectively). GSH level was significantly reduced in the D group compared to the CB group ($P < 0.005$). In the A+D group, the diabetic group treatment with antioxidants resulted in a significant increase in GSH level and noticeable decreases in XO activity and LPO level compared to the D group ($p<0.0001$; $p<0.005$; $p<0.0001$ respectively).

SOD, CAT, and GR results are presented in Table 2. Significant decreases were observed in SOD, CAT, and GR activities of the D group compared to the CB group ($p<0.05$; $p<0.0001$; $p<0.0001$ respectively). These decreases were negated by dietary antioxidants for SOD, CAT, and GR enzyme activities ($p<0.05$; $p<0.05$; $p<0.01$ respectively).

GPx, GST, and GGT activities in the liver tissue are shown in Table 3. In the D group, the activities of GPx and GST were decreased while GGT activity was increased compared to the CB group ($p<0.005$; $p<0.005$; $p<0.01$, respectively). Administration of antioxidants to the diabetic mice significantly increased GPx and GST activities in

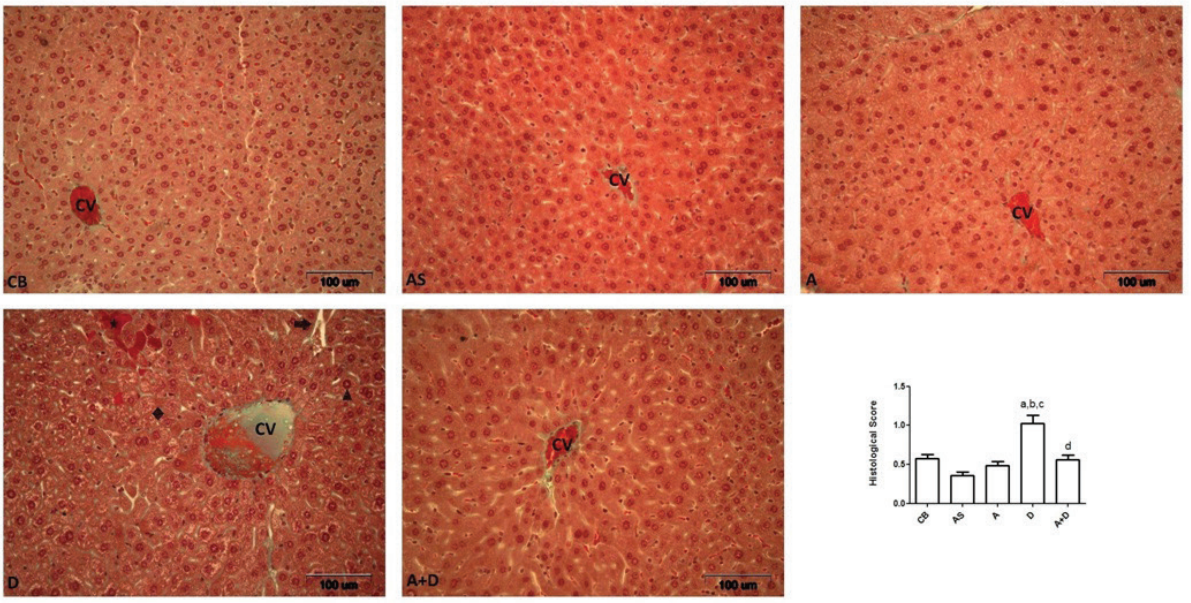

Figure 1. Histological appearance of the liver tissues of given citrate buffer (CB), antioxidants solvent (AS), antioxidants (A), STZ-induced diabetes (D), and Antioxidants+ Diabetes (A+D) groups. Necrotic areas (◆), hyperemia (★), pyknotic nucleus (▲), and sinusoidal dilatation (→) can be seen in diabetic mouse liver. CV: central vein. Masson's trichrome. [a]P<0.01 versus CB group, [b]P<0.001 versus AS group, [c]P<0.001 versus A group, [d]P<0.001 versus D group.

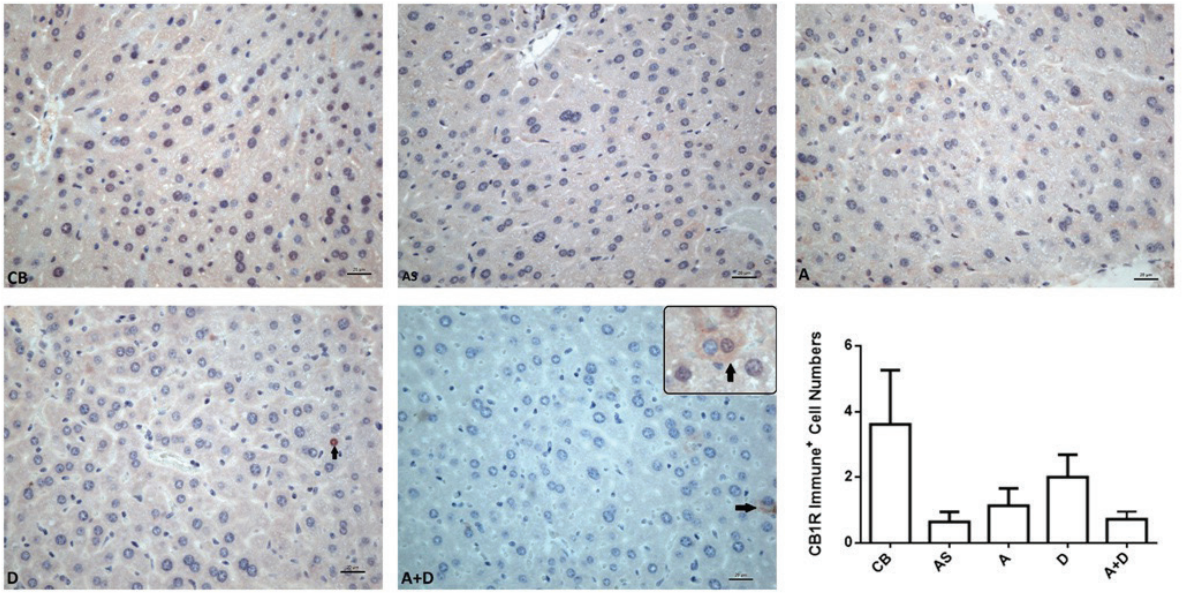

Figure 2. Immune+ cells (arrow) and the number of immune+ cells for cannabinoid 1 receptor (CB1R) in the liver of Citrate buffer (CB), the solvents of antioxidants (AS), antioxidants (A), STZ-induced diabetes (D), and Antioxidants+Diabetes (A+D) groups. Streptavidin-Biotin-Peroxidase technique, counterstain Hematoxylin. Scale bar = 20 µm.

the liver, while significantly decreased GGT activity compared to the D group ($p<0.05$; $p<0.005$; $p<0.0001$ respectively).

PON and G6PD activities, PCC, and AOPP levels are presented in Table 4. In the D group, the enzyme activities of PON and G6PD activities were reduced ($p<0.0001$; $p<0.0001$), PCC and AOPP levels were increased ($p<0.001$; $p<0.05$). Antioxidants supplementation administered to the diabetics resulted in an important increase in PON and G6PD activities ($p<0.05$; $p<0.0001$) whereas a reduction in PCC and AOPP levels was observed ($p<0.0001$; $p<0.05$).

Liver tissue glycoprotein levels are shown in Table 5. The glycoproteins containing sialic acid, fucose, hexose, and OH-proline levels were significantly increased in the D groups compared to the CB group ($p<0.01$; $p<0.05$; $p<0.05$; $p<0.01$ respectively).

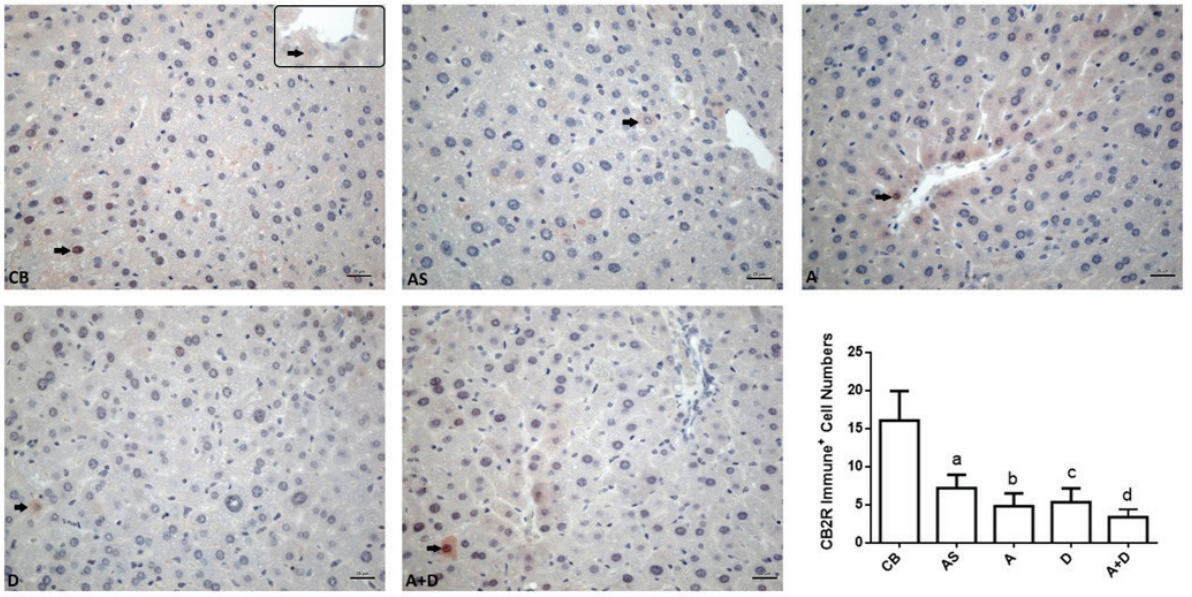

Figure 3. Immune+ cells (arrow) and the number of immune+ cells for cannabinoid 2 receptor (CB2R) in the liver of Citrate buffer (CB), the solvents of antioxidants (AS), antioxidants (A), STZ-induced diabetes (D), and Antioxidants+ Diabetes (A+D) groups. Streptavidin-biotin-peroxidase technique, counterstain hematoxylin. Scale bar = 20 µm. [a]P<0.05 versus CB group, [b]P<0.05 versus CB group, [c]P<0.05 versus CB group, [d]P<0.05 versus CB group.

Table 1. Liver tissue xanthine oxidase (XO) activity, glutathione (GSH) and lipid peroxidation (LPO) levels of all groups.

Group	XO (U/g protein)*	GSH (nmol GSH/mg protein)*	LPO (mmol MDA/mg protein)*
Citrate Buffer (CB)	0.45 ± 0.08	10.60 ± 3.05	4.03 ± 1.56
Antioxidants (A)	0.32 ± 0.13	10.03 ± 1.18	3.70 ± 1.32
Solvents of antioxidants (AS)	0.26 ± 0.04	11.58 ± 1.83	4.92 ± 0.85
STZ-induced diabetes (D)	0.87 ± 0.31[a]	4.49 ± 1.73[c]	8.05 ± 1.55[e]
Antioxidants+Diabetes (A+D)	0.22 ± 0.06[b]	12.53 ± 1.60[d]	2.80 ± 0.61[d]
P_{Anova}	0.0001	0.0001	0.0001

*Mean ± SD; [a]p<0.05 versus CB group; [b]p<0.005 versus D group; [c]p<0.005 versus CB group; [d]p<0.0001 versus D group; [e]p<0.01 versus CB group

Table 2. Liver tissue superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) activities of all groups.

Group	SOD (U/mg protein)*	CAT (U/mg protein)*	GR (U/g protein)*
Citrate Buffer (CB)	14.35 ± 5.71	76.92 ± 12.38	643.28 ± 78.12
Antioxidants (A)	13.39 ± 2.15	43.94 ± 14.65	374.20 ± 56.69
Solvents of antioxidants (AS)	15.24 ± 3.14	46.17 ± 14.32	576.62 ± 28.99
STZ-induced diabetes (D)	4.97 ± 1.94[a]	28.99 ± 3.15[c]	174.62 ± 45.86[c]
Antioxidants+Diabetes (A+D)	8.17 ± 1.40[b]	32.21 ± 10.40[b]	343.97 ± 88.17[d]
P_{Anova}	0.0001	0.0001	0.0001

*Mean ± SD; [a]p<0.05 versus CB group; [b]p<0.05 versus D group; [c]p<0.0001 versus CB group; [d]p<0.01 versus D group

Table 3. Liver tissue glutathione peroxidase (GPx), glutathione-S-transferase (GST) and γ-glutamil transferase (GGT) activities of all groups.

Group	GPx (U/mg protein)*	GST (U/g protein)*	GGT (U/g protein)*
Citrate Buffer (CB)	144.75 ± 32.72	274.09 ± 37.48	11.13 ± 2.33
Antioxidants (A)	135.49 ± 20.80	251.61 ± 30.12	9.10 ± 1.81
Solvents of antioxidants (AS)	159.94 ± 22.04	300.69 ± 35.57	13.40 ± 1.64
STZ-induced diabetes (D)	74.81 ± 10.41[a]	210.95 ± 19.87[a]	30.02 ± 7.88[d]
Antioxidants+Diabetes (A+D)	97.59 ±12.37[b]	256.70 ± 25.46[c]	5.54 ± 1.68[e]
P_{Anova}	0.0001	0.0001	0.0001

*Mean ± SD; [a]p<0.005 versus CB group; [b]p<0.05 versus D group; [c]p<0.005 versus D group; [d]p<0.01 versus CB group; [e]p<0.0001 versus D group

Table 4. Liver tissue paraoxonase (PON), glucose-6-phosphate dehydrogenase (G-6-PD) activites and protein carbonyl content (PCC) and advanced oxidation protein product (AOPP) levels of all groups.

Group	PON (U/g protein)*	G-6-PD (U/g protein)*	PCC (nmol PCC/mg protein)*	AOPP (nmol/g protein)*
Citrate Buffer (CB)	11.24 ± 2.08	13.50 ± 4.55	4.26 ± 1.26	2.09 ± 0.95
Antioxidants (A)	11.31 ± 3.55	14.21 ± 3.57	4.06 ± 1.38	1.60 ± 0.48
Solvents of antioxidants (AS)	6.17 ± 0.98	15.62 ± 2.32	5.22 ± 0.79	2.06 ± 1.40
STZ-induced diabetes (D)	5.84 ± 0.38[a]	4.62 ± 1.72[a]	9.61 ± 1.30[d]	3.85 ± 0.75[e]
Antioxidants+Diabetes (A+D)	11.54 ± 4.08[b]	12.11 ± 2.27[c]	3.39 ± 1.24[c]	1.84 ± 1.10[b]
P_{Anova}	0.02	0.0001	0.0001	0.05

*Mean ± SD; [a]p<0.0001 versus CB group; [b]p<0.05 versus D group; [c]p<0.0001 versus D group; [d]p<0.001 versus CB group; [e]p<0.05 versus CB group

Table 5. Liver tissue sialic acid, fucose, hexose and OH proline levels of all groups.

Group	Sialic Acid (μmol sialic acid/g protein)*	Fucose (μg fucose/mg protein)*	Hexose (μg glucose/mg protein)*	OH-Proline (μg OH-Proline/ g protein)*
Citrate Buffer (CB)	23.95 ± 6.03	4.74 ± 0.74	12.43 ± 2.44	211.47 ± 32.18
Antioxidants (A)	21.34 ± 6.19	5.53 ± 1.25	12.60 ± 1.54	182.77 ± 54.32
Solvents of antioxidants (AS)	26.84 ± 7.24	6.02 ± 2.99	18.11 ± 2.43	233.62 ± 47.88
STZ-induced diabetes (D)	42.89 ± 5.83[a]	9.12 ± 2.76[c]	21.90 ± 5.86[c]	400.72 ± 62.10[a]
Antioxidants+Diabetes (A+D)	21.82 ± 3.85[b]	4.81 ± 1.51[d]	11.56 ± 1.74[d]	227.99 ± 35.43[e]
P_{Anova}	0.001	0.05	0.002	0.0001

*Mean ± SD; [a]p<0.01 versus CB group; [b]p<0.0001 versus D group; [c]p<0.05 versus CB group; [d]p<0.05 versus D group; [e]p<0.001 versus D group

In the A+D group which was given STZ, glycoprotein components (sialic acid, fucose, and hexose) and OH-proline levels were significantly decreased after the administration of the antioxidants (p<0.0001; p<0.05; p<0.05, p<0.001 respectively).

DISCUSSION

Diabetes mellitus is an important disease and affects many people all around the world annually and is characterized by hyperglycemia development. Fasting blood glucose levels increased significantly in diabetes and were partially prevented with administered ALA + Se + Vit E combination (Karatug & Bolkent 2013). Long-term administration of ALA to diabetic rats has been reported to cause low blood glucose levels in the literature (Lateef et al. 2005; Kojima et al. 2007). Vit E and Se acting in critical metabolic roles are essential substances for humans. As a result of the literature review, the application of Vit E and Se to diabetic rats had different effects on the blood glucose levels. While some studies show that administration of Vit E and Se has no significant effect on blood glucose level (Mukherjee et al. 1998; Gocmen et al. 2000; Barbosa et al. 2008), some studies show that blood glucose level decreases significantly (Shirpoor, Ansari, Salami, Pakdel, & Rasmi, 2007; Karatug & Bolkent 2013).

The liver is an important organ for the metabolism of carbohydrates, protein, and fat. It works to make fewer toxic or harmless foreign substances and molecules such as drugs (Ozougwu 2017). Free radicals cause oxidative stress in diabetes-induced liver damage (Zhou et al. 2015; Asmat, Abad & Ismail, 2016; Masarone et al. 2018). In some studies, pyknotic nuclei and necrotic areas were shown in the liver tissue of diabetic rats, which were created by the administration of STZ, similar to our study (Al-Attar & Alsalmi 2019; Faddladdeen 2021). STZ shows toxic effects in pancreatic beta cells and causes hyperglycemia. In this case, oxidative stress increases with the decrease of the antioxidant defense system (Maritim, Sanders, & Watkins, 2003; Papaccio, Pisanti, Latronico, Ammendola, & Galdieri, 2000). In the present study, the occurrence of these negativities, which increased in the liver due to the diabetic condition, was prevented by the combined antioxidants given to mice before diabetes. In previous studies, the effect of these triple combined antioxidants was unknown. In this context, the effects of these combined antioxidants on the liver are shown for the first time in terms of diabetic status.

In one study, the levels of cannabinoid receptors expressions in different rat tissues, CB1R and CB2R mRNA levels showed very high expression in the liver as compared to peripheral organs. The expression of CB1R and CB2R mRNA levels depends on diurnal variations. Furthermore, the researchers suggested that the deficiency of insulin due to increased glucose concentration in the blood may cause a loss of CB1R and CB2R in the liver of STZ-induced rats with daily fluctuations (Bazwinsky-Wutschke, Zipprich, & Dehghani, 2017). Moura et al. (2019) reported that in forebrain glucose metabolism, the reduced or absent CB1R expression is sufficient to mimic hyperglycemia-induced impairment and/or insulinopenia. According to the study by Sena, Cipriano, Botelho, & Seiça, (2018) on diabetes, ALA prevents hepatic steatosis by decreasing inflammation and oxidative stress. Similarly, Daniel, Adeoye, Ojowu, & Olorunsogo, (2018) suggested that Vit E can lead to the protection of hepatic tissue from diabetes-induced oxidative stress. The findings of Jacobs et al. (2019) did not support the effect of Se supplementation on insulin action and/or secretion in diabetes. In contrast, a population study exhibited a positive association between Se level and diabetes (Moon et al. 2019). Collectively, our immunohistochemistry findings showed that the number of CB2R immune^{+} cells significantly decreased in the diabetic liver, whereas CB1R did not. The treatment of the three antioxidant agents ALA, Se, and Vit E had no effect on either the CB1R or the CB2R in the diabetic liver. Salinthone et al., (2011) suggested that lipoic acid activates some G-protein-coupled receptors but not others. According to our findings, the combination of ALA, Se, and Vit E did not behave as a ligand for the cannabinoid receptors, one of the G-protein-coupled receptors in liver tissue.

ALA modulates the redox potential since ALA has the ability to match the redox status between different subcellular compartments as well as extracellularly. ALA is a potential antioxidant for humans (Rochette et al. 2013). Se and Vit E are also potential antioxidants for animals (Liebler 1993; Tinggi 2008).

Due to the antioxidant properties of all these substances, they can play a crucial role in the prevention of oxidative stress caused by diabetes. Several clinical and experimental studies have identified a relationship between diabetes, hyperglycemia, and oxidative stress. Also, oxidative stress is characterized by a change in the enzymatic or non-enzymatic antioxidative system and/or increased LPO levels. These systems prevent oxidative stress and reduce the concentration of ROS (Kanbagli, Balkan, Aykac Toker, & Uysal, 2002). GSH is a non-enzymatic antioxidant and prevents the harmful effects of free radicals. Diabetes studies showed that GSH level is generally low value in diabetic humans and animals (Gezginci-Oktayoglu, Sacan, Yanardag, Karatug, & Bolkent, 2011). In this study, the GSH level was reduced in the diabetic group and the administration of ALA, Se, and Vit E was reversed back to normal levels when compared to the control group. LPO is an important marker associated with oxidative stress. Due to the increase in free radical amount, polyunsaturated fatty acids in the cell membrane react with free radicals and as a result of this reaction, the amount of LPO increases in diabetic groups (Girotti, 1985). In our study, the LPO value increased in diabetic rats and treatment with the combination of ALA, Se, and Vit E decreased the LPO value. XO is the key enzyme in purine metabolism and can be an important biological source of ROS. For that reason, XO is an important marker for oxidative stress, and it has been shown that the activity of XO is increased experimental diabetes (Matsumoto, Koshiishi, Inoguchi, Nawata, & Utsumi, 2003). Increased XO activity means there is oxidative stress in the liver due to diabetes. The application of the antioxidant combination before the formation of diabetes protected the liver significantly against XO-derived ROS formation.

Free radicals may play an important role in the complications and causation of diabetes mellitus. Free radical production levels and oxidative stress increase in diabetic patients and animals. For that reason, antioxidant systems have a very critical role in organisms. CAT, SOD, GR, GPX, GST, and endogenous antioxidant enzymes in organisms, play a key role in reducing the harmful effects of reactive oxygen species and free radicals (Harini & Pugalendi 2010; Sacan et al. 2016). In this study, CAT, SOD, GR, GPX, and GST antioxidant enzymes activities were decreased in diabetic rats and the results were reversed when antioxidants were used. The results show that the antioxidant combination may have helped increase the activity of antioxidant enzymes by reducing free radical formation. The combination of ALA, Se, and Vit E might be a scavenger for free radicals and might reduce the activities of endogenous antioxidant enzymes in that way. GGT is a marker for oxidative stress, and it contributes to the extracellular catabolism of glutathione (Whitfield 2001). Higher GGT levels are associated with diabetes (Sabanayagam, Shankar, Li, Pollard, & Ducatman, 2009). GGT levels increased significantly in diabetic rats in this study. The enzyme level decreased with the administration of a combination of antioxidants.

The activity of the PON enzyme has been linked to many diseases such as inflammation, stroke, myocardial infarction, diabetes, and Alzheimer's. Decreased PON activity is responsible for inflammation in patients who have cancer and diabetes (Camps,

Marsillach, & Joven, 2009). The study has shown that PON1 activity is reduced in Type 1 and Type 2 diabetic patients (Durrington, Mackness, & Mackness, 2001). In our study, PON activity was decreased in the diabetic group and this activity might be associated with hyperglycemia and oxidative stress (Nair, Shah, Taggarsi, & Nayak, 2011; Jamuna Rani, Mythili, & Nagarajan, 2014). The first enzyme of the pentose phosphate pathway is G6PD. It provides NADPH production. NADPH levels impact the entire antioxidant system and make tissues very vulnerable to oxidative damage. In our study, PON and G6PD activities were found significantly decreased in diabetic rats and were reversed back when a combination of antioxidants was administered. Oxidized amino acid residues in diabetes cause protein damage and PCC levels are used as a biomarker for this protein damage. Several studies showed that PCC levels increased in the diabetic group when compared to the control group (Dayanand, Kumar Vegi, & Kutty, 2012). AOPP is a marker that shows oxidative stress-based protein damage. Some researchers have reported that there is a relationship between ATP levels and diabetes (Baskol, Gumus, Oner, Arda, & Karakucuk, 2008). Treatment of diabetic rats with the antioxidant combination reversed increased PCC and AOPP levels in diabetic liver tissues. As a result, it can be concluded that the use of combined antioxidants before diabetes occurs is very effective in protecting liver tissue from any damage that might occur and combined antioxidants can be used as a hypoglycemic agent.

Glycoproteins are the main structures of the matrix, and they have many functions as hormones and enzymes. The metabolism of glycoproteins is very critical in diabetes. The amount of glycoprotein increases in high blood glucose levels. The carbonyl groups of amino acids react slowly with glucose and form Schiff-base. In this study, we observed increased levels of sialic acid, fucose, and hexose in the liver tissue of STZ-induced hyperglycemic rats when compared to the control group. Collagen is one of the proteins which contains hydroxyproline. Some authors consider hydroxyproline as a marker of collagen content. Hydroxyproline levels increased in the liver tissues of diabetic rats. This indicates that diabetes has a negative effect on liver collagen. Antioxidant administration reverses this increase showing the restoration of collagen damage (Wangoo et al. 2000).

CONCLUSION

Triple antioxidant treatment reversed the negative effects of diabetes, both morphologically and biochemically. In contrast, the combined treatment of antioxidants does not affect the regulation of cannabinoid receptors expressions.

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Original Article

Quantification of MMP-2 and TIMP-1 expressions in breast cancer

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ABSTRACT

Background and Aims: *MMP-2* and *TIMP-1* are vital molecules in the remodeling of the extracellular matrix, and they have a critical role in the metastatic process of breast cancer. This study aimed to detect expression levels of *MMP-2* and *TIMP-1* genes by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) and identify their potential roles in breast cancer prognosis.
Methods: *MMP-2* and *TIMP-1* gene expression levels in 17 breast cancer tumor tissues and normal breast tissue were examined. The expression levels of *MMP-2* and *TIMP-1* were analyzed by qRT-PCR. The association between the expression levels of *MMP-2* and *TIMP-1* and clinicopathological manifestations of breast cancer was examined.
Results: Lower gene expression levels of *MMP-2* and *TIMP-1* were detected in tumors compared to the controls. A statistical correlation was not observed between the expression level of *MMP-2*, *TIMP-1*, and clinicopathological parameters (tumor grade, lymph node involvement, hormone receptor status).

Conclusion: Our findings have been suggesting that expression profiles of *MMP-2* and *TIMP-1* might be independent prognostic and predictive biomarkers for breast cancer. These biomarkers are candidate molecules for personalized therapy. *MMP-2* and *TIMP-1* expression patterns will also aid in the identification of more precise and targeted subgroups of breast cancer.

Keywords: Breast cancer, *MMP2* metalloproteinase, *TIMP-1*

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INTRODUCTION

Breast cancer (BC) is the most frequent cancer adversely affecting women. According to GLOBOCAN, It accounts for 24.9% of cancer cases and is the first cause of cancer-related deaths among women (Ferlay et al., 2019).

The main clinical features for predicting the BC prognosis are age, primary tumor size, histological grade of tumor, distant metastasis, lymph node involvement, ER, and PR status (Ünçel et al., 2015). Despite the development of early detection techniques and advancements in therapy, metastasis from BC continues to be a major cause of mortality and morbidity (Ünçel et al., 2015). One of the key molecules in metastasis is extracellular matrix (ECM) elements which act as a primary set to block the accumulation of tumor cells. Degradation of the basement membrane enhances the metastatic process. For tumor cells to invade and metastasize, this barrier should be digested via MMPs (Matrix metalloproteinases) (Öncel, 2012).

MMP-2 is a member of the MMP family, and it can degrade type IV collagen, a component of basement membranes (Yadav et al., 2014). *MMP-2* facilitates tumor invasion and metastasis by digesting the basement membrane, which separates tumors from surrounding tissue. (Guo, Wu, Hathaway & Hartley, 2012). *MMP-2* has been associated with a variety of cancers, including breast, colon, skin, and lung cancers. Its expression has also been linked to tumor invasion, lymph node metastasis, and survival rates. One of the most effective BC prognostic indicators is *MMP-2* (Jezierska & Motyl, 2009).

Inactive MMPs are activated by proteolytic cleavage and are inhibited specifically by metalloproteinases (TIMP) tissue inhibitors. Up-to-date, four different TIMPs are described: TIMPs 1, 2, 3, and 4 (Arpino, Brck &, Jill, 2015). *TIMP-1* is a natural inhibitor of the *MMP-9*, which also plays an essential role in both normal physiological processes and carcinogenesis (Würtz, Schrohl, Mouridsen&Brünner, 2008) it, has a cancer-promoting effect via stimulating growth and inhibiting apoptosis. High *TIMP-1* expression has been associated with a poor prognosis in several cancers. *TIMP-1* has been suggested as a prognostic and predictive biomarker for BC (Würtz, Schrohl, Mouridsen&Brünner, 2008).

The association between MMP2 and BC's tumor growth, invasion, and metastasis has been corroborated (Guo, Wu, Hathaway & Hartley, 2012). *TIMP-1*, the inhibitor of *MMP-9*, has been extensively studied as a potential biomarker in BC. Overexpression of *TIMP-1* is associated with aggressive tumor behavior in many cancer types and breast cancer (Mahmood, Fakhoury, Yaseen & Moustafa, 2015).

Different techniques can be used to explore MMPs and their inhibitors at the transcriptional and protein levels. Gelatin zymography, ELISA, immunohistochemistry, in situ hybridization, and qRT-PCR is the most frequently used techniques in research (Hadler-Olsen, Winberg, &Uhlin-Hansen, 2013).

MMP-2 and *TIMP-1* were found to be upregulated according to the pooled microarray data (unpublished) from another study of our group by Cine et al. (Cine et al, 2014)

In this study, we analyzed the expression levels of *MMP-2* and *TIMP-1* by qRT-PCR in the same primary BC and adjacent non-tumor samples individually. We aimed to observe the potential biomarker role of *MMP-2* and *TIMP-1* for the prognosis of BC.

MATERIALS AND METHODS

Tissue collection

Seventeen samples from malign tumors and normal breast tissues were collected from patients who underwent surgery during diagnosis for BC at the Department of General Surgery, Kocaeli University, 2009-2010. This study was approved by the Kocaeli University Ethics Committee. (Approval no: 2008/76 IAEK 11/9.). Clinicopathological features of patients were retrieved from medical reports.

Total RNA isolation

Frozen tissue samples were divided for RNA isolation. Total RNA was extracted from tissue samples using the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) and treated with DNase I (Qiagen, Hilden, Germany), according to the manufacturer's instructions. A260/280 ratios were measured to detect the purity of samples. The quality of RNA was confirmed by RNA LabChip (Agilent Technologies, Waldbronn, Germany) and analyzed by Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). RNA integrity value of ≥6.10 was considered acceptable.

cDNA synthesis and qRT-PCR

Complementary DNA (cDNA) synthesis was performed with a commercial cDNA synthesis kit (Transcriptor First Strand cDNA synthesis kit, Mannheim, Germany, Roche). qRT-PCR (Syber Green, Mannheim, Germany, and Roche) experiment was applied as described previously to measure *MMP-2* and *TIMP-1* gene expression (Savlı, Aalto, Nagy, Knuutila & Pakkala, 2002; Savlı et al., 2003). Beta-actin was used as a housekeeping gene for normalizing gene expression values. Sequences of *MMP-2*, *TIMP-1*, and Beta-actin primers (Integrated DNA Technologies, Illinois, and USA) are shown in Table 1. Gene expression ratios were compared in tumor and non-tumor tissue relative expression software tools (REST ©, 2009, Qiagen, Hilden, Germany).

Statistical analysis

All data analysis was performed using GraphPad Prism version 7.3(GraphPad Software Inc., San Diego, CA). Statistical differences of at least $p<0.05$ were accepted as statistically significant.

Table 1. The sequences of primers used for RT-PCR.

GENE	PRIMER SEQUENCE
TIMP-1	
	(F) 5′ – CTT CTG GCA TCC TGT TGT TC- 3′
	(R) 5′ –AGA AGG CGG TCT GTG GGT – 3′
MMP-2	
	(F) 5′- CGC TCA GAT CCG TGG TGA G- 3′
	(R) 5′- TGT CAC GTG GCG TCA CAG T- 3′
BETA-ACTIN	
	(F) 5′-TGA CTT TGT CAC AGC CCA AGA- 3′
	(R) 5′-AAT CCA AAT GCG GCA TCT TC- 3′

The association between the expression of *MMP-2*, *TIMP-1*, and clinicopathological features was analyzed with the One-Way ANOVAs variance test.

RESULTS

Seventeen patients were aged from 38 to 73 years, with a mean of 52 years old. Of the 17 BC, 9 (52%) were classified as infiltrative ductal carcinoma, 5 (29%) were invasive ductal carcinoma, 1 (%5.8) was invasive micropapillary carcinoma, and 1 (%5.8) was ductal carcinoma in situ. One tumor sample remained unclassified. The clinical and pathological features of the patient group are listed in Table 2.

MMP-2 expression levels and clinical significance

We observed decreased *MMP-2* expression levels more frequently in tumor tissues than in non-tumor tissues (10/17). Relative expression values of all samples are shown in Figure 1. The relative expression levels of *MMP-2* with clinicopathologic parameters were compared and calculated p values were greater than 0, 05 for all parameters and are shown in Figure 2. Our study showed no statistical correlation between tumor grade, lymph node involvement, and hormone receptor status.

MMP-2 levels were compared within molecular subtypes, and Figure 4 shows the relative *MMP-2* levels of each molecular grade.

TIMP-1 expression levels and clinical significance

We observed decreased *TIMP-1* expression levels more frequently in tumor tissues than in non-tumor tissues (12/17). Relative expression values for all samples are shown in Figure 1. The relative expression levels of *TIMP-1* with clinicopathologic parameters were compared. Calculated p values were greater than 0, 05 for all parameters and are shown in Figure 3.

There was no statistically significant correlation found between the main clinical parameters and *TIMP-1* levels. *TIMP-1* levels were compared within molecular subtypes, and Figure 4 shows the relative *TIMP-1* levels of each molecular grade.

DISCUSSION

This study focused on detecting expression levels of *MMP-2* and *TIMP-1* in BC patients individually using qRT-PCR in breast

Table 2. *MMP-2* and *TIMP-1* expression levels and patients' clinicopathological data.

Pathological Data	Number of Patients	*MMP-2* Expression		*TIMP-1* Expression	
		Low	High	Low	High
Age					
≤50	9	4 (%44)	5 (%56)	5 (%56)	4 (%44)
≥50	8	5 (%62,5)	3 (37,5)	6 (%75)	2 (%25)
Histological Grade					
Grade 1	3	3 (%100)	-	2 (%66)	1 (%33)
Grade 2	3	1 (%33)	3 (%66)	2 (%66)	1 (%33)
Grade 3	7	4 (%57)	3 (%43)	5 (%71)	2 (%29)
No Information	4	1 (%25)	3 (%75)	4 (%100)	-
Lymph Node Metastasis					
Negative	7	5 (%71)	2 (%29)	4 (%57)	3 (%43)
Positive	10	4 (%40)	6 (%60)	8 (%80)	2 (%20)
ER Status					
Negative	7	3 (%43)	4 (%57)	6 (%84)	1 (%16)
Positive	10	6 (%60)	4 (%40)	6 (%60)	4 (%40)
PR Status					
Negative	11	5 (%45)	6 (%55)	8 (%72)	3 (%28)
Positive	6	4 (%66)	2 (%34)	2 (%34)	4 (%66)
CerbB2 Status					
Negative	8	4 (%50)	4 (%50)	4 (%50)	4 (%50)
Positive	9	5 (%56)	4 (%44)	8 (%87,5)	1 (%12,5)
Molecular Subtypes					
Luminal A	5	3 (%60)	2 (%40)	2 (%40)	3 (%60)
Luminal B	4	1 (%33)	2 (%66)	3 (%100)	-
Triple Negative	3	2 (%66)	1 (%33)	2 (%66)	1 (%33)
Her2/Neu	4	1 (%25)	3 (%75)	3 (%75)	1 (%25)

cancer and aimed to see if *MMP-2* and *TIMP-1* are prognostic biomarkers of BC.

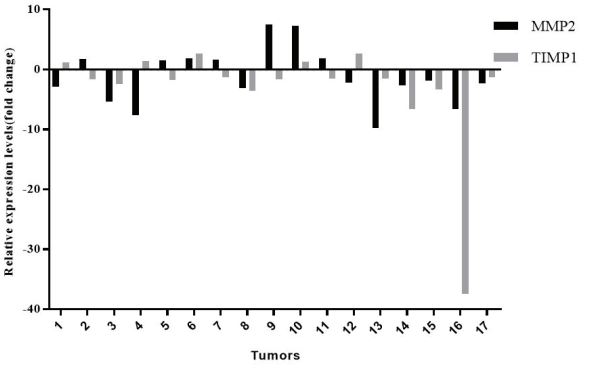

Figure 1. Column graphs demonstrate relative expression trends of *MMP-2* and TIMP-1 of each tumor.

In a systematic meta-analysis study by Chen et al., a considerable number of studies reported high expression levels of *MMP-2* in breast cancer tumors (Chen, Wang, Chen, Dong & Zhang, 2015). A study of qRT-PCR expression analyses revealed that *MMP-2* levels were significantly higher in breast cancer stages II-III than in benign breast tumor tissues (Mahmood et al., 2015). Figuera et al. observed high levels of *MMP-2* in tumor samples according to adjacent non-tumor tissue (Figueira et al., 2009). However, in our study, the mean expression levels of tumors were lower than controls.

Studies focused on the overexpression of *MMP-2* correlation with prognostic factors reported different association statuses with clinicopathological parameters. Chen et al. concluded in a systematic meta-analysis study that *MMP-2* overexpression was associated with lymph node metastasis and poor survival (Chen et al., 2015). Mahmood et al. showed that *MMP-2* expression levels were correlated with tumor grade, tumor stage,

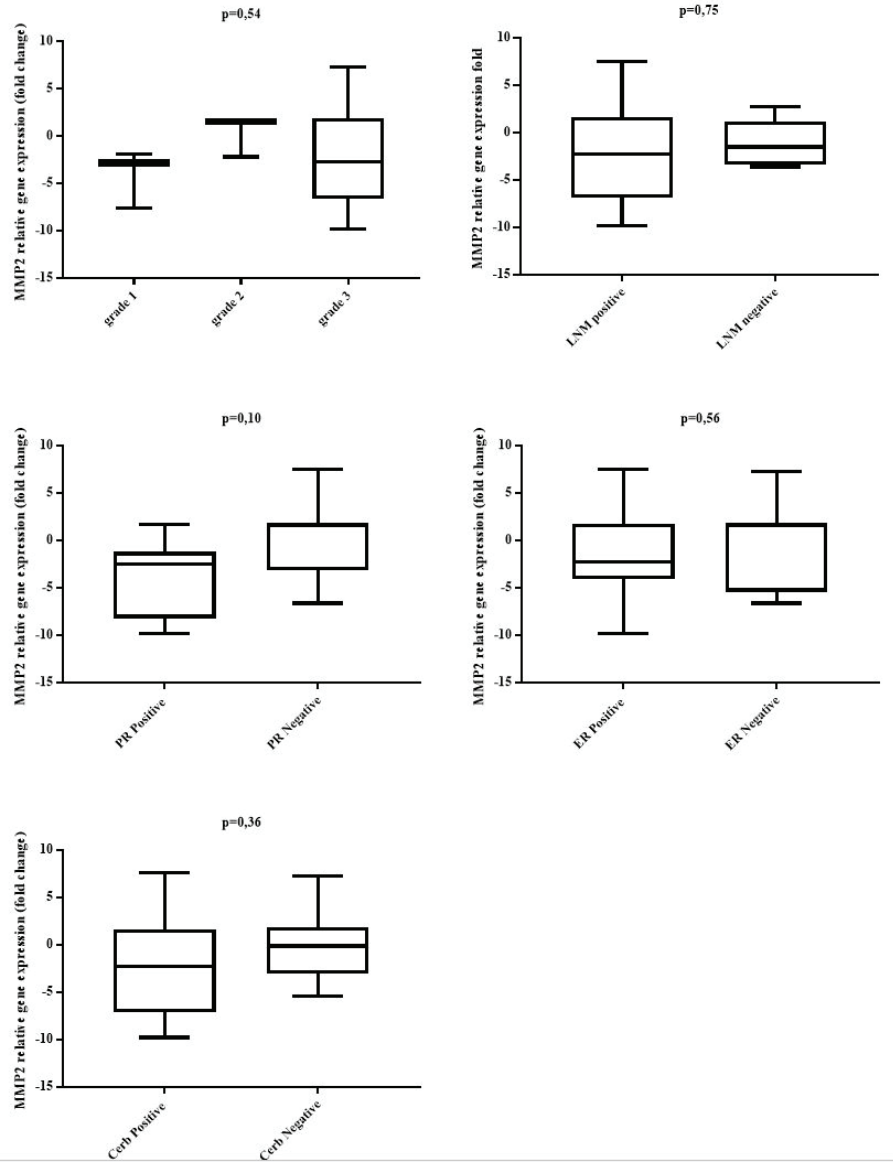

Figure 2. Box plots showing relative *MMP-2* expression trends and the clinicopathological parameters.

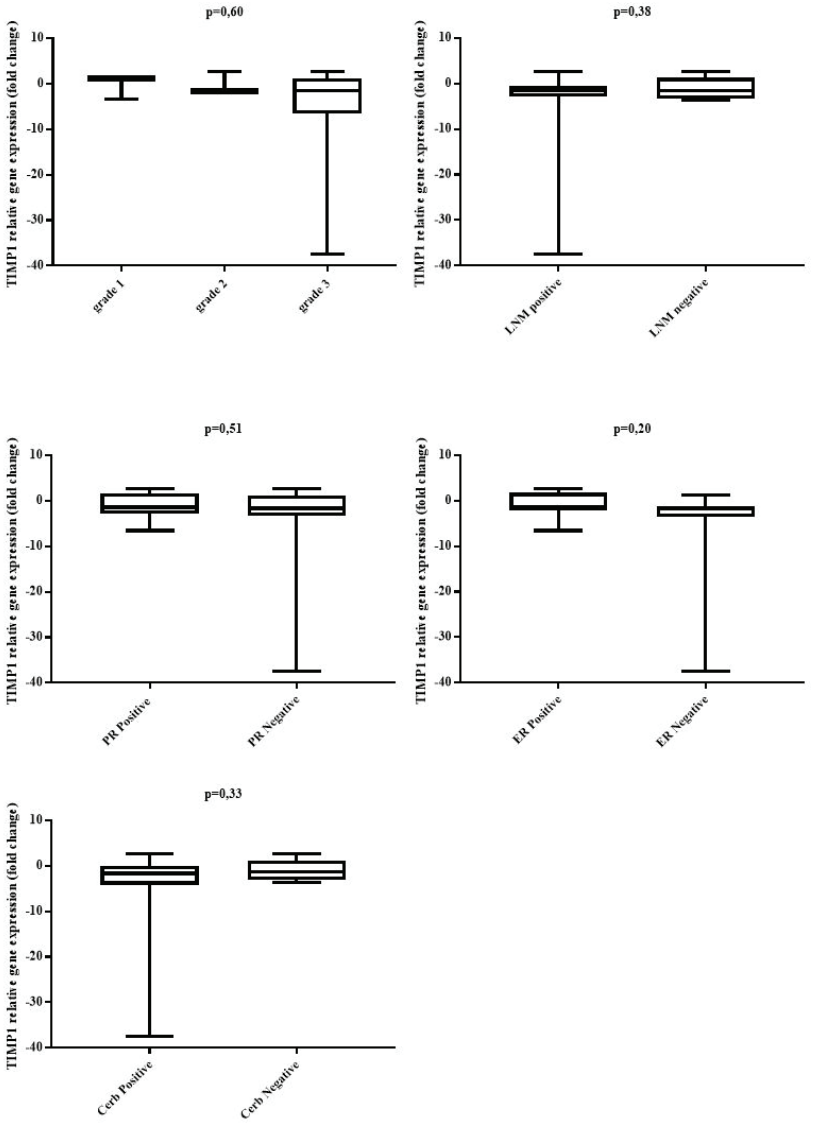

Figure 3. Box plots showing relative *TIMP-1* expression trends and the clinicopathological parameters.

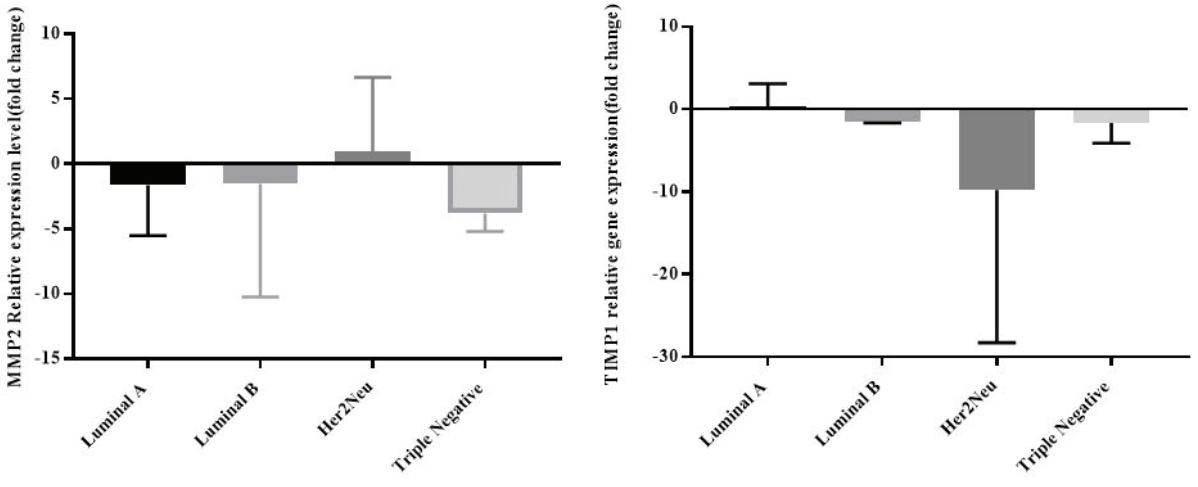

Figure 4. Box plots showing relative *MMP-2* and *TIMP-1* levels in each molecular grade. The line within the box plot represents the median value, and the lines extending from the box indicate the maximum and minimum expression levels.

and lymph node metastasis (Mahmood et al., 2015). Huang et al. measured serum *MMP-2* levels and reported a correlation between *MMP-2* and lymph node metastasis and higher TNM stage (Huang et al., 2014). Our study showed no statistical correlation between tumor grade, lymph node involvement, and hormone receptor status. Similar to our results, no correlation between *MMP-2* level and tumor grade, lymph node involvement, and ER/PR/Cerb-B2 status (Decock et al., 2005). These controversial reported results suggest that MMPs may provide independent prognostic foresight for BC progression.

MMP-2 has been extensively considered as a predictive biomarker for metastasis in BC. Both at the transcriptional level and protein level, most of the studies have reported an association between increased levels of *MMP-2* and lymph node metastasis (Huang et al., 2014). Similarly, to these reports, we found that the lymph node-positive group had higher *MMP-2* levels than the negative group. Daniele et al. looked at *MMP-2* expression in sentinel lymph nodes and serum in patients with metastatic and non-metastatic breast cancer. (Daniele et al., 2016). Their results showed that *MMP-2* was significantly decreased in the non-metastatic and control group compared to the metastatic group. These results emphasize the involvement of *MMP-2* in the metastatic process.

Despite the mean expression value of *MMP-2* being higher in the lymph node-positive group, a low level of *MMP-2* was observed in 4 patients.

An enzyme-linked immunosorbent assay (ELISA) study in lymph node-positive patients was performed by Leppaet al. (Leppa, Saarto, Vehmanen, Blomqvist&Eloma, 2004). They measured postoperative serum levels of *MMP-2* and followed five-year survival rates. They observed better overall survival (OS) and disease-free survival (DFS) rates in patients with low *MMP-2* levels; additionally, the frequency of bone and visceral metastasis was lower in the low *MMP-2* group than in the high *MMP-2* group. Their results suggested that the *MMP-2* level may be a predictive factor for DFS and OS and also may aid in dividing the node-positive group into two subgroups low risk and high risk. Thus, additional subgroups may be useful in identifying patients with a potentially favorable prognosis who could avoid toxic therapies. Although the negativity of lymph node involvement is a favorable prognosis factor in BC, some still suffer from metastasis. New prognostic biomarkers or subgroups are needed to overcome this dilemma. *MMP-2* has been considered a candidate prognostic biomarker and is associated with a favorable prognosis in node-positive BC (Daniele et al., 2016). Hirvonen et al. evaluated *MMP-2* expression by immunohistochemistry (IHC) staining in node-negative BC patients, and postoperative survival rates analysis was performed (Hirvonen, Talvensaari-Mattila, Pääkkö, &Turpeenniemi-Hujanen, 2003) Obtained data underlined potential correlation to MMP2 negativity and favorable prognosis in node-negative BC. Our sample group included seven lymph node-negative tumors, and 5 showed a low level of *MMP-2*. According to the study, these five patients should have better disease progression or relatively long survival than patients with high *MMP-2* levels. In the study of Lu et al., *MMP-2* expression levels were analyzed in primary tumors with metastasized lymph node samples by qRT-PCR in patients suffering invasive ductal BC (Lu, Chen, Ding, Li K, & Wu, 2012). The expression level of *MMP-2* in metastasized lymph nodes was higher compared with their primary tumors. These findings suggest that determining the level of *MMP-2* expression in metastatic tissue may help to clarify the role of *MMP-2* in lymph node metastasis. In all groups, the mean *TIMP-1* level was lower in tumors than in control tissues. Similar to our study, Figuera et al. found lower *TIMP-1* gene expression levels in tumors than in adjacent non-tumor tissues (Figuera et al., 2009)

Contrary to our study, Kousidou et al. and Zhang et al. reported higher expression levels of *TIMP-1* in BC tumors (Kousidou, Roussidis, Theocharis, &Karamanos, 2004; Zhang et al., 2013)

The correlation between main clinical parameters and *TIMP-1* levels was examined in this study, and no statistically significant correlation was discovered. Some authors reported an association between *TIMP-1* level and several clinicopathological parameters (Lipton et al., 2008; Sieuwerts et al., 2007, Figuera et al., 2009, Nakopoulou et al., 2002, Abdollahi et al., 2019). Lipton et al. showed the correlation between serum *TIMP-1* level and Cerb-B2 status, liver metastasis, and soft tissue metastasis (Lipton et al., 2008; Sieuwerts et al., 2007) found the relationship between tissue *TIMP-1* mRNA level and tumor size, lymph node involvement, tumor grade, age, ER/PR. Figuera et al. reported a significant correlation between *TIMP-1* and only PR status (Figuera et al., 2009). Nakopoulou et al. reported that high *TIMP-1* mRNA expression levels were associated with lymph node metastases and increased c-erbB-2 expression (Nakopoulou et al., 2002). Abdollahi et al. found *TIMP-1* gene expression levels in patients with lymph node metastasis and without metastasis using RT-PCR. They also emphasized the significant role of upregulated *TIMP-1* in lymph node involvement. (Abdollahi et al., 2019)

TIMP-1 levels in different samples, including tumor and serum, have been previously linked to a poorer outcome in BC at both transcriptional and protein levels (Nakopoulou et al., 2002; Würtz et al., 2008). In 2003, in contrast to those reports, they reported that an increased level of *TIMP-1* may be a sign of a favorable prognosis in BC (Nakopoulou et al., 2003).

Several publications investigated the prognostic value of *TIMP-1* in BC and showed a correlation between recurrence-free survival (RFS) (Dechaphunkul et al., 2012; Wu et al., 2008), DFS (Nakopoulou et al., 2003), OS (Nakopoulou et al., 2003; Dechaphunkul et al., 2012; Wu et al., 2008). In our study, increased *TIMP-1* expression was also observed in grade 1, lymph node-negative, and ER/PR/Cerb-B2 positive tumors, which were expected to present a better prognosis. Positivity of hormone receptors (ER/PR/Cerb-B2), low grade of the tumor (grade 1), and absence of lymph node involvement are considered favorable prognostic factors in BC. Patients with low risk are given less aggressive treatments. Talvensaari et al. conducted a prospective study on *TIMP-1* levels in serum using ELISA before surgery in primary node-negative patients (Talvensaari-Mattila &Turpeenniemi-Hujanen, 2005). Results suggested that cases with preoperative low TIMP-1 levels had longer RFS time than patients with high *TIMP-1* levels. Additionally, they found that

preoperative high serum *TIMP-1* elevated the risk of recurrence in primary node-negative breast carcinoma. In another study, Dechaphunkul et al. *TIMP-1* level was analyzed by IHC in early-stage primary BC patients treated with standard adjuvant therapy (Dechaphunkul et al., 2012). Increased *TIMP-1* levels in early-stage tumors were linked to early recurrence and short survival, according to their findings. These findings suggest that new independent prognostic factors for low-risk BC should be used to identify patients who require more aggressive treatment. The coexistence of high histological grades (grades 2 and 3) and lymph node involvement is considered a sign of high risk and poor prognosis, and more aggressive treatments are applied to patients with high risk.

To reduce the side effects of toxic chemotherapeutics, further effective prognostic biomarkers for the high-risk group are needed. Paula Kuvajaa and coworkers performed IHC staining of *TIMP-1* in node-positive and high-grade tumors (Kuvaja, Talvensaari-Mattila, Pääkkö, &Turpeenniemi-Hujanen, 2005). They discovered that the *TIMP-1* negative group had better disease-specific survival than the *TIMP-1* positive group. They hypothesized that lack of *TIMP-1* is associated with a better prognosis in patients with breast cancer due to *TIMP-1's* pro-apoptotic function. Our group examined low-level *TIMP-1* in 2 of 7 high-grade lymph node-positive samples. A more favorable prognosis is expected for this group.

CONCLUSION

In conclusion, alternative prognostic biomarkers for BC are required to clarify disease risk in both high-risk and low-risk groups. These prognostic markers may help enlarge subgroups related to previously defined risk groups. Our study and relevant reports have supported that *MMP-2* and *TIMP-1* are promising independent markers for proper risk stratification and predicting prognosis. Additionally, inhibition of metastasis-related biomarkers such as *MMP-2* and *TIMP-1* via drug-based inhibitors is a novel strategy for targeted therapy. Furthermore, we suggest that analyzing *MMP-2* and *TIMP-1* in different tissues with various methods at different times can be a part of the methodology approach for the diagnosis and follow-up.

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Original Article

Determination of sildenafil and tadalafil adulteration by LC-MS/MS and 23 elements by ICP-MS in food supplements

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ABSTRACT

Background and Aims: Since the increasing prevalence of erectile dysfunction disease, the use of phosphodiesterase type 5 (PDE-5) inhibitors is widespread in the current era. The first and the most known molecule of PDE-5 inhibitors is sildenafil. It is widely used in the market. The second and more developed one is tadalafil. It is known that these substances are found in sexual stimulant food supplements as adulteration. In addition, food supplements can contain metals that can cause hazardous activity by accumulating. In our study, 11 randomly chosen sexual stimulant known products are studied which were bought from different stores in Edirne.
Methods: Adulteration with sildenafil and tadalafil was determined by LC-MS/MS because of the enhanced accuracy and precision, general applicability, and higher selectivity. Also, the determination of 23 metals, including known heavy metals, was made with ICP-MS.
Results: In the study, 7 of 11 samples detected at least 1 compound of sildenafil (up to 92.44 mg in one serving) or tadalafil (up to 23.62 mg in one serving). Neither of the samples exceeded the limits of metal daily intakes that are shown in Table 4.
Conclusion: In past studies, adulteration was determined in food supplements, and it is still detected today. The results we obtained are engrossing for the regulations of sexual stimulant food supplement sales. Their additional effects on total daily intake raise health concerns. The data obtained from this study will contribute to inform consumers and health professionals about the status of food supplements in the market.

Keywords: Adulteration, dietary food supplements, metals, PDE-5 inhibitors, sildenafil, tadalafil

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INTRODUCTION

Erectile dysfunction is a widespread disorder that affects quality of life (Kessler, Sollie, Challacombe, Briggs, & Van Hemelrijck, 2019). The incidence of erectile dysfunction increases with advancing age, and its incidence may increase due to the increasing world average age. The public is interested in food supplements that are stated to be natural because they think they are safe and reliable. Recently, the increase in the variety of supplements and fear of the undesirable effects of the drugs, and the ease of access with online shopping, ensure the growth of this food supplements market. Some brands are likely to adulterate their products for enhancing the sexual stimulant effect. The adulterating compounds are mostly Phosphodiesterase-5 (PDE-5) inhibitors (Tseng, & Lin, 2002; Tucker, Fischer, Upjohn, Mazzera, & Kumar, 2018).

PDE-5 is an enzyme that specifically cleaves and degrades cGMP to 5′-GMP in the corpus cavernosum smooth muscle. PDE-5 inhibitors have a structure that is comparable to that of cGMP; they bind to PDE-5 competitively and prevent cGMP hydrolysis, which improves the effects of nitric oxide. Prolongation of erection time is maintained by an increase in cGMP in smooth muscle cells. The relaxation of corpus cavernosum smooth muscles is not directly impacted by PDE-5 inhibitors. Therefore, for an erection to occur after ingestion of these drugs, there must be sufficient sexual stimulation (Huang & Lie, 2013).

Sildenafil and tadalafil the PDE-5 inhibitors (Figure 1) are used in the treatment of erectile dysfunction and pulmonary hypertension. Tadalafil is also used for the treatment of benign prostatic hyperplasia. Tadalafil causes inhibition of PDE-11 concentrated in the testis, prostate and skeletal muscle, causing pain and myalgia (Huang & Lie, 2013). Since PDE-5 inhibitors are metabolized by the CYP3A4 enzyme, they may interact with substances that cause CYP3A4 inhibition and induction. In addition, concomitant administration of alpha-blockers and PDE-5 inhibitors may cause additive vasodilator effects. Moreover, since the use of nitrates increases the production of cGMP, the use of PDE-5 inhibitors will reduce the degradation of cGMP and cause a synergistic vasodilator effect. Sildenafil could cause myocardial infarction in patients with unknown cardiac disease history; there have been some cases already reported (Feenstra, van Drie-Pierik, Lacle, & Stricker, 1998; Kekilli, Beyazit, Purnak, Dogan, & Atalar, 2005). Flushing, nasopharyngitis, headache, dyspepsia and nasal congestion are the most frequent adverse effects observed with PDE-5 inhibitors. PDE-5 inhibitors have also been linked to rare but dangerous cases of long lasting erections (about more than 4 hours) and priapism (erections lasting painfully more than 6 hours) (Huang & Lie, 2013).

Heavy metals are a type of contaminant that may be found in soil, water, air, plants, food, etc. Exposure to heavy metals may cause DNA damage, oxidative stress and lipid peroxidation on humans and animals (Wu et al., 2016). The presence of metals in food supplements can have significant impacts on human health. Moreover, some of these metals, such as lead, are toxic even at low levels of exposure. This is particularly concerning because food supplements are often taken over a long period

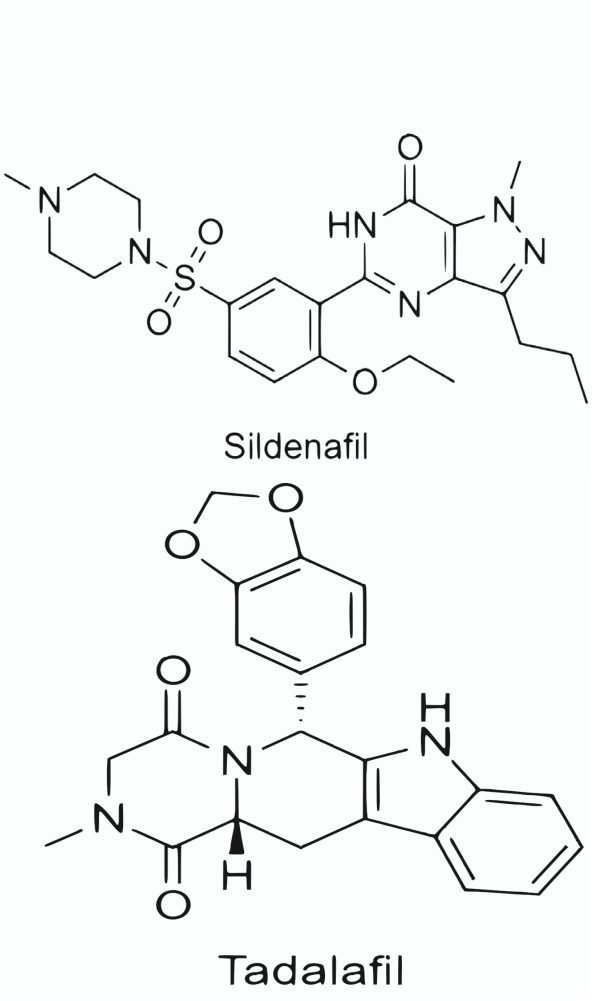

Figure 1. Chemical structures of sildenafil and tadalafil.

of time, which increases the risk of cumulative exposure. Accumulating evidence shows that exposure to heavy metals could raise the risks of obesity, diabetes, kidney dysfunction, cardiovascular diseases, neurodevelopmental disorders, and cancer (Wang et al., 2023). It is possible for dietary supplements that are advertised as herbal or natural to contain heavy metals (Clemens & Ma, 2016). This is because certain herbs and plants can absorb heavy metals from the soil in which they are grown, and thus, these metals can then be concentrated in the supplement. In addition, metals can be found in food supplements due to a variety of sources, such as contamination of raw materials, processing equipment or packaging materials. The public commonly believes that natural products pose no risk to health and that, even in the absence of sufficient evidence of medical benefits, there have no adverse effects.

ICP-MS was performed for the determination of 23 metals in 11 samples in this study. Elements of Li, B, Na, Mg, Al, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Sr, Cd, Sb, Ba, Tl, Pb and Bi were determined. Among them, heavy metals, particularly As, Cd and Pb are of main concern because of their hazardous activity. Maximum levels for certain contaminants in foods are set in Commission Regulation No. 1881/2006 by European Commission (European Commission, 2006). These regulations are

also available in the Turkish Food Codex Legislation (Turkish Food Codex, 2011).

In addition to setting maximum levels for other contaminants such as dioxins, mycotoxins, and nitrates, these regulations also specify limits for certain metals. Specifically, lead, cadmium, arsenic, mercury, and tin are among the contaminants primarily mentioned. Literature shows that high concentrations of these metals sometimes appear in food supplements (Dolan, Nortrup, Bolger, & Capar, 2003; Ernst, 2002).

Elements are usually determined by techniques, such as inductively coupled plasma optical emission spectrometry (ICP-OES) (Altundağ, Yildirim, & Altıntığ, 2019), atomic absorption spectrometry (Ahmad et al., 2019), and inductively coupled plasma mass spectrometry (ICP-MS) (Avula, Wang, Smillie, Duzgoren-Aydin, & Khan, 2010). For a convenient result, a sensitive analysis technique such as ICP-MS, which is more advanced than the generally used atomic absorption spectroscopy, is performed in this study.

This study aims to investigate the potential adulteration of sexual stimulant products with sildenafil and tadalafil, to determine the levels of 23 metals, including known heavy metals, and to assess the potential health risks associated with the use of these products. The objectives of the study will be achieved by identifying the presence and concentration of sildenafil and tadalafil in sexual stimulant products sold in Edirne, evaluating the levels of metals in these products, and informing regulators, health professionals, and consumers about the potential risks associated with the use of sexual stimulant food supplements in order to ensure their safety.

MATERIALS AND METHODS

ICP-MS analysis of heavy metals

Analysis of ICP-MS performed with an Agilent 7700 mass spectrometry (Agilent Technologies, Waldbronn, Germany). The EPA Method 200.8 guideline was followed to perform the analysis (U.S. EPA., 1994).

An amount of 0.5 grams of each sample was weighed using an analytical balance. The weighed samples were treated with 5 mL of concentrated HNO_3 (%65, Merck) and subjected to microwave digestion to dissolve the elements into an inorganic environment. The dissolved samples were then placed in Teflon microwave vials, sealed, and evenly placed in the microwave. Sensors were used for pressure and temperature adjustments, and the device parameters were set from the menu. After a 35-minute digestion process, the samples were transferred to storage containers and refrigerated until analysis. Then, a 100-fold dilution was made, and the samples were centrifuged. All dilutions were made with double-deionized water produced by the MilliQ water purification system (Millipore). The element standard solutions were created by diluting a 1000 mg/L stock solution (ICP standard CertiPUR, Merck, Germany). The samples were analyzed by using ICP-MS. Three readings were taken for each sample to obtain results with calculated standard deviations. The standard addition method was used to verify the accuracy of the analysis by preventing interference from the sample matrix.

ICP-MS conditions

The ICP-MS device has a power of 1550 watts and a sample acquisition rate of 0.3 revolutions per second (rps). It operates with an octupole radio frequency (OctP RF) at 180 volts and has a deviation of 13 volts and a depth of 8 millimeters. The maximum blank concentration is 100%, and it requires a stabilization time of 50 seconds. The device has an OctP slope of negative 8 volts, a nebulizer pump rate of 0.1 rps, and a matching voltage of 1.80 volts. It uses an energy separator of 5 volts and has a calibration curve confidence interval of 0.95. The Omega lens operates at 10 volts, and the carrier gas flow rate is 1.05 liters per minute (L/min). The sample acquisition time is 50 seconds, and the relative standard deviation is 5%. The device operates with a cell output of negative 50 volts, sample-to-cone (S/C) temperature of 2 degrees Celsius, layer slope of negative 40 volts, and a cell input of negative 30 volts.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of sildenafil and tadalafil

Reagents

All of the chemical solvents and standards used were of analytical quality. Sildenafil drug tablets were obtained from Biofarma (Istanbul, Türkiye), Tadalafil drug tablets were obtained from Nobel Türkiye (Istanbul, Türkiye), Hyper grade acetonitrile, methanol, dimethyl sulfoxide (DMSO), acetic acid, and formic acid were obtained from Merck (Darmstadt, Germany) for LC-MS.

Sampling

The samples were obtained from pharmacies, gas stations, and herbal stores located in the Edirne province of Türkiye. All the products were of Turkish origin. The samples were stored at room temperature until analysis. For liquid samples, the samples were directly diluted and prepared using the sample preparation procedure below. For solid samples, the drugs were pulverized by using a mortar and pestle to a powder form, and other samples were homogenized in a solvent medium. A total of eleven samples were analyzed, including eight solid and three liquid samples.

Extraction methods

A 1 g sample (liquid 1 mL) was taken into a falcon tube and 10 mL DMSO were added. The solution was vortexed 30 seconds and waited sonicator over 10 minutes at 50 ºC. Samples centrifuged at 13500 rpm for 5 min and 20 µL clear supernatant mixed with 980 µl methanol after extracting the samples filtered with a 0.22 micron nylon filter, and then injected to the LC-MS/MS system (dilution factor is 500).

LC-MS/MS conditions

The Agilent 1260 (Agilent Technologies, Waldbronn, Germany) LC system equipped with a Poroshell EC C18 4.6x150 mm 2.7micron (Agilent Technologies, Wilmington, DE, USA) column set at 40 ºC and a mobile phase flow rate of 0.6 mL/min was used for LC analysis. Gradient elution mobile phases consisted of 2 mM ammonium formate, 0.1 % formic acid in water (solvent A) and 1 % formic acid in methanol (solvent B). The gradient was started with a ratio of 90:10 (A:B). It was held constant for 1 minute. From 1 to 3 minutes, the ratio of A:B was decreased to 20:80. The ratio was kept constant at 20:80

for 6 minutes. At 6.1 minutes, the ratio was reduced to 5:95 and maintained for 10 minutes. At 10.1 minutes, the ratio was returned to the initial concentration of 90:10 (A:B).

The total method lasted 14 minutes. Sample temperature was stabilized at 4 ºC in the autosampler prior to analysis. A 5 µL sample volume was placed into the analytical column for analysis.

Analyses of MS/MS were successfully accomplished on an electrospray ionization (ESI) interface equipped Agilent 6460 triple quadruple LC-MS system (Agilent Technologies, Waldbronn, Germany). Electrospray ionization was executed in the positive ionization mode. The MS was performed with a 500 ms cycle time. For finding the optimal parameters of ion path and ion source of the studied compound, the quantitative optimization was applied by direct injection of standards utilizing an HPLC Agilent 1260. Multiple reaction monitoring (MRM) mode of the dominant product ion for each solution was realized using the optimal conditions. The ion source parameters were as follows: Gas Temperature: 275 ˚C; Gas Flow: 10 l/min; Nebulizer: 40 psi; Sheat Gas Heater: 375 Sheat Gas Flow: 10 l/min; Capillary (positive): 3000V. Multiple reaction monitoring mode consisted of comparison of pair ion (precursor and product ion m/z values) and LC retention times with standards served to confirm the identification of the analyte in the samples (Figure 3). Ion pairs were 475.0/100.0 and 475.0/50.0 for sildenafil, and 390.1/268.0 and 390.1/135.0 for tadalafil. MassHunter (Agilent LC-MS software) was used for both the data collecting and processing.

Calibration curve and quantification

All of the calibration curves were prepared in a 5 point calibration range 5, 10, 25, 50, 100 ng/g and injected 3 times for all points. Repeatability calculations were performed based on the standard deviation of three injections. Results within the range of 2-10% were obtained as repeatable. All the analytes curve linearity is $r^2 \geq 0.995$ (Figure 2). Limit of detection (LOD) and limit of Quantification (LOQ) values of the analytes (calculated over signal to noise ratio) are in Table 1. Because of the sugar content and chocolate samples, tadalafil and sildenafil standards were spiked with blank-chocolate, and recovery values were calculated. Since the standard deviation is below 5%, the precision value of the method is appropriate. When considering the recovery values and standard deviation, it has been determined that the method is highly accurate.

RESULTS AND DISCUSSION

The samples were quickly prepared for analysis because of the simple sample preparation technique that was developed in our study. Solid phase extraction (SPE) is an expensive method and takes a long time to perform. In our study, we applied only solid liquid extraction with DMSO. Our sample preparation time is nearly ten minutes. Song et al. have longer procedures and filtering stages in their study than ours (Song, El-Demerdash, & Lee, 2012). With the standard addition method, 25 and 75 ng/g recoveries were made at low and high concentrations on commercially purchased blank chocolates, and method verification was provided (Table 1). The developed method has been applied to matrices such as herbal products, food products and drug substances, and reproducible results have been obtained. In the literature, different sample preparation methods have been used for each matrix (Oh, Zou, Low, & Koh, 2006).

According to previous studies, several methods can be utilized to determine sildenafil and tadalafil in food supplement products. The most used methods are thin layer chromatog-

Table 1. The partial validation parameters of Tadalafil and Sildenafil in blank chocolate.

Compound	[M+H]+ m/z	R2 (Linearity)	LOD (ng/mL)	LOQ (ng/mL)	Recovery Low (%)	Recovery High (%)	%RSD
Tadalafil	390.1	0.9992	0.06	0.18	82	93	3.55
Sildenafil	475	0.9997	0.07	0.20	86	97	4.37

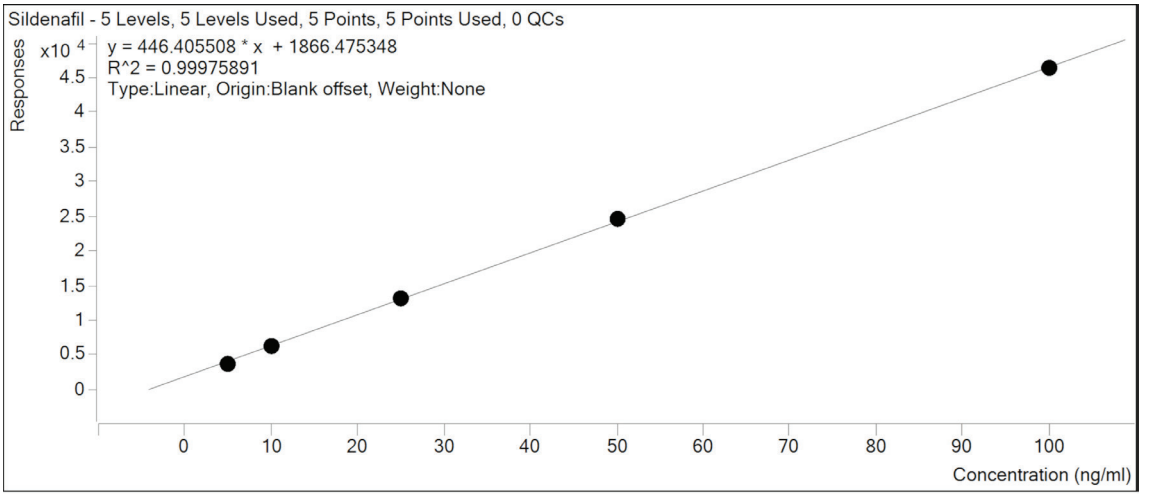

Figure 2. Linearity of sildenafil.

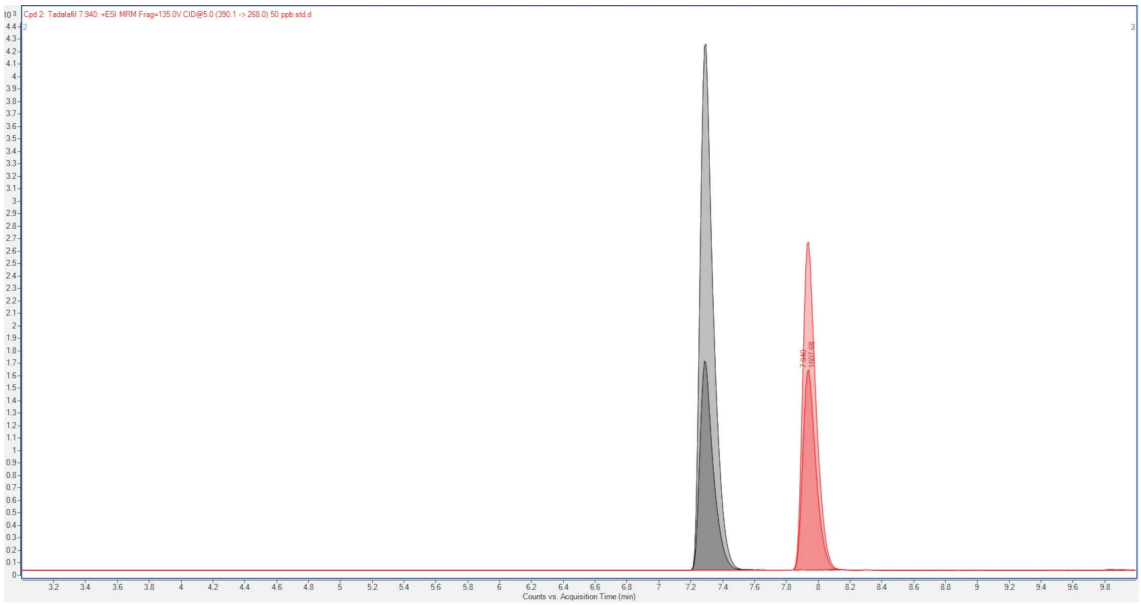

Figure 3. Chromatographic Separation of Tadalafil (Red, rt:7.29) and Sildenafil (Grey, rt:7.95).

Table 2. Quantitation results.

Sample no and Form	Compound	Final Concentration	Compound Dose Per Serving	One Serving Size
1 Liquid (Plant Based Drop)	SDF	0 ng/g	ND	5 mL
	TDF	0 ng/g	ND	
2 Liquid (Plant Based Drop)	SDF	0 ng/mL	ND	5 mL
	TDF	0 ng/mL	ND	
3 Liquid (Energy Drink)	SDF	206157.61ng/mL	30.91 mg	150 mL
	TDF	0 ng/mL	ND	
4 Paste (Herb and Honey Mixture)	SDF	3469060.12 ng/g	41.62 mg	12 g
	TDF	0 ng/g	ND	
5 Paste (Herb and Honey Mixture)	SDF	0 ng/g	ND	12 g
	TDF	0 ng/g	ND	
6 Paste (Herb and Honey Mixture)	SDF	4622334.76 ng/g	92.44 mg	20 g
	TDF	0 ng/g	ND	
7 Chocolate (Plant Based Chocolate)	SDF	1493053.67 ng/g	35.83 mg	24 g
	TDF	984203.35 ng/g	23.62 mg	
8 Chocolate (Plant Based Chocolate)	SDF	2152374.75 ng/g	38.74 mg	18g
	TDF	0 ng/g	ND	
9 Chocolate (Plant Based Chocolate)	SDF	2520986.17 ng/g	45.37 mg	18 g
	TDF	12660.51ng/g	0.22 mg	
10 Capsule (Plant Based Capsule)	SDF	8067844.26 ng/g	3.63 mg	450 mg
	TDF	575.81 ng/g	0.00025mg	
11 Effervescent Tablet (Multivitamin)	SDF	0 ng/g	ND	5.5 g
	TDF	0 ng/g	ND	

TDF, Tadalafil; SDF, Sildenafil; ND, Not Detected.

raphy (TLC) (Miller & Stripp, 2007; Moriyasu et al., 2001), micellar electrokinetic chromatography (MEKC) (Berzas, Rodrguez, Castaneda, & Rodrguez, 2002), nuclear magnetic resonance (NMR) (Venhuis, Blok-Tip, & de Kaste, 2008), flow injection analysis (Lopes Júnior et al., 2012), spectrophotometry (Harikrishna, Nagaralli, & Seetharamappa, 2008), high-performance liquid chromatography (HPLC)-ultraviolet (UV) (Daraghmeh, Al-Omari, Badwan, & Jaber, 2001; Miller & Stripp, 2007), HPLC-diode array detection (Venhuis, Blok-Tip, & de Kaste, 2008), gas chromatography (GC)-MS (Miller & Stripp, 2007), liquid chromatography (LC)-MS (Miller & Stripp, 2007; Venhuis, Blok-Tip, & de Kaste, 2008), electrospray tandem (ESI)-MS, and combinations of these methods can be applied together (Singh et al., 2009; Zhu et al., 2005).

TLC assays lack analytical sensitivity in typical assays due to extended analysis periods and quantification issues. GC-MS lacks analytical sensitivity because of its chemical characteristics, namely its low transition to the gas phase (Dural, 2020). Due to chemical interactions with molecules with similar chemical compositions in the absence of pre-separation using a column, spectrophotometric analysis might provide false positive results. Analytical procedures based on HPLC-UV give appropriate analytical sensitivity and repeatability to determine substances (Dural, 2020).

The developed and applied LC-MS/MS method provides higher accuracy and precision for LOD and LOQ value than the HPLC-UV systems (Table 1) (Dural, 2020). In the LC-MS/MS system that we used, calibration started at 5 ng/g, while in the HPLC-UV system used by Dural, it started at 20 ppb. Additionally, due to the possibility of matrix effect and interference, the LC-MS/MS system is a much more advanced analysis technique compared to the HPLC-UV system. Obtained method parameters are compatible with other methods developed in the literature (Lee et al., 2021).

It was determined that there was 30.91 mg of sildenafil in one serving (150 mL) in Sample 3 which is an energy drink (Table 2). The determination is 38.7 mg of sildenafil in Sample 8, which is a chocolate product for women as written on the package. The addition of an active ingredient approved only for men by the FDA to a product specifically marketed for women indicates a lack of pharmacological knowledge regarding adulteration by the producers of this product. Sample 4, which is a mixture of herbs and honey in paste form, has 41.62 mg sildenafil in one serving (12 g), making its effect close to the recommended daily dose of sildenafil. Sample 6, which is also a mixture of herbs and honey in paste form, has 92.44 mg of sildenafil in one serving (20 g), that is around the maximal dosage of sildenafil. Additionally, the presence of these active substances is not stated on the product packaging. The recommended daily dose of sildenafil is 50 mg as a single dose before sexual activity, and the recommended maximum daily dose is 100 mg (Bethesda, 2012). According to dosage information, Sample 3 and 4 are close to the recommended daily dose amount. Sample 6, on the other hand, has exceeded the recommended daily dose amount and approached the maximum daily dose of 100 mg (Bethesda, 2012).

Sample 7 is a chocolate bar containing 23.62 mg of tadalafil and 35.83 mg of sildenafil in one serving (24g). There is no information on the presence of these active substances on the package. The amount of tadalafil in one serving of this sample is above the maximum daily dose for erectile dysfunction treatment. In addition, 35.83 mg of sildenafil in its content will create a synergistic effect and potentially cause a hazardous activity. Due to the combination of these substances in high levels, this sample is the unhealthiest supplement among the samples. In sample 9, which is a chocolate bar, 45.37 mg of sildenafil and 0.22 mg of tadalafil were determined in one serving. In sample 10, which is an herb containing capsule, 3.63 mg of sildenafil and a trace amount of tadalafil were found in one serving. It should be noted that although the substances are at very small amounts, adulteration is still illegal. Tadalafil is recommended to be taken as a single 10 mg dose 1 hour before sexual activity for erectile dysfunction. Although the dose of tadalafil can be adjusted according to its effect and developing tolerance, it is recommended that the daily dose should not exceed 20 mg (Bethesda, 2012).

Data observed from ICP-MS (Table 3) has shown that none of the samples used in the metal content evaluation exceeded the daily metal limits (Table 4) in their one size serving. It is important to note that the size of the serving can impact the amount of metal present in a sample. Based on the results of the study, none of the samples exceeded the daily limit for metals when measured per serving size. However, it is still possible that there could be a risk of accumulating too much metal over time if the samples are consumed frequently or in larger quantities.

Na^+ and K^+ are the electrolytes to maintain blood volume and fluid. Still, consuming too much sodium and too little potassium may raise blood pressure. Sample 11 has a 187.95 mg of sodium and 0.43 mg of potassium in one serving (Table 3), which can be hazardous to an individual with hypertension on a sodium diet.

As, Cd, Cr and Pb are the most toxic heavy metals studied in this study. According to the data observed in the Table 3, the maximum values of these metals determined from the samples are 3.1514 µg, 0.6300 µg, 4.9701 µg and 6.3272 µg, respectively. These values can affect the daily heavy metal intake especially for arsenic and chromium in a certain sense. Using the food supplement several times, contaminated water and food consumption could increase the amount of arsenic and chromium intake. Therefore, total intake may exceed the daily usage limits and cause toxic effects (Table 4).

Additionally, effects of combined metal exposure on human health are still being studied. When metals are combined, their interaction can produce additive, synergistic or antagonistic effects due to the ability of certain metals to either facilitate or hinder the absorption of other metals (Xiao et al., 2021). Previous studies have suggested that the toxicity of multiple metals with the same or similar organ toxicity may be additive, but it still needs to be studied (Hong, Jin, & Zhang, 2004; Madden, & Fowler, 2000). Currently, there is insufficient information to establish science-based limits for specific products based on the combined exposure to metals with similar toxicities. Therefore, in this study, metal restrictions will be evaluated separately, given the lack of conclusive evidence on the effects of combined exposures.

Table 3. Total amount of metals in each sample (µg).

Samples	Li	B	Na	Mg	Al	K	Ca	V	Cr	Mn	Fe
1	6.9840	4.2614	755.1255	41.4579	2.9421	521.0734	42.1655	0.0193	0.2047	0.1485	15.8606
2	6.6354	2.6533	837.9692	60.0059	1.9259	1271.1705	16.6941	0.0406	0.1609	0.1249	6.9382
3	148.0858	101.0386	21998.2178	4756.1353	350.8711	3378.3841	4748.6677	0.5958	4.9701	0.6379	58.8998
4	10.2785	42.6031	1283.1488	2802.1419	58.2739	13914.6667	1223.6129	0.1780	0.6352	28.5669	100.1891
5	12.0561	25.0213	1558.8974	1888.8992	47.7539	10988.9256	789.3358	0.3056	0.6347	9.7524	89.1118
6	12.2510	22.0481	417.5935	1120.0842	53.5080	4942.4064	460.6529	0.1174	1.3322	9.5444	58.5257
7	18.8839	77.4876	1289.0675	22577.4927	49.8642	59665.6799	1852.9611	0.2449	3.9881	62.2178	294.2305
8	11.6535	46.2160	9634.0883	10873.0405	87.7392	50145.9809	2800.9252	0.2681	4.5369	25.7412	204.0241
9	14.6336	36.2459	10350.3930	8339.9514	47.9354	34156.1806	2002.5262	0.2190	3.2233	20.3172	154.8425
10	0.2835	0.8499	51.6186	187.4495	1.5047	402.4890	337.1979	0.0179	0.0143	1.3743	5.2842
11	9.5994	1.7393	187953.3994	693.5467	11.0453	439.6487	252.6896	0.1946	0.4221	0.9843	6.6853

Li, Lithium; B, Boron; Na, Sodium; Mg, Magnesium; Al, Aluminum; K, Potassium; Ca, Calcium; V, Vanadium; Cr, Chromium; Mn, Manganese; Fe, Iron.

Samples	Ni	Co	Cu	Zn	As	Sr	Cd	Sb	Ba	Tl	Pb	Bi
1	3.5752	0.0095	1.1184	6.1986	0.0921	0.3310	0.0148	0.3684	0.5689	0.0029	0.2331	10.9755
2	2.3969	0.0147	0.3491	2.1761	0.1199	0.3688	0.0171	0.0567	0.5455	0.0026	0.1965	10.9131
3	54.2766	0.2638	6.3977	20.1586	3.1514	59.6470	0.4258	0.8153	48.7270	0.0545	6.3272	331.5763
4	6.4942	0.2849	9.8288	27.8385	0.4526	22.0836	0.1359	0.1772	18.1771	0.0175	1.2112	32.3222
5	5.7549	0.3330	7.9699	27.9315	0.8579	9.1445	0.0806	0.1341	6.8655	0.0050	0.7746	21.9694
6	6.6812	0.0870	3.2459	7.3262	0.4350	4.5277	0.0949	0.1455	6.0016	0.0080	1.1405	46.6552
7	29.3361	2.5960	105.0657	161.0723	0.3973	46.4253	0.6300	0.5656	48.4686	0.0326	1.0039	50.8705
8	13.9507	1.3154	38.0474	66.8492	0.2779	25.8148	0.5336	0.6673	26.3749	0.0184	0.9218	39.0154
9	11.1158	0.8524	29.1357	45.4345	0.4584	19.1460	0.2732	0.7816	18.4763	0.0149	0.7191	35.4141
10	0.1595	0.0104	0.7047	2.1596	0.0058	1.1065	0.0041	0.0190	0.4268	0.0001	0.0274	1.0030
11	0.9742	0.0820	0.3719	1.9498	0.1033	5.2018	0.0267	1.2485	4.8535	0.0012	0.2272	9.9099

Ni, Nickel; Co, Cobalt; Cu, Copper; Zn, Zinc; As, Arsenic; Sr, Strontium; Cd, Cadmium; Sb, Antimony; Ba, Barium; Tl, Thallium; Pb, Lead; Bi, Bismuth.

Table 4. Minimum risk levels/recommended dietary allowances/no observed adverse effect levels of the elements/day (Doses may differ between authorities).

Elements	AI/RDA/NOAEL/ MRL/DI/PTDI	References
Li	RDA: 1 mg/day for adults	(Voica, Roba, & Iordache, 2021)
B	Estimated DI: 13mg	(Nielsen, 1997)
Na	AI: 1500 mg for adults	(Avula, Wang, Smillie, Duzgoren-Aydin, & Khan, 2010)
Mg	RDA: 420 mg for males and 320 mg for females	(Avula, Wang, Smillie, Duzgoren-Aydin, & Khan, 2010)
Al	DI: 0.10-0.12 mg of Al/kg/day for adults	(Avula, Wang, Smillie, Duzgoren-Aydin, & Khan, 2010)
K	AI: 4700 mg for adults	(Avula, Wang, Smillie, Duzgoren-Aydin, & Khan, 2010)
Ca	AI: 1000 mg for adults	(Meyers, Hellwig, & Otten, 2006)
V	MRL: 210 µg for adults	(Avula, Wang, Smillie, Duzgoren-Aydin, & Khan, 2010)
Cr	RDA: 35 µg for males and 25 µg for females	(Meyers, Hellwig, & Otten, 2006)
Mn	RDA: 2.3 mg for males and 1.8 mg for females	(Meyers, Hellwig, & Otten, 2006)
Fe	PTDI: 48 mg	(Akinyele & Shokunbi, 2015)
Co	DI: 0.005-1.8 mg	(Avula, Wang, Smillie, Duzgoren-Aydin, & Khan, 2010)
Ni	DI: 100-300 µg	(Akinyele & Shokunbi, 2015)
Cu	RDA: 900 µg for adults	(Meyers, Hellwig, & Otten, 2006)
Zn	AI: 11 mg for males and 8 mg for females	(Meyers, Hellwig, & Otten, 2006)
As	MRL: 21 µg	(Meyers, Hellwig, & Otten, 2006)
Sr	DI: 2-4 mg	(Nielsen, 2004)
Cd	MRL: 14 µg	(Avula, Wang, Smillie, Duzgoren-Aydin, & Khan, 2010)
Sb	MRL: 0.4 and 6 µg/kg/d	(Raab, Stiboller, Gajdosechova, Nelson, & Feldmann, 2016)
Ba	MRL: 0.2 mg/kg/d	(Raab, Stiboller, Gajdosechova, Nelson, & Feldmann, 2016)

Table 4. Continue.

Elements	AI/RDA/NOAEL/ MRL/DI/PTDI	References
Tl	DI: <5 µg	(Filipov, 2015)
Pb	DI: 490 µg	(Kumar et al., 2020)
Bi	RDA: 0.6-0.8 g	(Bradley, Singleton, Po, 1989)

Abbreviations: AI, Adequate intake; DI, Daily intake; RDA, Recommended dietary allowance; PTDI, Provisional tolerable daily intake; NOAEL, No observed adverse effect level; MRL, Minimum risk level. Non-specified intakes are for healthy adults of 70kg.

In our study, we analyzed 23 metals, and some of them have not been assigned limit values by official authorities. The Turkish Food Codex Regulation on contaminants specifies limit values for Sn, Pb, Hg, and Cd, while the European Union Commission Regulation on contaminants specifies limit values for Sn, Pb, Hg, As, and Cd. Although EFSA suggests limit values, it is not legally obliged.

CONCLUSION

The levels of sildenafil, tadalafil and 23 metals in 11 food supplements from a Turkish market were studied, and their potential risks to health are discussed. The results show that the amounts of the concerning toxic metals in the studied food supplements were not as high as to set an individual hazardous activity. However, their mechanism of additive, synergistic and potentiation effects requires further studies. The result of the sildenafil and tadalafil determination analysis is a more serious concern. 7 of 11 samples were found to contain sildenafil and tadalafil separately or in combination.

The active pharmaceutical substances and heavy metals in food supplements with unknown concentrations can be a risk to health of an individual. Unintentional misusing, overusing, or interaction of the supplement with other medications, existing health problems, or other pharmaceuticals within the same food supplement have the potential to cause significant adverse effects on health. Our results represent the fact that there must be frequent and developed control to these products.

It is important to note that the lack of official limit values for certain metals does not necessarily mean that they are safe or that they do not pose a risk to human health. Further research is needed to determine safe exposure levels for these metals, and regulatory authorities should consider establishing limit values for them.

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Original Article

Enhancement of dissolution of *Prosopis africana* stem bark extract by solid dispersion technique

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ABSTRACT

Background and Aims: Literature reveals many benefits and potentials of the stem bark extract of *Prosopis africana* but like other plant extracts, its use in the oral pharmaceutical formulation is limited due to its poor aqueous solubility, reduced absorption, and bioavailability. The aim of this study is to develop oral solid dispersion formulations of *Prosopis africana* extract to improve its dissolution and possible bioavailability.

Methods: Crushed stem bark of *Prosopis africana* was macerated in methanol for 72 h at room temperature. The resulting extract (PREx) was incorporated into polyethylene glycol 4000 (PEG 4000) by the solvent-evaporation and melt-fusion methods using different ratios (1:1, 1:2, 1:4) of extract to polymer; physical mixtures of the same ratio of extract to polymer were also prepared. The crystallinity of the formulations was characterized by differential scanning calorimetry (DSC), possible interaction/incompatibility between the extract and polymer was determined by Fourier transform infra-red spectroscopy (FT-IR). *In vitro*, release and modeling of the release profile were also determined.

Results: No interaction was found between materials used for the preparations while DSC thermograms showed a reduction in crystallinity of PREx in the solid dispersion formulations. *In vitro* release profile of the formulation prepared with the highest polymer concentration by the melt-fusion method (M3) showed the greatest enhancement in dissolution.

Conclusion: This study shows solid dispersion prepared by the melt-fusion method as an effective technique for the enhancement of dissolution of poorly water-soluble *Prosopis africana* extract.

Keywords: Melt-fusion, *Prosopis africana* extract, PEG 4000, solid dispersion

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INTRODUCTION

The use of medicinal plants is as old as mankind in the treatment of various diseases. Parts of plants such as roots, leaves, fruits, buds, stems, flowers, and barks contain biochemically active constituents which are thought to be responsible for the cure and management of various diseases.

In African nations, the utilization of these plants as the primary source of or an integral part of healthcare is well-documented (WHO, 2004; Bandaranayake, 2006, Sofowora, Ogunbodede, & Onayade et al., 2013). This is understandable because in developing climes like Africa, with high poverty rates, access to and affordability of the usual conventional drugs is a challenge. Moreover, herbal medicines are believed to be readily accepted due to their purported claim of relative safety.

Although plant extracts are considered to have remarkable *in vitro* properties, the *in vivo* efficacy is usually limited due to low aqueous solubility resulting in reduced absorption and bioavailability (Gunasekaran, Haile, Nigusse, & Dhanaraju, 2014; Nikam, Shete, & Khapare, 2020). To address such solubility problems, formulation with suitable polymers has proved positive (Ansari & Parveen, 2016).

Solid dispersion is a technique of dispersing one or more active hydrophobic ingredients in an inert hydrophilic carrier or matrix in a solid state resulting in improved solubility and better bioavailability (Nikam et al., 2020; Enose, Dasan, Sivaramakrishnan, & Shah, 2014). This technique converts the crystalline form of drugs into the amorphous form which enhances its bioavailability and improves its dissolution kinetics (Wani et al., 2021). When solid dispersion formulations come in contact with aqueous media, the carrier dissolves and the drug is released as very fine colloidal particles or oily globules of submicron sizes.

The solid dispersion technique is beneficial in overcoming the solubility, dissolution, and bioavailability complications of poorly soluble phytoconstituents. These can be achieved by optimizing the surface area available for dissolution through the reduction of particle size of the solid compound and/or by enhancing the wetting characteristics of the drug compound surface, to decrease the boundary layer thickness and ensure sink conditions for dissolution (Sharma, Sahoo, Agrawal, & Kumari, 2019; Rahman, 2020; Singh, Baghel, & Yadav, 2021). Apart from improving the dissolution of poorly soluble phytoconstituents, the solid dispersion technique has been used to mask disagreeable tastes, odor, and smell and in reducing gastric irritation (Cai et al., 2014; Qusa, Siddique, Nazzal, & El Sayed, 2019). In addition, the technique has been exploited to control the release of phytoconstituents (Cid, Simonazzi, Palma, & Bermúdez, 2019), protect phytoconstituents from degradation, and improve constituent's stability (Parikh, Kathawala, Song, Zhou, & Garg, 2018; Saidan, Kaus, Aisha, Hamil, & Ismail, 2020) while also reducing therapeutic dose and possible side effects (Rahman, 2020).

Methods used in the preparation of solid dispersions include solvent evaporation which involves the dissolution of the drug and carrier in a suitable solvent. Evaporation of the solvent leaves a film or mass which can be dried to constant weight. Another method is the melt method which involves heating the drug and suitable carrier at temperatures above their eutectic points until both are melted. The melted mixture is rapidly cooled to obtain a congealed mass which can be pulverized and sieved (Kumar & Kumar, 2017). Other methods include co-precipitation, kneading, co-grinding, gel entrapment, spray-drying, and freeze drying.

Literature reveals reports of studies on the formulation of poorly water-soluble herbal constituents and/or, extracts by solid dispersion techniques. The study by Onoue et al., (2010) showed that the pharmacokinetic behavior and photo-stability of curcumin were significantly enhanced when prepared as a solid dispersion. Another study reported increased solubility of quercetin in solid dispersion formulations (Costa et al., 2011). The hepatic activity of poorly water-soluble silymarin was observed to increase when developed as solid dispersion (Balata & Shamrool, 2014). In a different study, a solid dispersion formulation of fat-soluble *Ginkgo biloba* extract significantly improved its dissolution (Wu et al., 2018). In another study, solid dispersion formulation of the ethanol extract of *Andrographis paniculata* enhanced its solubility, furthermore, compression of the solid dispersion into tablets showed enhanced dissolution and oral bioavailability of the extract (Nitave, Chougule, & Koumaravelou, 2018).

In a different study, alginate encapsulation of turmeric extract solid dispersions showed stronger antibacterial activity than other formulations of the extract (Bangun, Arianto, Bangun, & Nainggolan, 2019). Formulation of solid dispersion of *Fagonia indica* extract showed significant anti-hepatotoxicity activity and complete recovery from hepatotoxicity when compared to the crude extract alone (Shehab, Shahiwala, Benouared, & Khan, 2020). The study by Tafu & Jideani, (2021) suggested that solid dispersions of the popular *Moringa oleifera* leaf powder may be useful in functional foods and beverages and in nutraceutical formulations. Bajracharya, Song, Lee, Jeong, & Han, (2022) reported that solid dispersion of *Isatis indigotica* and *Juglans mandshurica* produced better dissolution and oral absorption of the combined extract than when used in their crude form.

Prosopis africana (*P. africana*) is the only tropical African *Prosopis* species, and belongs to the family Fabaceae, sub-family *Mimosoideae*. It is a leguminous tree native to Africa and Asia and is found abundantly in the middle belt regions of Nigeria. It is popularly called the Locust beans tree, or Ironwood tree and in Nigeria, it is known as "kiriya" by the Hausas, "okpehe" by the Idoma, "ubwa" by the Igbos and "ayan" by the Yorubas (Bello, Madusolumuo, & Igbokwe, 2016; Odoh et al., 2018).

Different parts of the tree have been used for the management of ailments ranging from toothache, sore throat, bronchitis, dysentery, gonorrhea, dermatitis, and skin diseases to malaria (Kolapo, Okunade, Adejumobi, & Ogundiya, 2009; Nnamani, Kenechukwu, Chika, & Otuu, 2012). Specifically, the fresh leaf buds and shoots have been used as fodder while the pods are consumed by cattle as well as been a rich source of potash

for the manufacture of local soap (Orwa, Mutua, Kindt, Jamnadass, & Anthony, 2009; Ajiboye, Agboola, Fadimu, & Afolabi, 2013). In the middle belt states of Nigeria, the fermented seeds of the plant are popularly used as a food seasoning; other parts are used in the preparation of soups and the making of cakes (Achi & Okolo, 2004). Laboratory investigations have confirmed that the extract possesses anti-inflammatory, antidiabetic, antibacterial, antifungal, anti-ulcer, anticancer, and anthelmintic properties among others (Okide, Odoh, & Ezugwu, 2003; Atawodi & Ogunbusola, 2009; Ayanwuyi, Yaro, & Abodunde, 2010; Ibrahim, Mohammad, Faisal, & Musa, 2018; Alimata et al., 2020).

In spite of the potential of *Prosopis africana* extract, its use in the pharmaceutical formulation is limited due to its poor aqueous solubility, reduced absorption, and bioavailability as with other plant extracts (Rohini, Muhammad, & Rabeta, 2021). Therefore, the aim of this work is to develop solid dispersion formulations of the methanol stem bark extract of *Prosopis africana* using a hydrophilic polymer (polyethylene glycol; PEG 4000). The novelty of this work is in the development of a solid dispersion formulation containing the methanol stem bark extract of *Prosopis africana* which has not been hitherto reported.

MATERIALS AND METHODS

Materials

Methanol extract of *Prosopis africana* stem bark, polyethylene glycol 4000 (Merck, Germany), methanol (Sigma, Aldrich, United Kingdom), and hydrochloric acid (Sigma Aldrich, United Kingdom).

Collection and preparation of plant material

The stem bark of *Prosopis africana* was deposited at the botanical garden of the National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria, and given the voucher number; NIPRD/H/7285. The dried stem barks were cleaned, air-dried, and pulverized using a mechanical grinder. The coarse powder obtained was packaged in an air-tight container and stored in a desiccator until further use.

Preparation of the extract of *Prosopis africana*

An earlier method was adopted with some modifications (Ayanwuyi et al., 2010). About 1 kg of the pulverized powdered stem bark was macerated in 70% methanol (v/v) in a ratio of 1:3 (powdered stem bark: methanol) for 72 h at room temperature with intermittent stirring. After soaking, the mixture was filtered using a muslin cloth. The extract was concentrated over a water bath (Karl Kolb, Germany) at 70 °C and the resulting extract (PREx) was pulverized and stored in an air-tight container until further use.

Preparation of solid dispersion formulations

Solvent evaporation method

Three (3) different batches of solid dispersion formulations were prepared using polyethylene glycol (PEG) 4000 at extract: polymer ratios of 1:1, 1:2, and 1:4 using methanol as the solvent of choice. Appropriate amounts of the extract (PREx) and polymer (PEG 4000) as displayed in Table 1 were used. The extract (2.5 g) and polymer (2.5 g) were mixed, placed in a beaker, and dissolved with sufficient volume (20 mL) of methanol with continuous stirring. The solvent was completely evaporated over a water bath at 40 ^{0}C while continuously stirring to obtain a dried mass. The dried mass was pulverized, screened through a sieve (A.S.T.M. E-11 Specification No. 60), packaged in an air-tight container, and coded as S1; this was stored in the desiccator until further use. The other batches were prepared similarly using 2.5 g of extract and 5 g of polymer for batch S2 and 2.5 g of extract and 10 g of polymer for batch S3 (Table 1).

Table 1. Composition of solid dispersion batches.

Batch	PREx (g)	PEG 4000 (g)
S1	2.5	2.5
S2	2.5	5
S3	2.5	10
M1	2.5	2.5
M2	2.5	5
M3	2.5	10
P1	2.5	2.5
P2	2.5	5
P3	2.5	10

Melt fusion method

Three (3) different batches of solid dispersion formulations were prepared using polyethylene glycol 4000 (PEG 4000) at extract:polymer ratios of 1:1, 1:2, and 1:4 using the melt-fusion method. As displayed in Table 1, 2.5 g of extract and 2.5 g polymer (PEG 4000) were mixed together, placed in a beaker, and heated directly on the hot plate at 50 - 55 °C while stirring at 50 rpm (until melted). The molten mixture was continuously stirred until the extract was completely melted into a homogenous mass. The melted mass was rapidly cooled on an ice pack under vigorous stirring. The solid mass was crushed, pulverized, and sieved through a sieve (A.S.T.M. E-11 Specification No. 60), packaged in an air-tight container, and labeled as M1, this was stored in the desiccator until further use. The other batches were prepared similarly according to the composition in Table 1 and coded M2 and M3.

Physical mixture method

Physical mixtures were prepared by manually mixing appropriate amounts of the extract and PEG 4000 according to the composition in Table 1. The extract (2.5 g) was mixed with 2.5 g of PEG 4000 in a porcelain mortar for about 3 min, the powdered mixture was packaged in an air-tight container and labeled P1. Batches P2 and P3 were prepared in the same manner using the appropriate quantities as stated in Table 1, packaged appropriately, and stored in the desiccator until further use.

Evaluation of solid dispersion formulations

Determination of product yield

The product yield of each batch was determined by the ratio of the weight of the dried dispersions and the sum of the starting materials (using amounts of PREx and PEG 4000 as displayed in Table 1) expressed in percentage.

Fourier transform-infrared (FT-IR) spectra studies

The solid dispersions were triturated with potassium bromide, made into pellets (1 ton/cm^2) and infrared (IR) spectra were obtained between scanning ranges of 4000 and 400cm^{-1} using the Cary 630 Fourier transform infrared (FT-IR) Spectrometer (Agilent Technologies, USA).

Differential scanning calorimetry (DSC) analysis

Samples of the optimized solid dispersion formulations; M3, PM3, and the extract alone (4.7 - 6.3 mg) were placed in aluminum pans of the differential scanning calorimetry (DSC) (Model DSC 204 F1Netzsch, Germany). The pans were crimped and heated between 60 and 300 °C at a scanning rate of 10 °C/min under constant nitrogen flow at a rate of 20 mL/min.

***In vitro* dissolution studies**

The calibration curve of the extract (PREx) was prepared using concentrations of 40-100 µg; scans of the extract and their corresponding absorbance were obtained from the UV-Visible spectrophotometer (Cary 60). *In vitro,* dissolution was conducted the United States Pharmacopeia (USP) apparatus II (paddle method) at a speed of 100 rpm. An amount of the solid dispersion formulation (50 mg) was placed in the dissolution vessel containing 0.1N Hydrochloric acid, maintained at 37 ± 0.5 °C. The dissolution test was carried out for 60 min, and aliquots of 5 mL were withdrawn from the vessels at predetermined intervals of 5, 15, 30, 45, and 60 min, and the same volume of medium was replaced into the vessel to maintain sink condition. The same procedure was repeated for all the other solid dispersion formulations and the extract alone. The absorbance of the withdrawn samples was determined using the Ultra-violet (UV)-Visible spectrophotometer (Cary 60) at 230 nm, and the content of PREx was determined from the calibration curve.

Modeling of release profile

The kinetics of extract release was determined by fitting the data obtained from *in vitro* release studies into the zero-order, first-order, Higuchi, and Hixson-Crowell models while the mechanism of release was determined by the Korsmeyer-Peppas model. The model with the highest coefficient value was selected as the appropriate model used to describe the possible kinetics and mechanism of the extract's release from the solid dispersion formulations. The equations that describe the model-dependent mathematical kinetics are as follows:

$$\text{Zero order} = Q_t = Q_0 + K_0 t \qquad Eq\ 1$$

$$\text{First order} = LnQ_t = LnQ_0 + K_1 t \qquad Eq\ 2$$

$$\text{Higuchi} = Q_t = K_h t^{1/2} \qquad Eq\ 3$$

$$\text{Hixson-Crowell} = Q_0^{1/3} + Q_t^{1/3} = K_d t \qquad Eq\ 4$$

$$\text{Korsmeyer-Peppas} = Q_t/Q_\infty = K_{kp} t^n \qquad Eq\ 5$$

where Q_t is the drug dissolved amount in time t, Q_0 is the initial quantity of drug in the solution, K_0 is the zero-order release constant, K_1 is the first-order release constant, K_h is the Higuchi rate constant, K_d is the dissolution constant of Hixson–Crowell kinetics, Q_t/Q_∞ is a proportion of drug released at time t, K_{kp} is the Korsmeyer-Peppas release rate constant.

RESULTS

Determination of product yield

Product yield for all the formulations was between 65.32 and 92.97 % with physical mixture formulations having the highest yield and solvent evaporation method having the least yield (Table 2).

Table 2. Product yield of solid dispersion formulations and physical mixture.

Formulation	Yield (%)
S1	65.32
S2	78.52
S3	81.69
M1	90.24
M2	76.80
M3	88.70
P1	84.12
P2	92.97
P3	83.96

Fourier transform-infrared (FT-IR) spectroscopy analysis

FT-IR spectroscopy analysis was carried out to investigate any physicochemical interactions between the crude extract (PREx) and the polymer used in the preparation of the solid dispersions. The spectra for PREx, the optimized batch for the physical mixture (P3), and solid dispersion prepared by the melt-fusion method (M3) are displayed in Figures 1A, 1B, and 1C respectively.

Characteristic peaks of PREx were observed at 3596.9cm^{-1}, 2113.4, 1599.0, 1312.0 cm^{-1}, 1021.3, and 708.2cm^{-1} (Figure 1A). The IR spectrum of the physical mixture (Figure 1B) and solid dispersion (Figure 1C) were observed to be similar. However, the peak at 2113.4cm^{-1} due to the extract was observed to be smoothened in the presence of the polymer (Figures 1B and 1C).

Differential scanning calorimetry (DSC) analysis

Differential scanning calorimetry (DSC) is a thermal analysis technique in which the heat flow into or out of a sample is measured as a function of temperature or time. Figure 2A shows the DSC thermogram of the pure extract (PREx), the optimized physical mixture (Figure 2B), and the optimized solid dispersion prepared by the melt-fusion method (Figure 2C). PREx showed an endothermic peak beginning at 59.99 °C and ending at 299.96 °C with a peak temperature of 177.74 °C corresponding to its crystalline melting peak as displayed in Table 3.

Thermal properties of the physical mixture and solid dispersion formulation were observed to differ from those of the crude extract (PREx). The endothermic melting peak of the extract was observed to be broadened in Figure 2B and completely absent in Figure 2C. Change in heat of enthalpy of PREx (779.45 J/g) displayed in Table 3 is seen to decrease in P3 (692.00 J/g) and considerably decreased in M3 (614.92 J/g).

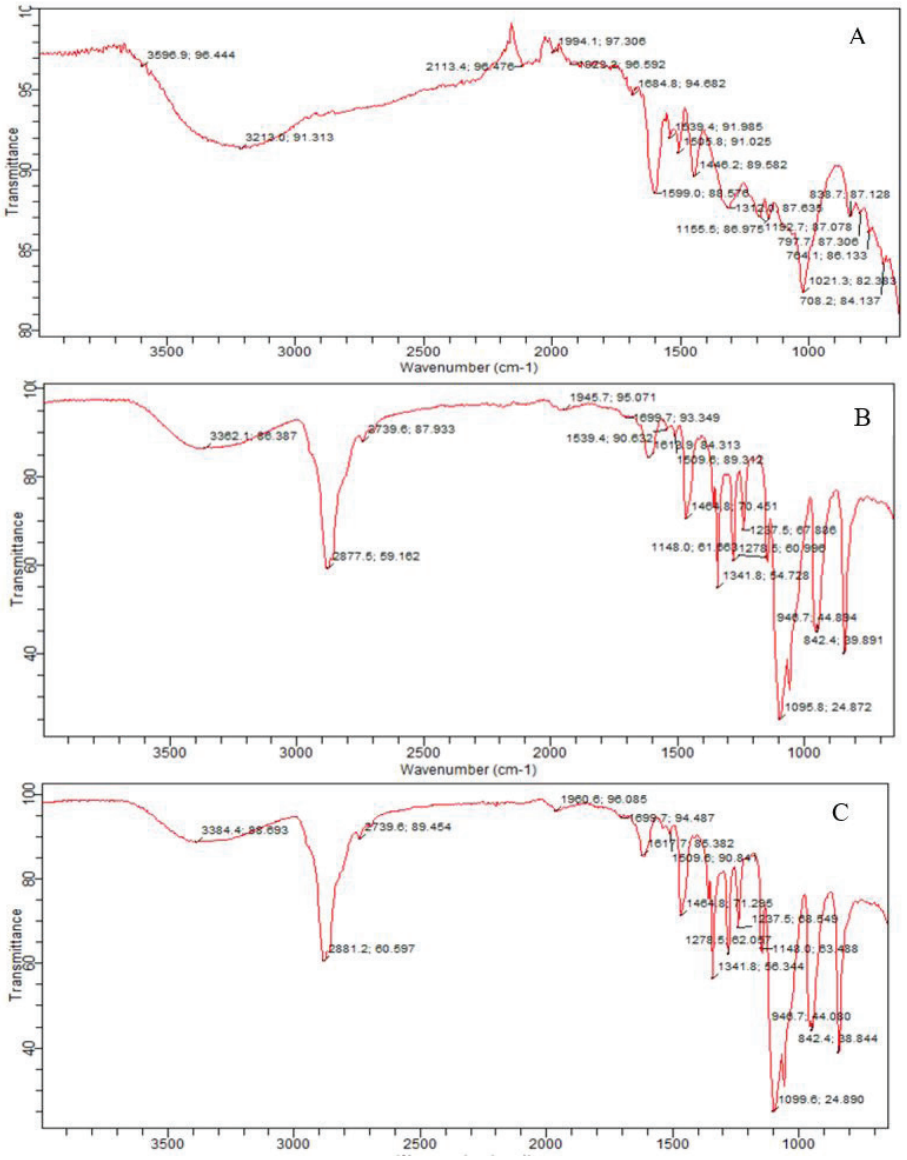

Figure 1. FT-IR spectra of PREx (A), physical mixture of PREx and PEG 4000 (B), solid dispersion of PREx and PEG 4000 prepared by melt-fusion (C).

Table 3. Thermal properties of PREx and solid dispersion formulations.

Parameter	PREx	P3	M3
Onset temperature (°C)	59.99	61.53	34.26
Peak temperature (°C)	177.74	86.07	75.88
Conclusion temperature (°C)	299.96	101.11	228.74
Enthalpy of crystallization (J/g)	779.45	692.00	614.92
Crystalline melting temperature range; ΔT (°C)	239.97	39.58	194.48

In vitro dissolution studies

Results of *in vitro* dissolution studies for the solid dispersion formulations prepared by the solvent-evaporation, melt-fusion method, physical mixture, and crude extract alone (PREx) are presented in Figure 3. Extract release from the formulations was observed to be polymer concentration-dependent irrespective of the method of preparation. However, the melt-fusion formulations showed the highest release across the formulations except for M1, containing the least polymer concentration, which had a lower release rate than S1 but greater than P1 and the crude extract.

Release of extract from all the formulations including PREx in the first 5 min was between 0.06 and 95% with the crude extract having the least release and M3 exhibiting the highest release. However, release of extract at the end of dissolution test time (60 min) from all the solid dispersion formulations and physical mixtures was observed to increase appreciably; each formulation showed an increase in extract release with time, the values ranged between 17.07 and 100%. Conversely, PREx was found to have released < 1% at the end of the dissolution time.

Visual inspection of the formulations during dissolution revealed M3 to be completely dissolved producing a clear solu-

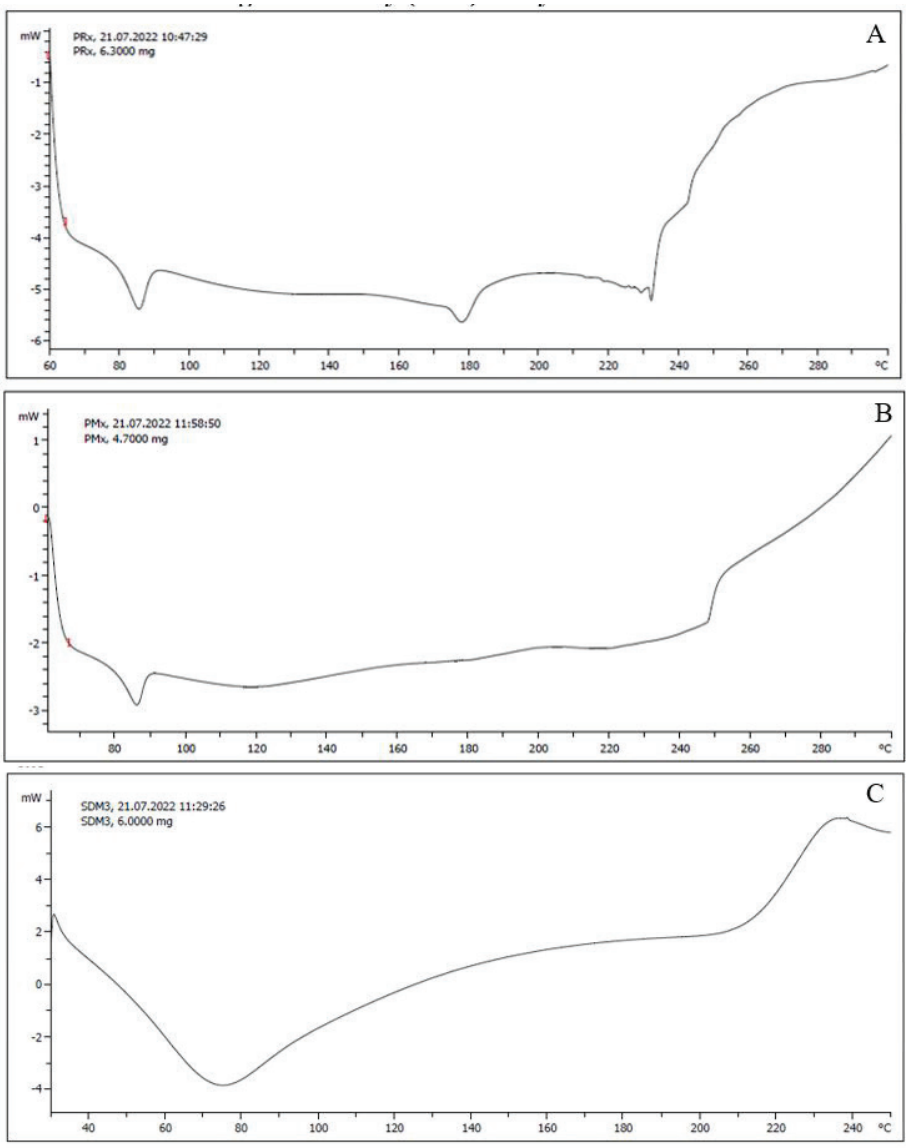

Figure 2. Differential Scanning Calorimetry thermogram of PREx (A), physical mixture of PREx and PEG 4000 (B), solid dispersion of PREx and PEG 4000 prepared by melt-fusion (C).

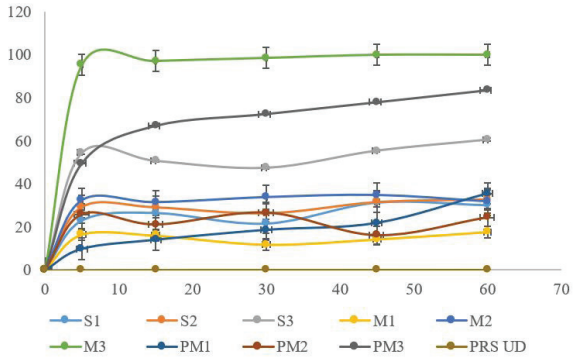

Figure 3. *In vitro* release profile of the different solid dispersion formulations, physical mixture, and extract alone.

tion while M1 and the crude extract showed very slow dissolution and produced residues due to incomplete solvation in the media. Formulation M1 (ratio 1:1) prepared by the melt-fusion method was observed to be sticky and formed agglomerates after preparation and did not completely disintegrate in the dissolution media.

At the end of the dissolution time, M3 was observed to have complete release (100%) while P3 had about 83% and S3 had about 61% release.

Modelling of extract release profile

The release kinetics used to extrapolate the mode of extract release from all the formulations were Zero order, First order, Higuchi, and Hixson-Crowell models. The *in vitro* release model with the highest coefficient regression factor was deemed the best model; Table 4 shows that the kinetic release was best expressed by the Higuchi model with the highest regression factors. The Korsmeyer-Peppas release coefficient (n) was used to determine the mechanism of release from the formulations. Table 4 shows that solid dispersion formulations containing higher polymer concentration (S3 and M3) and the physical mixture with corresponding high polymer concentration (P3)

Table 4. Kinetics and mechanism of PREx release from solid dispersion formulations.

Formulations	Zero order	First order	Higuchi	Hixson-Crowell	Korsmeyer-Peppas	
	r^2	r^2	r^2	r^2	r^2	n
S1	0.4958	0.2699	0.7123	0.2618	0.7275	0.9870
S2	0.3889	0.2784	0.6187	0.2741	0.6911	0.9035
S3	0.3903	0.2546	0.6100	0.2435	0.6851	0.7097
M1	0.2812	0.2880	0.4708	0.2875	0.6408	1.1134
M2	0.3703	0.2866	0.5620	0.2856	0.6806	0.8990
M3	0.3284	0.2948	0.5822	0.2397	0.6821	0.6856
P1	0.9209	0.2354	0.9220	0.2130	0.8586	0.9296
P2	0.1678	0.2956	0.3572	0.2985	0.6134	0.9489
P3	0.6287	0.0887	0.8622	0.0735	0.7648	0.7821
PREx	0.6174	0.2890	0.8542	0.2889	0.4900	0.1837

had "n" values between 0.6856 and 0.7821 while all the other formulations (S1, S2, M1, M2, P1, P2, PREx) had 'n' values between 0.8990 and 1.1134.

DISCUSSION

The product yield from all the formulations was substantial and shows the efficiency of the preparation process. However, low product yields observed from the solvent-evaporation method could be attributed to the dissolving action of the solvent.

The spectrum of PREx (Figure 1A) was characterized by principal absorption peaks and broad characteristic peaks at 3596.9cm^{-1} which corresponds to the stretching vibration band of O-H. This band is associated with polyphenolic compounds like tannins, flavonoids, and glycosides (Nagalakshmi & Anuradha, 2017; Olorunsola, Adedokun, Olisakwe, & Alozie, 2022). Peaks at 2113.4, 1599.0, and 1312.0cm^{-1} reveal the presence of other functional groups; alkanes, ketones, and aromatic groups. A characteristic identity of the extract which is its peculiar fingerprint is revealed between 1021.3 and 708.2cm^{-1}. The characteristic peak of the extract disappeared at 3213.9.0cm^{-1} in Figures 1B and 1C (spectra of P3 and M3) but peaks at 2877.5 and 2881.2cm^{-1} were observed which is attributable to the polymer (PEG). An earlier report has shown that PEG has a characteristic peak between 2800 and 2900cm^{-1} which is associated with C-H stretching (Khairuddin et al., 2016) and this characteristic peak was observed in our results. Smoothening of the extracted peak in the presence of the polymer observed in Figures 1B and 1C suggests possible strong integration of the extract and the polymer in both the physical mixture (P3) and solid dispersion prepared by the melt-fusion method (M3). Although slight shifting, broadening, and reduction in peak intensity were observed in the solid dispersion mixtures (Figures 1B and 1C), no additional peaks were observed indicating that there was no chemical interaction between the extract and excipients used in the preparation of the solid dispersion formulations.

Differential scanning calorimetry (DSC) thermogram showed the thermal characteristics of the investigated samples. DSC is capable of providing data about the molecular transition of molecules, conversions of different crystalline phases, and thermal stability of materials (Fernandes et al., 2013). The DSC thermogram shown in Figure 2 shows the differences in thermal characteristics which in turn demonstrates the impact of solid dispersion formulation on the physical characteristic of the extract. The lower onset melting temperature of M3 which was remarkably lower than that of PREx or P3 indicates rapid mobility of amorphous chains. Similarly, the low peak temperature and conclusion temperature of M3 compared to PREx point to the fact that the crystalline nature of the extract has been greatly diminished. Broadening of the extract's endothermic melting peak in P3 could be attributed to the melting of small quantities of the extract signifying that a large percentage of the extract was incorporated in the polymer carrier (Bikiaris et al., 2005). Conversely, the absence of the extract peak in M3 could suggest the dissolution of crystalline extract into the molten carrier resulting in transformation into an amorphous form or a lack of crystallinity of the extract in M3 (Xie et al., 2009). This confirms the dispersion of the extract at the molecular level in the polymer carrier matrix (Ghanem, Ali, El-Shanawany, & Ibrahim, 2013).

Our result is in tandem with the literature where the absence of the melting peak of an active drug correlated with the amorphous nature of the drug (Xie et al., 2009; Weerapol et al., 2017; Yen, Liang, Cheng, Hsu, & Wu, 2020). It also corroborates an earlier report that the decrease in intensity and shifting of sharp melting peaks of drugs in solid dispersion is an indication that the amount of crystallinity is considerably reduced, and the drug is present in an amorphous form (Sharma, Jain, & Tanwari, 2013).

The reduction in enthalpy of crystallization observed in the thermogram infers that the energy required to solubilize the extract in P3 is lower than that required to solubilize the crude extract (PREx) itself and much lower for M3. ΔH is directly related to the crystalline nature of a material; it reflects the melting of the crystalline region in a material. A low crystalline melting temperature range (ΔH) indicates a reduction in crystalline linkages (Bhupender, Rajneesh, & Baljeet, 2013; Olayemi et al.,

2021; Unnisa et al., 2022). This shows that the extract in solid dispersion formulation had lost its crystallinity which is a precursor to the desired effect of improving the extract's aqueous solubility.

In vitro, the release profile of the formulations shows the extent of improvement in the rate of dissolution of the extract. As observed, agglomerates in formulation M1 did not readily get dispersed upon contact with water as such, M1 did not readily go into the solution. Thus, the entrapped extract in the polymer-drug matrix in M1 had a very low dissolution rate (Kale, Hapgood, & Stewart, 2009). On the other hand, the highest dissolution profile seen with M3 could be attributed to its amorphous nature as observed in the DSC thermogram which may be responsible for its rapid release. This is because lower kinetic energy was required to break any crystal lattices within the dispersion mixture leading to rapid release and improved bioavailability (Ghaste, Chougule, & Shah, 2009). Generally, the poor dissolution profile of the crude extract (PREx) was observed to be improved in the physical mixture and much more improved in the solid dispersion formulations. Improved dissolution of the extract is attributable to the hydrophilic properties of the carrier; PEG 4000 which includes increasing wettability, dispersibility of the poorly soluble extract, and surface area available for dissolution by reducing the interfacial tension between the extract and medium (Jigar, Jayvadan, & Jain, 2012). These features are thought to have led to possible molecular dispersion of the extract in the dissolution mixture leading to a marked dissolution rate of all the solid dispersion formulations. Furthermore, the incorporation of higher polymer concentrations produced a remarkable increase in extract release across the formulations.

The method of preparation of the solid dispersions was seen to greatly influence the rate of release from these solid dispersions. Solvent-evaporation method of solid dispersion is known to solubilize the drug and carrier at the molecular level while the fusion technique is said to improve molecular mobility between the drug and the carrier molecules, especially at the melting points of the components of the dispersion mixture (Pawar, Mundhe, Deshmukh, Pandhare, & Nandgude, 2021). Both methods are known to be good strategies for improving drug release but our result shows that the melt-fusion method was better at improving the solubilization of the crude extract of *Prosopis africana* stem bark. This is an advantage in further processing and probable commercialization because the melt-fusion method is a simple and economical process devoid of possible residual solvent in the formulation and high cost of preparation as a result of the solvent-evaporation method (Tran et al., 2019).

The coefficient of correlation (r^2) from the plots was used to indicate the degree of curve fitting and values approaching 1 were used to determine the predominant dissolution profile fitting to the mathematical equation. Interpretation of the *in vitro* release data as determined by the Higuchi model which produced the highest r^2 values suggests that the kinetic release was accomplished by diffusion from the porous matrix system of the polymer and extract in the solid dispersions upon contact with the dissolution fluid (Hamid, Harris, Jaweria, & Rabia, 2006; Azadi, Hamaid, & Rouini, 2013). On the other hand, the Korsmeyer-Peppas model was applied to determine the mechanism of release from the formulations and the release diffusion coefficient (n) was used to characterize the mechanism of release. When the "n" value is 0.45, it indicates the release is diffusion -controlled which is also known as Fickian diffusion. Values ≥ 0.89 indicate swelling-controlled release (Case II or Super-case II transport) while "n" values between 0.45 and 0.89 indicate Non-Fickian or anomalous diffusion which is a superimposition of the other two mechanisms (Siepmann & Peppas, 2001). The extract release for M3, P3, and S3 was found to be Non-fickian/anomalous diffusion which was characterized by the movement of the dissolution fluid into the dispersion matrix at constant velocity leading to an increase in the amount of fluid absorbed with time. This corresponds to the fact that extract release was controlled by simultaneous diffusion out of and erosion of the dispersion matrix (Azadi et al., 2013; Gouda, Baishya, & Qing, 2017). The fast dissolution of these formulations could be ascribed to the possible conversion of the crystalline extract into its amorphous form thus making dissolution faster as shown by the DSC thermograms. Extract release from the other formulations (S1, S2, M1, M2, P1, P2, PREx) was indicative of super case II transport signifying that drug release was primarily by swelling of the polymer matrix, which is accompanied by slow drug release. This could be the reason why drug release from these formulations was slower than those of S3, M3, and P3 as observed in the *in vitro* dissolution test. This suggests that release of the extract was complicated by molecular relaxation and functional interaction between the fluid and the dispersion matrix (Odeku, Okunlola, & Lamprecht, 2013; Camelo, Franceschi-Messant, Perez, Girod, & Ré, 2016). The result shows that release from the optimized formulation (M3) was by super-imposition of swelling and then diffusion mechanism.

CONCLUSION

In this study, solid dispersion formulations of the methanol stem bark extract of *Prosopis africana* (PREx) were successfully prepared by solvent evaporation and melt-fusion using the hydrophilic polymer; polyethylene glycol 4000.

DSC analysis results showed the transformation of extract crystallinity into its amorphous form, dissolution studies also indicated improved solubility of PREx. Significant enhancement of dissolution of the extract was obtained from formulations prepared by the melt-fusion method containing the highest polymer concentration; 98%release was achieved at the end of 30 min. This shows the possibility of enhancing dissolution of the poorly water-soluble *Prosopis africana* extract by solid dispersion however, substantiating this effect by clinical evaluation could broaden its application as a potential therapeutic agent.

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Review Article

In vitro dissolution testing methods for inhaled drugs

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ABSTRACT

Mimicking the lung environment has always been a challenge with regards to dissolution testing of inhaled drugs from dry powder inhalers (DPIs). The aim of this review is to critically appraise the literature currently available on the in vitro test methods for dissolution of orally inhaled drug particulates. Reasons for the lack of standardised testing methods are discussed. Currently, there is not one test that fully represents the situation that occurs in the lungs in vivo, and this is the reason for the lack of a dissolution test recommendation by the pharmacopoeia. The importance of dose collection as a prerequisite to dissolution testing is also discussed using the Andersen cascade impactor as an example. Moreover, a study was carried out to determine the most robust method for testing the dissolution of fluticasone. Three different testing methods were used, i.e., the Transwell system, the paddle-over-disk method and DissolvIt. The results of this study determined that the paddle-over-disk method had the fastest dissolution rate. However, the data showed that there was a lack of similarity between all three tests. This lack of similarity between dissolution methods contributes to the reason why there is no standardised recommended dissolution method listed in the pharmacopoeia. Whilst the paddle-over-disk method yielded the fastest dissolution rate, it does not mean that it is reflective of in vivo dissolution.

Keywords: Pulmonary drug delivery, particle dissolution, inhalation biopharmaceutics

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INTRODUCTION

In recent years, the pulmonary route has become an increasingly favourable route for the delivery of medication to treat local pulmonary conditions (e.g., asthma, chronic obstructive pulmonary disease) and systemic diseases (e.g., diabetes mellitus). Reasons for the latter include, the highly perfused nature of the lungs in addition to the large surface area (>100 m^2) and ultra-thin epithelium (0.2 – 0.7 µm) of the alveoli (Agu et al., 2001). Despite these advantages, drug delivery via the pulmonary route still poses some challenges for pharmaceutical scientists.

Drug administration via the pulmonary route is non-invasive and allows for the absorption of macromolecules and small drug molecules. The lungs provide an increase in drug permeability than that of the gastrointestinal route. Drugs administered via this route also avoid the process of first-pass metabolism by the liver (Patton & Byron, 2007).

The speed of absorption via the pulmonary route is faster than any other non-invasive route of drug administration. This allows for drug absorption in seconds, and therefore, can be very useful in treating symptoms that require a rapid response (Patton et al., 2004).

The human lungs are extremely complex in nature, and can be subdivided into the conducting zone and the respiratory zone. Whilst the lungs are an invaluable route for drug administration, innate clearance mechanisms can sometimes limit their benefit. The epithelium within the conducting zone is lined with cilia and mucus designed to clear particles that are present here via mucociliary clearance (Henning et al., 2010). Thus, pulmonary drug dissolution is influenced by a range of factors including inhalation of the drug particles, deposition, absorption, mucociliary clearance (MCC) and phagocytosis by macrophages (Figure 1).

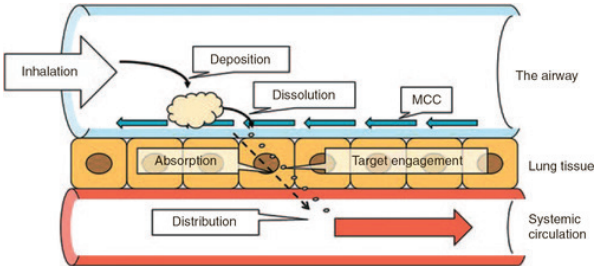

Figure 1. Schematic diagram of drug deposition, dissolution, mucociliary clearance (MCC), absorption and distribution within the lung (from Bäckmann et al., 2014).

The dissolution properties of a drug are a critical determinant of its bioavailability and therapeutic efficacy. They represent the process by which a solute disintegrates in a solvent to yield a solution. Dissolution testing is routinely used in testing solid oral dosage forms. It serves as a promising tool for predicting the release and dissolution of drugs *in vivo*. It can also be used to obtain a waiver for *in vitro* bioavailability studies in the Biopharmaceutics Classification System (BCS) (Klein, 2019). The US Food and Drug Administration (FDA) have set out a guidance for the dissolution testing of solid oral dosage forms. This guidance includes the standardised dissolution testing methods that must be used (basket method USP 1 and paddle method USP 2), the conditions that must be maintained during testing and the acceptance criteria that is required (US FDA, 2018). However, such guidance does not yet exist for orally inhaled drug products.

When a drug is inhaled orally from a dry powder inhaler (DPI), it is deposited onto the epithelium of the lung and must dissolve into the lung fluid before it can exert its action. The process is vital and acts as the rate-limiting step for absorption into systemic circulation as well as a prerequisite for local action (Rohrschneider et al., 2015).

The dissolution behaviour of a drug can be assessed using a number of different *in vitro* dissolution tests. Such tests can give us an indication of how this process occurs *in vivo*. However, to date, there is no standardised *in vitro* dissolution test for assessing the dissolution behaviour of orally inhaled drug products (US FDA, 2018). This lack of predictive *in vitro* dissolution tests makes it harder to determine the dissolution behaviour that an orally inhaled drug is likely to possess *in vivo* (Floroiu et al., 2018). According to the European Medicines Agency (EMA), there are a number of pharmaceutical development studies that a drug must initially undergo. These include aerodynamic particle size distribution testing, physical characterisation testing and testing of the rate at which the drug is delivered. Dissolution testing, however, is not a requirement set out by the EMA or the US FDA (EMA, 2006).

This lack of standardised dissolution testing makes it difficult to analyse the fate of drug particles in the lung after they are deposited. Limitations in the testing methods and biological clearance is the main reason for this. In this review, we aim to first outline the barriers which prevent the development of a reliable dissolution testing method for orally inhaled drug particles. We then review the importance of dose collection as a prerequisite to dissolution testing. Next, the benefits and limitations of the various methods used to carry out dissolution tests on orally inhaled drug particles are assessed, and finally, the results of a short study which was carried to compare the different dissolution profiles of fluticasone propionate are discussed.

STATE OF THE ART

Although, there is no pharmacopeial method for dissolution testing of orally inhaled drugs, there are still a wide variety of methods that have been developed for this purpose. This section of the paper aims to discuss these methods as well as discussing the factors that influence drug dissolution in the lungs.

Barriers to dissolution testing of orally inhaled drug particles

There are many limitations and barriers that occur when trying to simulate a dissolution test for orally inhaled particles. Some of these limitations relate to physiological conditions in the body that are extremely difficult to replicate *in vitro*. Others relate to pitfalls in the testing apparatus that prevent them from

being reflective of conditions *in vivo*. This lack of *in vitro-in vivo* correlations (IVIVC) means that *in vitro* testing is not fully predictive for the outcome of pharmacokinetic studies for orally inhaled formulations and there is, therefore, a need for the assessment of drug dissolution using *in vivo* testing techniques (Fröhlich, 2019).

Mimicking the lung environment in still a major limitation to the development of a reliable dissolution testing method for orally inhaled drugs (Radivojev et al., 2019). Mucociliary clearance of drug particles from the lung to the oral cavity and clearance by phagocytosis are parameters that occur *in vivo* but cannot be represented *in vitro*. These clearance mechanisms can also act as a barrier to the therapeutic effectiveness of the drug (Labiris & Dolovich, 2003). Mucus is secreted on top of the epithelium by goblet cells and submucosal glands. Cilia present in the trachea extend to the terminal bronchioles where they are in contact with the mucus. This thick mucus acts as a physical and chemical barrier to drug particles. When insoluble drug particles get trapped in the mucus, they are moved to the oropharyngeal region by mucociliary clearance and cough (Figure 2). These particles are mixed with saliva and are swallowed. This mechanism means that not all of the administered drug is dissolved and absorbed (Patton et al., 2010). Mucociliary clearance is a natural biological response and is one that may be difficult to replicate *in vivo*. Therefore, an *in vitro* dissolution test that does not take into account these clearance mechanisms cannot accurately reflect the conditions that occur *in vivo*.

Another clearance mechanism in the lungs is phagocytosis by alveolar macrophages in more peripheral regions. Once particles are internalised by phagocytosis, they under degradation by lysosomal enzymes. The extent of this mechanism is dependent on the number of particles deposited and the number of alveolar macrophages. This clearance mechanism affects dissolution and makes it more difficult for a robust *in vitro* dissolution test to be developed (from Ruge et al., 2013).

Another limitation to dissolution testing of orally inhaled drugs is the lack of sink conditions that occur in the lung. Sink conditions must be maintained during standard dissolution testing to maximise biological relevance (Radivojev et al., 2019). According to the European Pharmacopoeia, sink conditions represent a volume of solvent or dissolution media that should be at least 3-10 times the volume present in the drugs saturated solution. The lungs have a large surface area (>100 m^2), however, the volume of liquid in the respiratory tract of a healthy human is between 10-20 ml. These figures vary in those suffering with pulmonary diseases (Floroiu et al., 2018). However, most dissolution apparatus use non-physiological volumes of dissolution ranging between 60-1000 ml in order to maintain sink conditions (May et al., 2015). These conditions are not reflective of conditions *in vivo* and contribute to the difficulty in developing a robust dissolution test for inhaled drugs. In recent years, this has been improved by the use of Transwell inserts which only require a small amount of dissolution media (May et al., 2015).

Agitation and stirring of the dissolution media are commonly seen in dissolution testing of orally inhaled drugs. The rate of stirring increases as the volume of the dissolution vessel decreases, i.e., smaller vessels need faster stirring rates (Radivojev

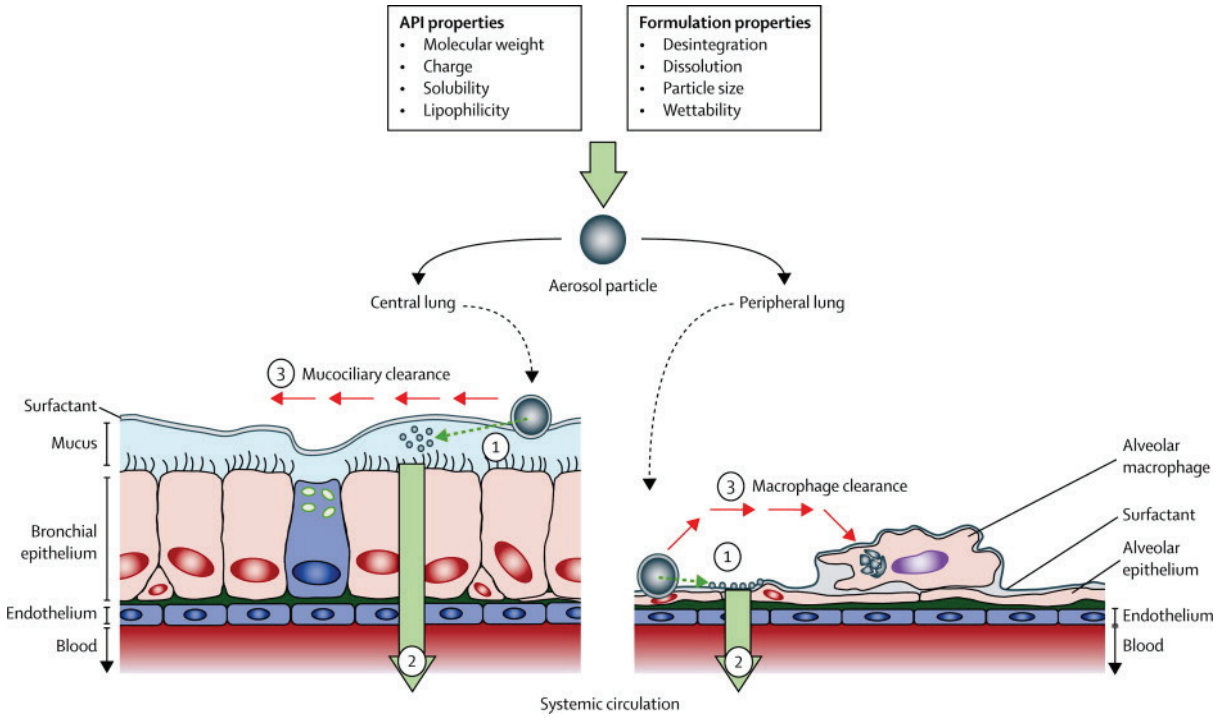

Figure 2. Biological clearance mechanisms of drug particles after inhalation. Another clearance mechanism in the lungs is phagocytosis by alveolar macrophages in more peripheral regions. Once particles are internalised by phagocytosis, they under degradation by lysosomal enzymes. The extent of this mechanism is dependent on the number of particles deposited and the number of alveolar macrophages. This clearance mechanism affects dissolution and makes it more difficult for a robust *in vitro* dissolution test to be developed (from Ruge et al., 2013).

et al., 2019). Stirring increases dissolution rates. However, the fluid in the lungs is not subjected to mixing forces. Therefore, the use of agitation *in vitro* is not reflective of the physiological conditions that occur *in vivo* (Shaji & Shaikh, 2016). The combined use of large vessels and agitation causes on overestimation of the amount of dissolution that is likely to occur *in vivo*.

In order to mimic *in vivo* conditions, the simulated lung fluid (SLF) used in the tests must be almost identical to the lung lining fluid. However, it is difficult to create an exact copy of SLF that contains the authentic mucus and the correct proteins present in the lung (Riley et al., 2012). The simulated fluid also needs be stable and easy to reproduce. Sometimes, different fluids may be needed to reflect the variations in the composition of the lining fluid along the respiratory system (Radivojev et al., 2019). However, it is difficult to produce SLF due to their complex formulations. They have been proven to be unsuitable for pH dependent drugs due to their low buffering capacity. For one dissolution test, the SLF caused an increase in pH from 7.4 to 8.8 over a 24-hour period (Floroiu et al., 2018).

Surfactants or lung surfactant preparations, for example, Survanta® is sometimes added to the dissolution media especially when analysing drugs with poor soluble drugs. The addition of these surfactants can increase the dissolution rate, solubility and wettability. One surfactant used is dipalmitoyl phosphatidylcholine (DPPC) which is present as the most abundant surfactant in the lungs. However, preparation of this surfactant is variable and time consuming, and so, synthetic surfactants are often used as an alternative (Riley et al., 2012).

Effect of aerodynamic particle size distribution (APSD) on dissolution

There are three main types of pulmonary drug delivery systems available to patients. These are: dry powder inhalers (DPIs), pressurised metered dose inhalers (pMDIs) and nebulisers. In DPI's, the drug particles are often bound to lactose carrier particles. This mix of particles are deagglomerated and the carrier particles are impacted on the walls of the upper airways while the drug particles move down into the lung. DPIs are generally the most favourable pulmonary delivery system as they are breath actuated and easier to use

(Kwon et al., 2020). Aerodynamic particle size is a crucial factor that effects drug dissolution. In order for a drug particle to reach the lungs, it must be small enough to avoid retention in the mouth but big enough to avoid being exhaled back into the environment. Aerosols with a mass median aerodynamic diameter (MMAD) of >10 µm are usually impacted in the oropharyngeal region by inertial impaction. Aerosols <1 µm remain suspended in the air and are therefore, moved out of the respiratory tract upon further exhalation (Shaji & Shaikh, 2016). Thus, the ideal particle size at which aerosols can be deposited in the lung and undergo dissolution is at a mass mean aerodynamic diameter of 1-5 µm (Labiris & Dolovich, 2003).

Examples of *in vitro* particle collection techniques accepted by the Food and Drug Administration (FDA) include the eight stage Andersen Cascade Impactor (ACI) at 60 L/min, the seven stage Next Generation Impactor (NGI) or the four stage Multi-stage Liquid Impinger (MSLI) at 60 L/min (Fröhlich, 2019). This usually represents the first step involved in *in vitro* dissolution testing and involves the collection of the appropriately sized drug particles on a membrane which can then be coupled to a chosen dissolution setup (Tay et al., 2018)

The ACI is an instrument consisting of eight stages. The apparatus is pressure sealed and a vacuum is applied throughout the system. There is a collection plate between each stage. There is a nozzle between each stage which narrows as you move down the impactor (Figure 3). If the particle diameter is too large, the particle will not continue to move in the airstream through the impactor, and will instead be impacted on the collection plate (Andersen, 1958).

The aerosolised dose will be present on the plate at the bottom of the chamber and can then be used for dissolution testing. Often, a polyvinylidene difluoride (PVDF) membrane filter is placed on the last collection plate in order to aid transfer of the aerosolised particles into the dissolution apparatus (Riley et al., 2012).

The next generation impactor (NGI) is another method of classifying aerosolised particles based on their size. It consists of seven stages with removable impaction cups that allow for assay (Yoshida et al., 2017). The impactor operates at an inlet flow rate between 30 and 100 L/min. The apparatus also contains a micro-orifice collector (MOC) which is used to capture extremely small particles which can then be coupled to the dissolution apparatus (Marple et al., 2003). Whilst the dose collec-

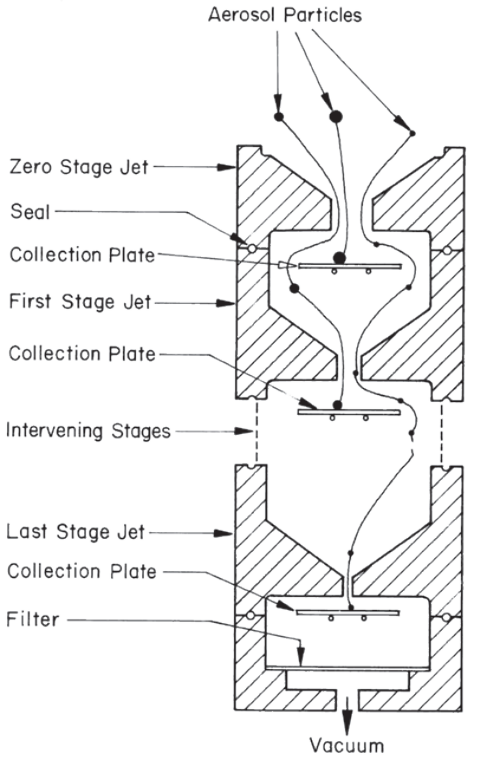

Figure 3. Schematic of an Andersen Cascade Impactor (ACI) (from USP)

tion step can be avoided and dissolution testing can be carried out using the active pharmaceutical product (API), performing tests on the aerosolised particles is the most accurate way to reflect the conditions that occur *in vivo*.

Dissolution testing methods for orally inhaled drug particles

After particle collection of the aerosolised drug using the ACI, dissolution testing can occur. The drug present on the membrane can then be transferred directly into the dissolution apparatus. Here, the drug will be released through the membrane and dissolution can be assessed (Frenning et al., 2020). There are different techniques used to carry out dissolutiontesting. Each technique has its own advantages and limitations.

USP 2 Paddle Apparatus

The Paddle Apparatus is the most common *in vitro* dissolution technique that has been used ever since it was founded in 1978 (Dokoumetzidis & Macheras, 2006). This method is easy to set up and can be used to the analyse dissolution behaviour of a range of different dosage forms. The basic apparatus consists of a semi-hemispherical vessel containing the dissolution medium at a volume up to 1000 ml. The vessel is immersed in a water bath, and inside the vessel, there is a paddle which agitates the system (Deepika et al., 2018). A common adaptation involves the placement of the membrane filter from the ACI into the dissolution vessel. The membrane is usually sandwiched with another membrane before it is placed in the vessel. This method is known as the "paddle over disk" method. The main advantage of this adaptation is that it allows different types of particle collection filters to be used in the dissolution vessel. However, the filter may act as a hinderance to wetting and can increase diffusion layer thickness (Riley et al., 2012). The large volumes of dissolution media in the vessel and the presence of agitation means that this technique, although robust, is not reflective of the conditions that occur in the lungs.

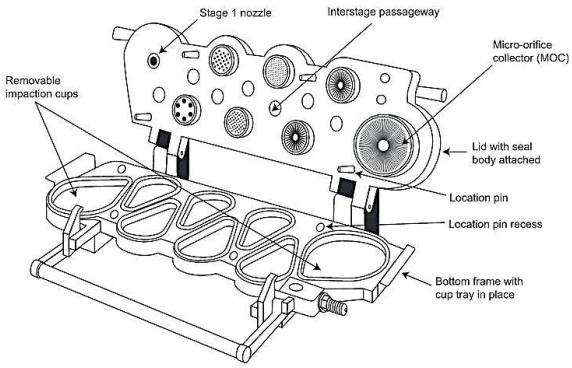

Figure 4. Schematic of a Next Generation Impactor (from Marple et al., 2003).

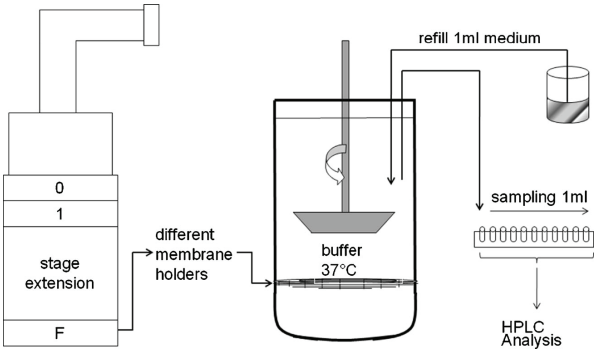

Figure 5. Schematic of an Andersen Cascade Impactor (ACI) coupled to Paddle Over Disk apparatus (from May et al., 2014).

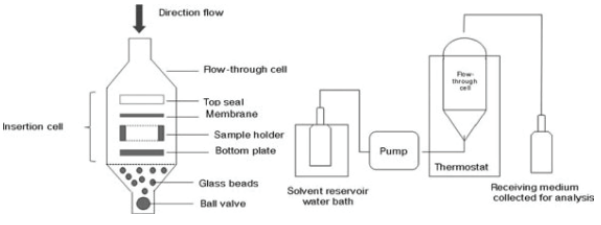

Figure 6. Schematic of an open flow through cell system (from Olejnik et al., 2012).

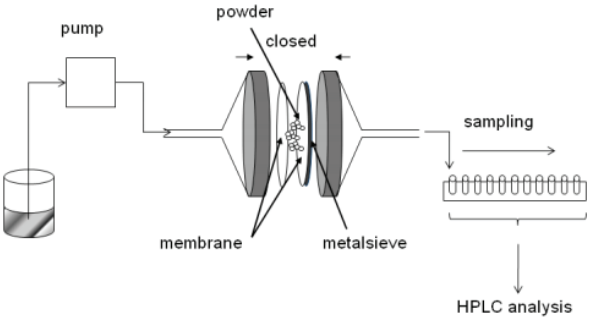

Figure 7. Schematic drawing of the modified flow through cell (from May et al., 2012).

USP 4 Flow Through Cell Apparatus

The flow through cell dissolution method is an analytical method that has been used for decades and was implemented into the European Pharmacopoeia in 2007 (29). It assesses dissolution based on the flow of the medium containing the drug through a cell. The medium is pumped through the flow through cell using a pulsating piston pump with a flow rate between 4 and 16 ml min^{-1} (McDonnell et al., 2018). The bottom of the cell contains small glass beads approximately 1 mm in diameter (Singh & Aboul-Enein, 2006). The cell mainly operates as an open system whereby fresh medium is continuously piped through the cell. The alternative is a close system where the medium is recycled through the cell (Fotaki & Reppas, 2005). This method is often used as the preferred method for dissolution testing of poorly soluble drugs (Eaton et al., 2012).

This technique has been modified by Boehringer Ingelheim to a flow through cell which features the particle collection membrane filter taken straight from the ACI. This modification occurred in order to ensure sink conditions, uniform flow and homogenous wetting (May et al., 2012). The filter is covered with another membrane filter and is placed into the cell where it is held in place using a filter holder. The dissolution medium is pumped through the cell by a HPLC pump and the fractions are collected. Whilst this technique is beneficial in terms of keeping the system homogenous, the high velocity of the

dissolution medium does not represent the behaviour of lung fluid *in vivo*. This system is also sensitive to entrapped air (Floroiu et al., 2018).

Franz Diffusion Cell

The Franz diffusion cell is a membrane type dissolution method that was first conducted by Thomas J. Franz in 1975. The original set up of the apparatus was used to test the permeability of a membrane, however, this is modified to allow for dissolution testing. This modification consists of dissolution media in a vessel with a volume of up to 1 litre to allow sink conditions. The membrane with the aerosolised particles from the ACI can be taken directly and placed on top of the vessel in a membrane holder (Radivojev et al., 2019). The system is agitated with a stirring bar that is located within the vessel. The system is heated to physiological temperature and condensed drops fall onto the particles on the membrane and stimulate dissolution. Samples are taken and the solvent removed is replaced with fresh dissolution medium in order to maintain a constant volume (May et al., 2012) This method is favourable as it takes into account the air liquid interface that is present in the lungs. However, there are still conditions that occur which do not represent *in vivo* dissolution such as the presence of agitation. It can also be challenging to distinguish between diffusion effects through the membrane and the dissolution rate (Riley et al., 2012).

Transwell® System

The Transwell system is another membrane type dissolution method, however, unlike the Franz cell there is no agitation present in the Transwell apparatus (Riley et al., 2012). The Transwell system only requires a small volume of dissolution medium which is more reflect of biological conditions present in the lung (Velaga et al., 2018). After aerosol deposition in the ACI, the membrane containing the required drug particles is removed and placed facing down onto the semi-permeable polyester membrane of the Transwell insert. One point 4 millimetres of the dissolution media is poured over the membrane to initiate the dissolution process. Aliquot samples of half a millimetre are taken and this is replenished with fresh dissolution medium in order to maintain the initial volume (Arora et al., 2010). The system must be maintained at a temperature of 37°C and a relative humidity of 100% (Floroiu et al. 2018). In order to determine the influence of mucus on dissolution, porcine tracheal mucus can be coated onto the Transwell insert (Cingolani et al., 2019). This method is robust; however, it does not take into account the sink conditions that occur in the lungs. In order to mimic the sink conditions that occur *in vivo*, a high diffusion coefficient and low retention must be present (Riley et al., 2012).

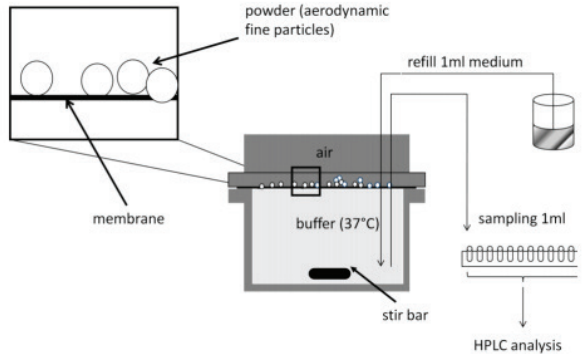

Figure 8. Schematic diagram of the modified Franz diffusion cell (from May et al., 2012).

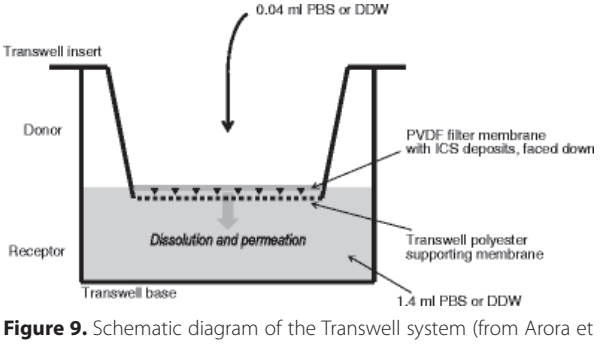

Figure 9. Schematic diagram of the Transwell system (from Arora et al., 2010).

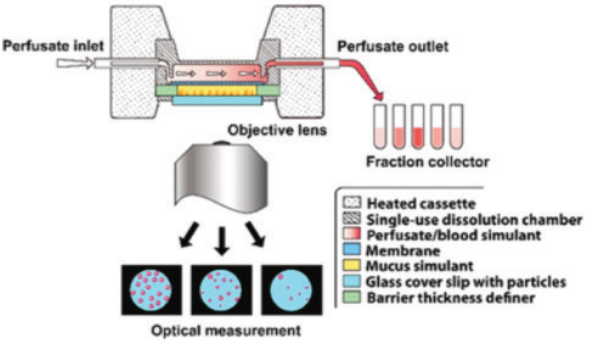

Figure 10. Schematic diagram of the DissolvIt dissolution apparatus (from Gerde et al., 2021).

DissolvIt Apparatus

This is a stimulation tool for dissolution and absorption testing of inhaled dry powders. Simulated blood acts as the dissolution medium and it is pumped through the system so that it flows across a membrane that is in contact with the aerosolised particles (Floroiu et al. 2018). These conditions mimic the air-blood barrier that exists in the upper airways. However, it must be noted that the air-blood barrier in the DissolvIt system is of greater thickness than the epithelium in the deep lung, which may increase the retention time *in vitro* (Floroiu et al. 2018). The particles are dissolved in a mucus stimulant and present on a glass cover slip. Over the mucus is a polycarbonate membrane which represents the basal membrane in the respiratory tract. As the simulated blood passes over the membrane, it absorbs the dissolved constituents of the particles and can then be analysed using mass spectroscopy. DissolvIt is capable of producing pharmacokinetic profiles of fluticasone propionate that resemble that in the rat lung. This shows that it may be useful for *in vivo in vitro* correlations in orally inhaled drugs dissolution testing (Börjel et al., 2015). Whilst this system looks promising, unfortunately, there is not a lot of reported data on its performance in dissolution testing (Hassoun et al., 2019).

A comparison of different dissolution testing methods using fluticasone propionate (FP) as an example

This study aimed to compare the dissolution profiles of FP using three different dissolution methods. The methods analysed include the Transwell system with a 0.4 µm polyester membrane, the paddle-over-disk method and the DissolvIt system.

MATERIALS AND METHODS

This study looked at the results obtained from dissolution testing of FP that have already been carried out. Various tests of the different dissolution testing methods were analysed. For the purpose of this study only the tests that used FP as the drug were used. This allows for comparison of the results obtained from each of the studies.

The Transwell system dissolution test, as described by Rohrschneider *et al.,* involved the use of a Flixotide DPI inhaler containing 100 µg of FP per actuation. The inhaler was actuated five times into an Andersen cascade impactor. The aerosolised dose present on the filter paper at the last stage was then transferred directly into the Transwell system. The Transwell system consisted of a 6-well plate and 1.5 mL of dissolution media (0.5% SDS in PBS). The dissolution test was initiated by pouring 0.1 mL of dissolution media over the filter paper. Samples were taken at various time points and the removed volumes were replaced with fresh medium. The samples were analysed using HPLC analysis (Rohrschneider et al., 2015).

The paddle-over-disk method as described by Price *et al.* involved the use of a Flixotide DPI inhaler containing 100 µg of FP per actuation. The next generation impactor was used to collect the aerosolised dose. The collected dose was then transferred onto a 50 mm diameter stainless steel disk with a 74-mesh screen. This disk was placed within a vessel containing 300 mL of dissolution medium (0.2% SDS in PBS). The paddle was set to a speed of 75 rpm. Samples were taken at various time points and the removed volumes were replaced with fresh medium. The samples were analysed using HPLC analysis (Price et al., 2020).

The DissolvIt method, as described by Hassoun *et al.,* involved the use of a Flixotide DPI inhaler containing 50 µg of FP per actuation. The aerosolised dose was collected using the US Pharmacopoeia Induction Port No.1 which is a standardised simulation of the throat. The aerosolised particles were placed on a glass cover slip. The dissolution media (5.7 µL of Survanta) was applied to the polycarbonate membrane. Perfusate containing phosphate buffer and 4% w/v albumin was streamed over the membrane at a flow rate of 0.4 mL/min. Samples were taken at various time points and the removed volumes were replaced with fresh medium. The samples were analysed using LC-MS analysis (Hassoun et al., 2019).

The numerical data for each of the three tests was found by extrapolation of graphs from each study. The points were extrapolated at the same time points for each of the studies. These data points could then be graphed as connected scatterplots using Microsoft Excel and were also combined in order to allow the various results to be compared (Figure 11).

The three sets of numerical data could then be analysed and compared by performing a one-way ANOVA test. This allowed for comparison of the three different means. It also gave a p value and F value for significance which allowed the null hypothesis to be rejected. In order to assess where exactly the differences occur between the three groups, a post-hoc test was conducted using a Bonferroni correction. The Bonferroni correction value can be determined using the following formula:

$$\text{Bonferroni corrected p value} = \alpha/n$$

where α is 0.05 and n is the number of tests being compared.

The data was also compared by determining the similarity (f_2) and the difference (f_1) factor. Similarity and difference factors are often used to compare two or more dissolution profiles. The difference factor (f_1) represents the percentage difference between two dissolution profiles at each timepoint. It is calculated using the following equation:

$$f_1 = \left(\frac{\sum_{t=1}^{n} |R_t - T_t|}{\sum_{t=1}^{n} R_t} \right) \times 100$$

where n is the number of time points, R_t is the mean dissolution value for the reference at time t, and T_t is the mean dissolution value for the test at the same time point. If f_1 is lower than 15 (0-15), there is no difference between the two dissolution profiles.

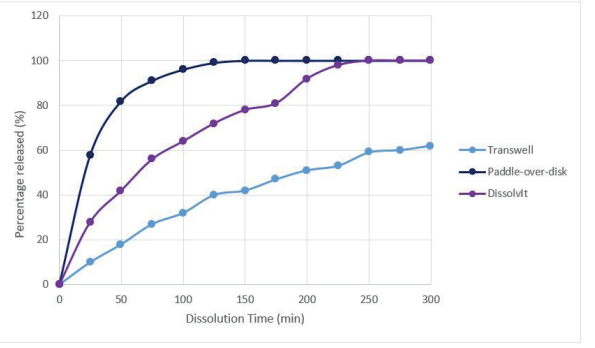

Figure 11. Comparison of fluticasone propionate dissolution profiles.

Table 1. Similarity and difference factor results.

	Transwell System vs. paddle-over-disk	Transwell System vs. DissolvIt	DissolvIt vs. paddle-over-disk
Similarity Factor (f_2)	13	23	30
Difference Factor (f_1)	144	88	23

The similarity factor measures the similarity that exists between two dissolution profiles at each timepoint. It can be measured using the following equation:

$$f_2 = 50 \times \log_{10}\left[\frac{100}{\sqrt{1+\frac{\sum_{t=1}^{n}(R_t-T_t)^2}{n}}}\right]$$

where n is the number of time points, R_t is the mean dissolution value for the reference at time t, and T_t is the mean dissolution value for the test at the same time point. An f_2 value of greater than 50 (50-100) indicates that two dissolution profiles are similar (Diaz et al., 2016).

Comparison of the three graphs show that the paddle-over-disk system has the fastest dissolution rate with all of the FP being dissolved by 150 min. DissolvIt also reaches 100% dissolution, however, at a slower rate than the paddle-over-disk set up. The Transwell system has the slowest dissolution rate with only 62% of the drug dissolved by the time the study was ceased at 300 min.

Calculation of dissolution similarity and difference factor:

The results for the similarity factor (f_2) calculation shows that all three dissolution profiles lack similarity. A f_2 value of greater than 50 indicates that the dissolution profiles of the two methods are similar. All of the results obtained are below 50, which indicates that the profiles are dissimilar.

The results for the difference factor (f_1) show that there is a great variation between the dissolution profiles. A f_1 of less than 15 indicates that there is no difference between the dissolution profiles. The results obtained are well above 15, thus indicating the variability that exists between the dissolution profiles.

The results of the ANOVA test show that there is a significant difference between the dissolution profiles obtained for the three different testing methods. For data to be statistically significant, it must yield a p-value of less than 0.05. The p-value in this study is 0.00024812. This result means that the null hypothesis must be rejected in favour of the alternative hypothesis.

Post hoc analysis

Bonferroni correction value: 0.05/3 = 0.0167

A post hoc was conducted to find out exactly where the differ-

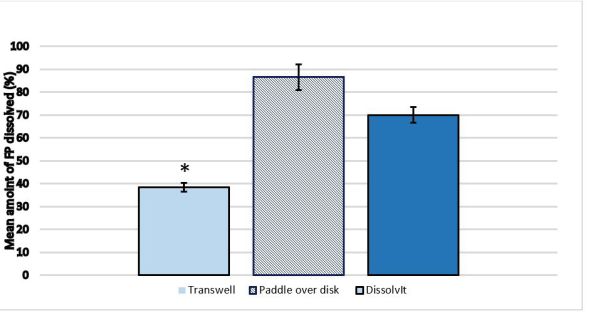

Figure 12. Comparison of the three mean FP dissolved with error bars representing the standard errors of means and an asterix (*) to show a difference in significance.

Table 2. ANOVA test results.

ANOVA: Single Factor					
SUMMARY					
Groups	**Count**	**Sum**	**Mean**	**Variance**	
Transwell	13	501	38.54	398.10	
Paddle over disk	13	1126	86.62	821.42	
DissolvIt	13	911	70.08	986.41	
ANOVA					
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**
Between Groups	15511.5	2	7755.7	10.5475901	0.00024812
Within Groups	26471.2	36	735.3		
Total	41982.7	38			

Table 3. Post hoc test results.

	P-value	**< 0.0167**	**Significant**
Transwell vs. Paddle Over Disk	0.0000455655	YES	YES
Transwell vs. DissolvIt	0.005429612	YES	YES
Paddle over disk vs. DissolvIt	0.173584067	NO	NO

ences noted from the ANOVA test lie. The p-value was determined using the Bonferroni correction. The results show the Transwell system is statistically different from both the paddle-over-disk and DissolvIt methods. Thus, the null hypothesis must be rejected. When comparing the paddle-over-disk to DissolvIt, the p-value obtained was greater than 0.0167. Thus, the null hypothesis can be accepted.

DISCUSSION

Whilst dissolution testing is a very important parameter for the quality control of solid dosage forms, there is currently no standardised dissolution testing method recommended for orally inhaled drugs (Floroiu et al., 2018). However, the importance of testing the rate of dissolution prior to drug absorption has been emphasised since the introduction of the Biopharmaceutics Classification System (BCS) (Rohrschneider et al., 2015).

Dissolution testing is highly beneficial in drug development and can often be used to obtain a biowaiver for *in vivo* bioequivalence studies (Ku, 2008). However, the lack of a fully validated dissolution method makes it difficult to produce an *in vitro - in vivo* correlation (IVIVC) for orally inhaled drugs (Fröhlich, 2019). The main reason for this is the lack of an *in vitro* dissolution testing method that accurately mimics the physiological conditions that occur *in vivo*. Each of the testing methods comes with its own limitations as discussed in this paper.

As well as limitations in the testing methods, there are also certain biological events that occur in the lung that make it difficult to develop a suitable dissolution test. The two main events being clearance by the mucociliary escalator and clearance by phagocytosis (Labiris & Dolovich, 2003). These clearance mechanisms do not occur *in vitro*. Many *in vitro* dissolution testing methods also feature non-sink conditions and agitation, both of which do not occur in the lungs. The presence of these conditions creates an overestimation of dissolution *in vitro,* and therefore, make it difficult to compare this to dissolution behaviours *in vivo*. The presence or absence of lung surfactant also affects *in vitro* dissolution. Ideally, dipalmitoyl phosphatidylcholine (DPPC) is the preferred surfactant as it is the same one that is present in the lungs. However, synthetic surfactants are often used as they are more reproducible (Riley et al., 2012).

A number of academics have previously carried out dissolution tests on orally inhaled drug particles. These studies analyse the dissolution profiles obtained when using the different methods individually (18), the influence of simulated lung fluid composition with or without surfactant (Kumar et al., 2017) and the effect that varying the membrane pore size has on dissolution (Frenning et al., 2020). Since data exists in these areas, it was decided that this study would look at the different methods previously tested, and compare them to each other to determine the most suitable method. Fluticasone propionate was chosen as the drug to study due to its prevalence in treating asthma and other respiratory conditions.

The results obtained from this study show a great deal of variability between the different methods. The results show that the paddle-over-disk method yielded the fastest dissolution rate (Figure 11). This method saw 99% of all fluticasone dissolved at 125 min. A separate study conducted by Velaga *et al.* similarly found that the paddle apparatus is advantageous for dissolution testing due to its discriminatory power and its reproducibility (Velaga et al., 2018). Despite this, it is hard to accept that this method is suitable for orally inhaled drugs. The paddle apparatus uses large volumes of dissolution media and stirring in order to maintain sink conditions (Floroiu et al., 2018). These conditions do not in any way reflect the *in vivo* dissolution process of orally inhaled drugs.

FP in the Transwell system and DissolvIt dissolves slower than it does in the paddle-over-disk apparatus (Figure 11). However, these methods are more reflective of the lung physiology due to the presence of membranes representing the air-liquid interface. The volumes (which appear to have a significant impact on the dissolution rate) of dissolution media are much lower, and thus reflect biorelevant conditions. However, these systems still have their own limitations such as the fact that diffusion acts as the rate limiting step which can make it difficult to distinguish between diffusion through the membrane and the drug dissolution rate (Riley et al., 2012).

The data obtained from the three different systems was then compared statistically by performing a one-way variance (ANOVA) test, where $p<0.05$ is significant. The results of this test produced a significant F and p value (Table 2). The p value obtained is less than 0.05 (i.e., 0.00024812), which means that the null hypothesis must be rejected. This indicates that there are differences that exist between the three dissolution profiles. This is expected as the conditions and the limitations vary between the different tests.

A post hoc test using the Bonferroni correction then had to be conducted to determine where exactly the difference lies between each of the systems. The results (Table 3) show the Transwell system is significantly different to the other two testing methods. The lack of similarity determined from these tests can be backed up with the results of the similarity (f_2) and difference (f_1) factor calculations (Table 1). All of the f_1 values above 15 indicate that there are major variations between the dissolution profiles. The f_2 values are also less than 50, which indicates that the dissolution profiles lack similarity. The results involving the Transwell system produced the greatest difference factor (f_1) values and the least similarity factor (f_2) values. This is expected as the post hoc test results show that the Transwell system is the one that is significantly different to the other testing methods.

Overall, the results show that despite the inaccuracy in reflecting the *in vivo* dissolution process, the paddle over disk apparatus appears to be the fastest and most discriminatory dissolution method. The Transwell system appeared to be the technique with the greatest amount of variability. It was also the slowest dissolution method, and had not reached completion by the time that the test was ceased at 300 min. However, a previous study noted that the use of Transwell inserts acted as a barrier to diffusion especially for poorly water-soluble inhaled corticosteroids. When the 0.4 µm Transwell polyester membrane was

switched for porcine mucus layers present on a glass microfiber with a pore size of 3.0 µm, there was a significant improvement in the dissolution rate (Alqahtani et al., 2020).

The Transwell insert used in this study was a polyester membrane with a pore of 0.4 µm. However, if the study is replicated using the same alteration as Alqahtani *et al*., more significant and comparable results may be seen.

The thickness of the particle/perfusate barrier could have also influenced the results obtained for the Transwell system and DissolvIt. The results for dissolution using DissolvIt showed that complete dissolution occurred, but at a slower rate than that of the paddle-over-disk apparatus. This slower dissolution rate is most likely attributed to the fact that the perfusate barrier has a thickness of 60 µm. This would cause retention of the drug particles, and thus, give rise to a slower dissolution rate. In the isolated perfused lung of a rat, this barrier has a thickness of 0.5-5 µm (Börjel et al., 21015). The paddle over disk system avoids this complication as the particles are in direct contact with the perfusate. As a result, retention of drug particles does not occur.

Taken together, the results show the degree of dissimilarity that exists between each of the systems. The paddle over disk method is the fastest and most reproducible, however, of the three, it least represents physiological conditions. DissolvIt acts as a good comparison to physiological conditions, however, it is limited by its overestimation of retention time as a result of a thick air-blood barrier (Floroiu et al., 2018). The Transwell system also serves as a good comparison, however, it is limited due to the varying membrane pore sizes and the lack of a concentration gradient that reflects *in vivo* conditions.

In order to be able to produce comparable dissolution tests, future areas of research need to be considered. These areas include:

- The use of lung cell lines in *in vitro* dissolution tests.
- The use of simulated lung fluid as the dissolution medium instead of phosphate buffered saline (PBS)
- The need for the development of *in vitro in vivo* correlation (IVIVC)
- The development of an artificial lung simulation where dissolution can be tested (Marques et al., 2011).

CONCLUSIONS

This paper aimed to critically review the available literature on dissolution testing of orally inhaled particles. It discussed the different testing methods available and touched on their benefits as well as their limitations. The methods were compared to each other and it was determined that the variability that exists between the methods makes them incomparable.

To achieve this aim, the dissolution profile of fluticasone propionate using different dissolution methods was determined and analysed. The methods studied were the Transwell system, the paddle-over-disk apparatus and the DissolvIt method. The results obtained showed us a lack of similarity between all three methods. This lack of comparability between dissolution methods contributes to the reason why there is no standard dissolution testing method set out by the pharmacopoeia.

In order for a standardised validated dissolution test to be developed for orally inhaled drug particles, certain parameters that accurately reflect *in vivo* conditions must be defined. These parameters are:

- Type of dissolution apparatus
- Composition and recommended volume of the dissolution medium
- Method of aerosolization and sample collection of particles
- Quantification of tested particles

This project also examined the method of aerosolised dose collection using the Andersen cascade impactor (ACI). It is essential that the particles are homogenous and of a mean aerodynamic diameter between 1 – 5 µm before dissolution commences. This is to ensure that the particles being tested are only those that would make it down into the lung and are capable of being dissolved here.

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Review Article

Importance and review of drug metabolite synthesis

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ABSTRACT

Phase I and Phase II metabolic reactions are involved in the pharmacokinetic properties of drugs after administration. These reactions mainly aim to make drugs more polar and eliminate them safely. However, some of these metabolites have the potential to exhibit a toxicological effect. Industry and/or academia have to consider these metabolites in terms of their pharmacodynamic and pharmacokinetic properties. These metabolites are not only residual intermediates from the synthetic process of the main drug but also unique structures produced by metabolic enzymes in the human organism. Thus, metabolite synthesis by synthetic or semi-synthetic methods is a key feature in the pharmaceutical industry. In this review, synthetic methods of the metabolites from all known metabolic pathways are reviewed from the literature. It was observed that both synthetic and semi-synthetic methods require more attention as they are as important and complex as drug synthesis. Moreover, it showed that there was much more research available for Phase I than Phase II in the literature.

Keywords: Drug metabolite, metabolism, synthesis, oxidation, Phase I, Phase II

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INTRODUCTION

Biotransformation reactions are divided into two main classes known as Phase I and Phase II. Phase I reactions are the functionalization of a parent compound by introducing polar chemical moieties, then making them more soluble in water media. Insertion of new polar functional groups into the parent compound is performed by oxidation, reduction, and hydrolytic reactions. Phase I reactions can be carried out by either enzymatic cytochrome P450 (CYP), Flavin-containing monooxygenases (FMO), esterase, and amidases or hydrolytic at the physiological pH. In the Phase II reactions, Phase I metabolites or endogenous polar molecules are conjugated with the large hydrophilic groups (Foti & Dalvie, 2016; Low & Castagnoli, 1991). These conjugative reactions are mediated by specific enzymes (glucuronosyltransferase, sulfotransferase, and *N*-acetyltransferase) that each lead to their specific conjugate such as sulfate, glucuronate, glycine, etc. (Mulder & Burchell, 1990). Drug metabolism is one of the most important steps in ADMET studies. Thus, the synthesis of metabolites is an important process in drug metabolite profiling, metabolite stability, as well as pharmacological activity testing, metabolite quantification, toxicity testing and metabolism-based drug interaction (Rollas, 2007). The US Food and Drug Administration (FDA) guidelines for metabolites in safety testing declare the acceptable metabolite/drug ratios in drug development (Food Drug Administration (FDA), 2008). Metabolites above the 10% parent drug should be subjected to safety testing in terms of tolerability according to the metabolites in safety testing (MIST) approach by a group of scientists (Baillie et al., 2002). The approach that finds the abundance is more important than the percentage causes a debate. In this discussion, scientists from the pharmaceutical industry proposed that the abundance approach should be considered as it is more reliable in terms of dose, chemical structure, and various parameters (Smith & Obach, 2005). The development of synthesis methods is needed to obtain authentic metabolites. Particularly, the synthesis of metabolites has a crucial role in pharmaceutical industry operations for producing a large number of pure metabolites to perform pharmacokinetic and pharmacodynamics studies. Biotransformation reactions, from the leading pharmacological activation of drugs, have been involved in aliphatic or aromatic carbon hydroxylation, epoxidation, heteroaromatic oxidation, reduction, glucuronidation, sulfation, acetylation, and other metabolic pathways. The synthesis of metabolites that are not easily carried out by chemical methods can be produced by microbial biotransformation (Asha & Vidyavathi, 2009; de Paula et al., 2015; Di Nardo & Gilardi, 2012), using the plant cultured cells as a biocatalyst (Ishihara, Hamada, Hirata, & Nakajima, 2003). Liver microsomes of various species have been used for *in vitro* metabolism studies and they are commercially available (Krebsfaenger, 2007). The biosynthesis of drug glucuronides may be performed using human liver microsomes in combination with uridine 5'-diphosphoglucuronic acid (Uldam, Juhl, Pedersen, & Dalgaard, 2011).

In this respect, this review reported several examples of the chemical and biotechnological (Schroer et al., 2010) synthetic methods of drug metabolites in favor of metabolism and pharmacologic activity studies of the pharmaceutical industry (Fox & Gibas, 1953; Fura et al., 2004; Genovino, Sames, Hamann, & Toure, 2016; Kuo et al., 2004; Lombardino, 1981; Obach, 2013) and the analysis method of drugs and their metabolites (Kostiainen, Kotiaho, Kuuranne, & Auriola, 2003; Protti et al., 2020).

The synthesis of Phase I metabolites

The purpose of Phase I reactions is to introduce a polar functional group –OH, -COOH, -NH_2, -SH into the drugs and other xenobiotic molecules. These functional groups can also be released by the hydrolysis of esters or amides and the dealkylation of ethers, thioethers, and secondary amines.

Oxidation

One of the most important reactions of Phase I metabolites is oxidation. Most of the drugs, xenobiotics, and dietary compounds are metabolized by CYP enzymes which are also known as the microsomal mixed-function oxidase system. CYP enzymes are located primarily in the endoplasmic reticulum.

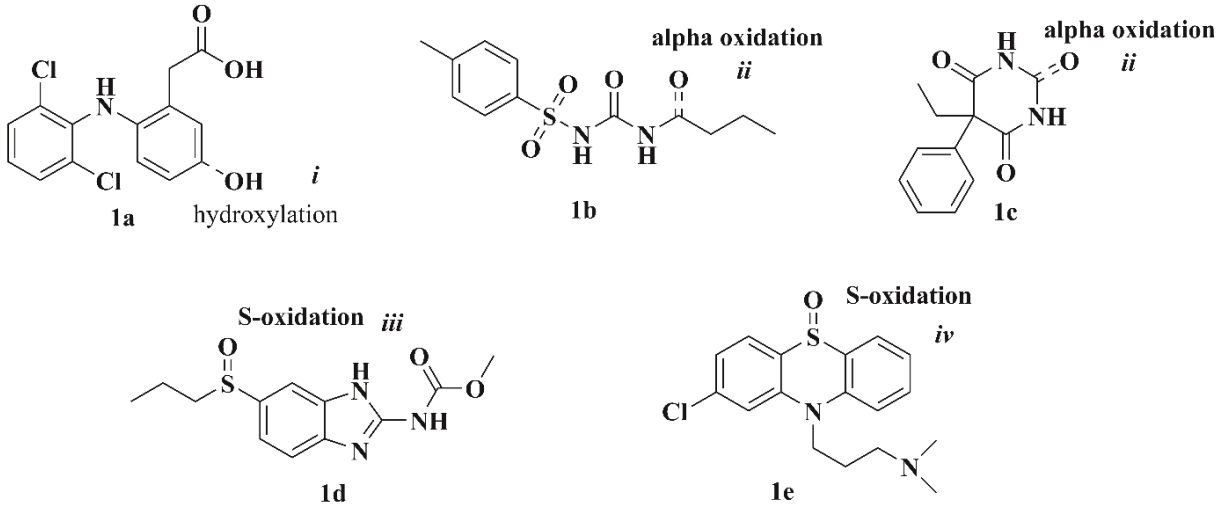

Figure 1. Structure of diclofenac (1a), tolbutamide (1b), primidone (1c), albendazole (1d), and chlorpromazine (1e) and their synthesized metabolites. (i: 10 F/mol, 4eq NaHSO3, ACN 1:1 H2O; ii: 6 F/mol, NaHCO3, ACN 1:1 H2O; iii: 1.5 F/mol, NaHCO3, ACN 3:1 H2O; iv: 4 F/mol, NaHCO3, ACN).

The largest amount of CYP enzymes is found in the liver and they can also be found in intestinal, adrenal, and other tissues (Schroer et al., 2010).

Flavin monooxygenases (FMOs) are involved in the Phase I drug metabolism of a nucleophilic hetero atom containing drugs, xenobiotics, and dietary compounds to their sulfoxide or N-oxide metabolites (Gao & Zheng, 2019; Geier et al., 2015). Phase I metabolites of some drugs such as diclofenac, tolbutamide, primidone, albendazole, and chlorpromazine (Figure 1) were synthesized via preparative scale continuous-flow electrosynthesis method (Stalder & Roth, 2013).

The oxidation of aromatic and aliphatic compounds

Aromatic hydroxylation is a major route of metabolism for many drugs. Generally, hydroxylation occurs at the 4- position of the aromatic ring. Most of the phenolic metabolites undergo further conversion to polar glucuronide or sulfate conjugates.

Patric et al. synthesized hydroxylated metabolites of methylphenidate (Patrick, Kilts, & Breese, 1981). As shown in Figure 2, the nitrile group of α-(2-pyridyl)-α-(4-methoxyphenyl)acetonitrile **(2a)** is partially hydrolyzed to the corresponding amide (**2b**) at room temperature. The direct hydrolysis reaction is not preferred because of the possibility of decarboxylation. Then **2b** is reduced by Adam's catalyst to obtain a 20:80 *erythro / threo* mixture of **2c**. At this stage, column chromatography or fractional crystallization manages only to isolate *erythro* configuration of **2c**. However, it is possible to separate *erythro /threo* mixture by fractional crystallization after hydrolysis using HBr, although the quantification for *threo* (**2d**) is quite low. Thus, first epimerization is made by using KOH to obtain *threo* compound (**2d**). **2d** and **2e** were subjected to Fischer esterification in methanol.

Acetaminophen is widely used drug as analgesic and antipyretic. Valero et al. (Valero, Lozano, Varon, & Garcia-Carmona, 2003) reported the enzymatic synthesis of catechol metabolite of acetaminophen that is not commercially available (Figure 3).

Toxic metabolites such as *N*-acetyl-p-benzoquinone imine (NAPQI) of acetaminophen synthesised by Dahlin and Nelson (Dahlin & Nelson, 1982) from acetaminophen and silver oxide (Figure 4).

Recombinant human CYPs expressed in *Escherichia coli* are suitable biocatalyst for the synthesis of drug metabolites. Vail et al. (Vail, Homann, Hanna, & Zaks, 2005) stated that the synthesis of anabolic testosterone metabolite 6β-hydroxytestosterone human cytochrome P450 3A4 with NADPH-P450 reductase (NPR) was expressed in *E. coli*.

The expected human drug metabolites have been used in several model systems. One of these is microbial transformation (Asha & Vidyavathi, 2009).

Moody et al. (Moody, Freeman, Fu, & Cerniglia, 2002) produced the metabolites of antidepressant mirtazapine using the fungus *Cunninghamella elegans* as a model of mammalian metabolism. As shown in Figure 5, 8-hydroxymirtazapine obtained as a major metabolite after 96 h.

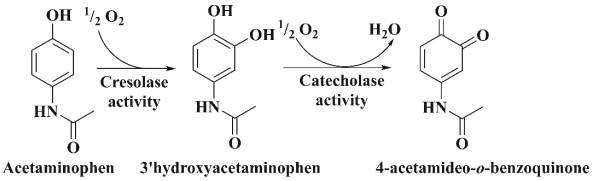

Figure 3. Representation of enzymatic synthesis of 3′-hydroxyacetaminophen from acetaminophen.

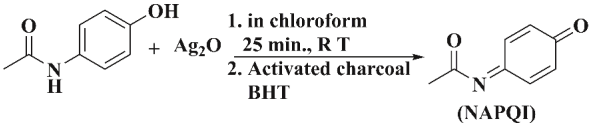

Figure 4. Synthesis of N-acetyl-p-benzoquinone imine (NAPQI) from acetaminophen.

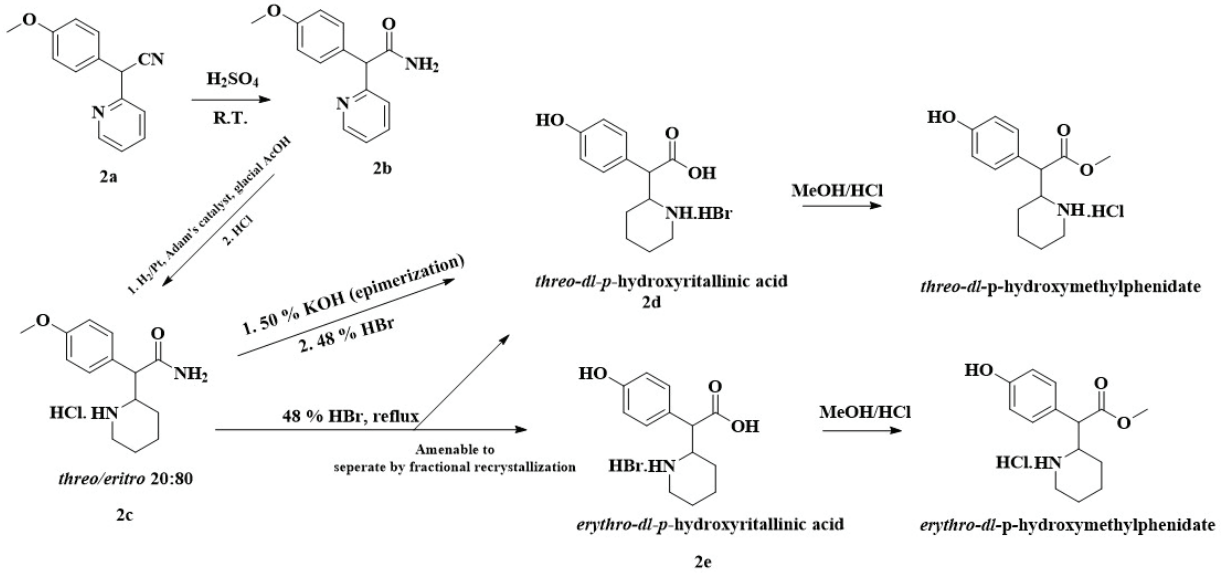

Figure 2. Synthesis pathway for hydroxylated metabolites of methylphenidate.

As shown in Figure 6, Otey et al. (Otey, Bandara, Lalonde, Takahashi, & Arnold, 2006) reported the hydroxylation of propranolol by variant P450 BM3 heme domain (BM3-H) 9C1. The variants of P450 BM3 have been evaluated from *Bacillus megaterium*.

Sawayama et al. (Sawayama et al., 2009) demonstrated a group of variants for cytochrome P450 BM3 from *Bacillus megaterium*. Verapamil and asterimizole metabolites have been produced by P450 BM3 variants.

Weis and coworkers achieved a hydroxylation reaction by using bifunctional cytochrome P450 P450 enzymes (Weis et al., 2009). This biotechnological application included the preparation of metabolites of diclofenac and clorzoxazone which are 4'-hydoxydiclofenac and 6-hydroxychlorzoxazone by biohydroxylation. The mentioned reaction of diclofenac and chlorzoxazone is shown in Figure 7.

Rinnofner et al. (Rinnofner, Kerschbaumer, Weber, Glieder, & Winkler, 2019) reported the hydroxymetabolites of ibuprofen using *Pichia pastoris* as a catalyst. In this study, the synthesis made in the presence of catalyst and then products were lyophilized. Subsequently preparative LC-MS analysis was performed. The spectral data of the products obtained in this way were compared with the previous studies and the metabolites were given as a percentage. Accordingly, 83% of 502 mg ibuprofen was converted into its metabolites. Of these, 30% is 2-OH ibuprofen, 37% is 1-OH ibuprofen and 16% is an unknown metabolite (Figure 8).

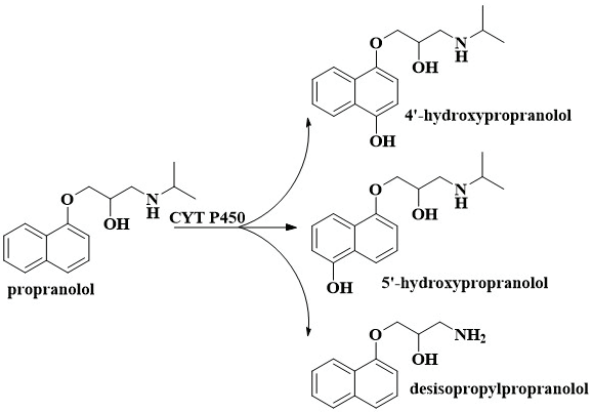

Figure 6. Three metabolites of propranolol formed by cytochrome P450.

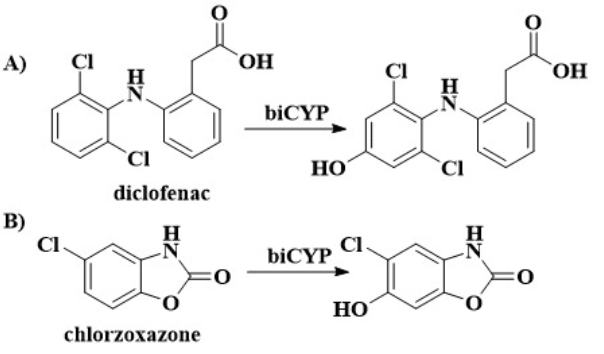

Figure 7. Biohydroxylation of A) diclofenac and B) chlorzoxazone by bifunctional P450s (biCYPs).

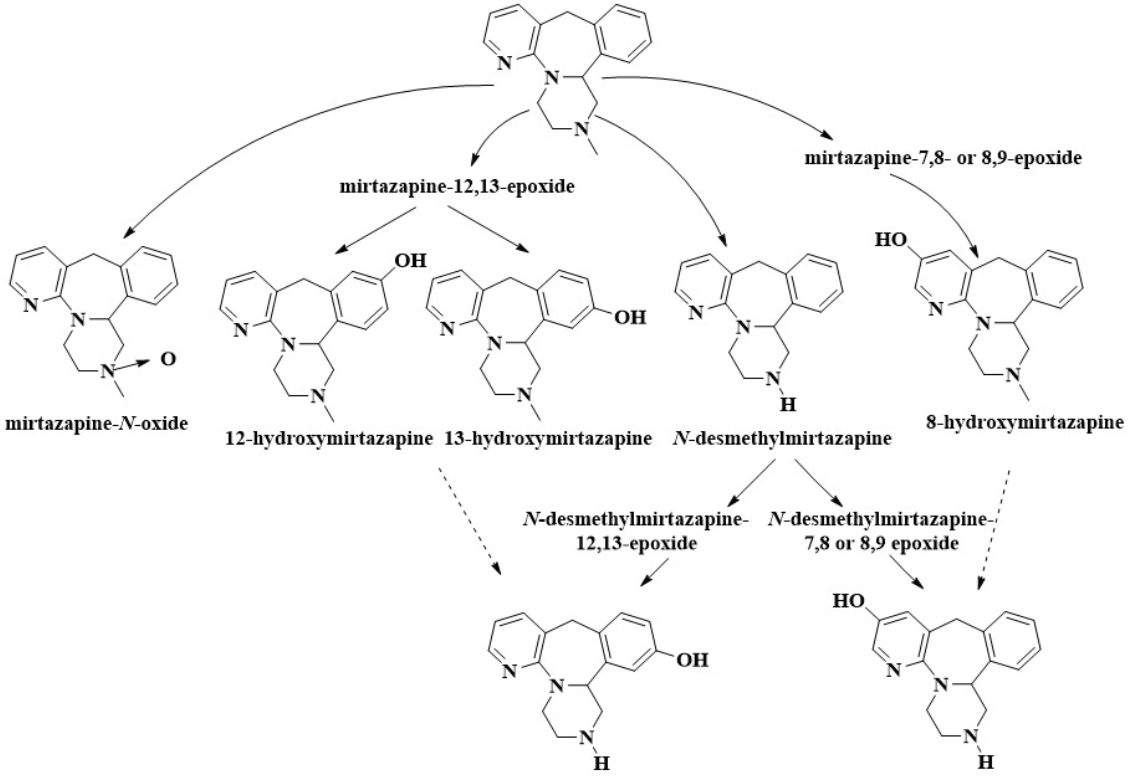

Figure 5. Schematic suggestion for biotransformation of a racemic mixture of mirtazapine to give it all metabolites after incubation with *Cunninghamella elegans* after 168 h.

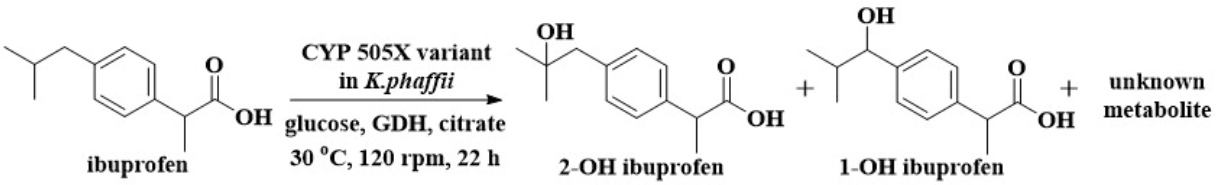

Figure 8. The summarized biooxidation reaction of ibuprofen.

Kuo et al. (Kuo et al., 2004) synthesized various putative Phase I and Phase II duloxetine metabolites. The major metabolite of duloxetine is hydroxylated metabolite in the naphthalene ring. 4/5/6-hydroxyduloxetine (Figure 9) has been synthesized with the same method. The synthesis of other metabolites of duloxetine was given as a reference.

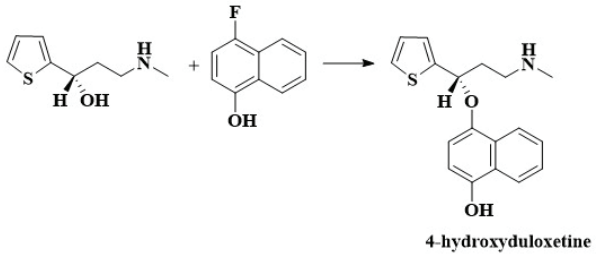

Figure 9. Synthesis and structure of 4-hydroxyduloxetine.

Lombardino reported the synthesis of piroxicam monohydroxylated metabolites using 2-amino-hydroxypyridines (Lombardino, 1981). The multistep pathway is summarized in Figure 10.

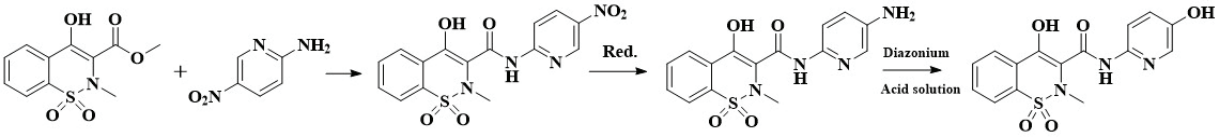

Figure 10. 4-The multistep synthesis pathway for monohydroxylated piroxicam metabolite.

Steinbrecht et al. (Steinbrecht et al., 2020) reported the UPOs for the metabolites synthesis of cytostatic drug cyclophosphamide. 4-Hydroxycyclophosphamide metabolite was performed by a peroxygenase from *Marasimius rotula* (Figure 11).

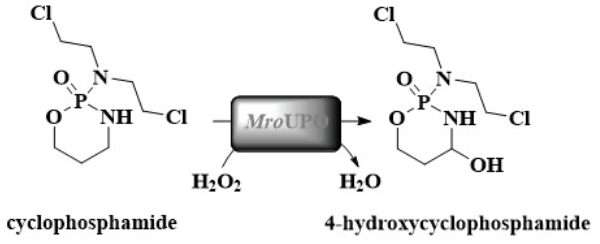

Figure 11. 4-Hydroxycylophosphamide metabolite of cyclophosphamide obtained by *MroUPO*

The oxidation of amines

As a result of metabolic *N*-dealkylation of secondary aliphatic and alicyclic amines, they produce primary amine and unsubstituted alicyclic amine metabolites. The tertiary amines and heterocyclic nitrogen compounds are biotransformed by oxidative dealklation and N-oxidation. Schematic represantation of N-oxydation of amines is given in Figure 12.

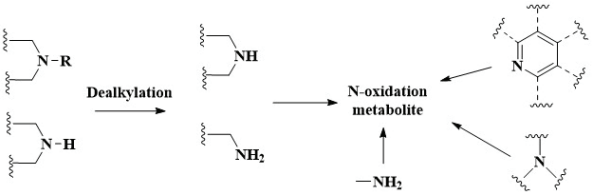

Figure 12. N-oxidation of amines.

Sun L. et al. (L. Sun, Huang, Liu, & Zhong, 2004) reported the major metabolites (N-demethylation, O-demethylation, and sulfate conjugation) of verapamil produced by *Cunninghamella blakesleeana*. Metabolites that are shown in Figure 13 were isolated via the semipreparative liquid chromatography-ion trap mass spectrometry method and identified by ^{1}H-NMR and ESI-MS analyses.

Fodi et al. (Fodi et al., 2018) reported the biomimetic synthesis of amiodarone metabolites. Metabolite of antiarrhythmic amiodarone and N-desmethylamiodarone were obtained by biomimetic oxidation as a major metabolite (Figure 14).

Another method was given for dealkylation by Çoruh. (Çoruh, 2012). As shown in Figure 15, the dealkylation of alkyl substituted 1,2,4-triazolethiones may be carried out with the cyclization of benzoyl substituted acylthiosemicarbazides in alkaline media.

The tertiary amine and heterocyclic nitrogen compound N-oxides are synthesized using molecular oxygen or other oxidants such as hydrogen peroxide, m-chloroperoxybenzoic acid, magnesium monoperphtalate, 2-sulphonyloxziridines, dioxiran, dimethyl dioxiran, and oxaziridines (Figure 16). Youssif (Youssif, 2001) and Cai et al. (Cai, Sha, Guo, & Pan, 2012) published excellent reviews about tertiary amine N-oxides.

Jaworski et al. (Jaworski et al., 1993) synthesized chlorpromazine-N-oxide and fluphenazine-N-oxide from chlorpromazine

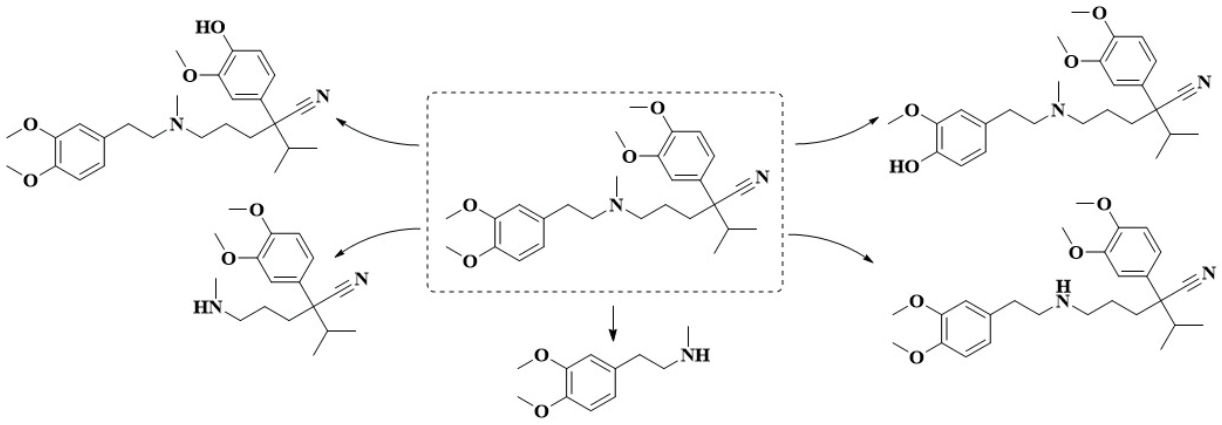

Figure 13. Five major metabolites obtained via transformation of verapamil by *C. blakesleeana*.

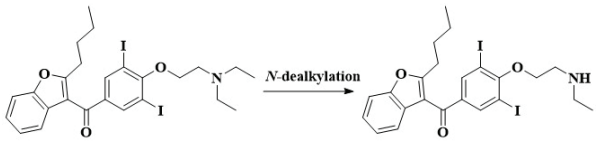

Figure 14. *In vitro* major biotransformation product of amiodarone by using human liver microsomes.

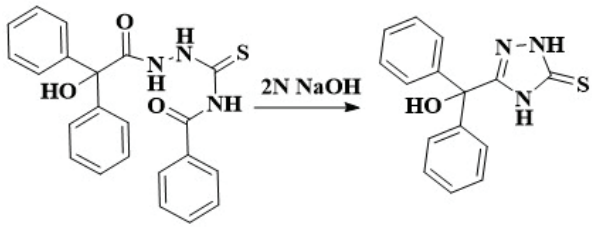

Figure 15. Nonsubstituted 1,2,4-triazole thiones synthesis.

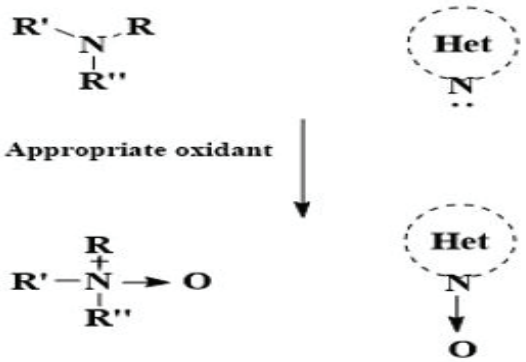

Figure 16. Synthetic N-oxidation of tertiary amines and heterocyclic nitrogen containing compounds.

and fluphenazine in the presence of m-chloroperoxybenzoic acid in tetrahydrofuran that yielded 67 % and 45 %, respectively (Figure 17).

Reddy et al. (Reddy, Mukkanti, Kumar, Babu, & Reddy, 2008) reported the synthesis of lansoprazole-N-oxide. As a starting material 2-chloromethyl-3-methyl-4-(2,2,2-trifluoroethoxy)pyridine hydrochloride was used to synthesize lansoprazole *N*-oxide in the presence of m-chloroperoxybenzoic acid in chloroform (Figure 18).

Lansoprazole-sulfone-N-oxide was prepared from lansoprazole sulfide in the presence of m-chloroperoxybenzoic acid in chloroform (Figure 19**).**

As shown in Figure 20, Doddaya and Peddakonda reported a synthesis method for chloroquine N-oxide which is a major degradation product of chloroquine and also metabolite of chloroquine (Doddaga & Peddakonda, 2013).

Hanlon et al. (Hanlon et al., 2012) prepared moclobemide N-oxide metabolite (65 mg) by using the FMO enzyme (Figure 21).

The oxidation of thioether

S-dealkylation, desulfuration, and S-oxidation (sulfoxide and sulfone) reactions are known as the metabolic pathways of thioether. A sulfur atom present in the cyclic ring is susceptible to S-oxidation. The sulfoxide functional group containing drugs and metabolites may be further oxidized to a sulfone group. Reddy et al. (Reddy, Mukkanti, Bhaskar, & Reddy, 2008) prepared rabeprazole sulfone from rabeprazole sulfide using m-chloroperbenzoic acid in a chloroform and methanol mixture (2:1, v/v) at -20 to -25 °C (Figure 22).

Zhang et al. (Zhang et al., 1996) investigated the *in vitro* metabolism of chlorpromazine using *Cunninghamella elegans* (*C. elegans* ATCC 9245). *C. elegans* biotransformed chlorpromazine to its potential metabolites. Chlorpromazine sulfoxide (Figure 23) and other metabolites were characterized by MS, UV, and NMR analyses.

Oxidative O-dealkylation

Oxygen functionality is found in many drugs and other xenobiotics. Drugs containing the ether functional groups are metabolized by oxidative dealkylation.

Antihypertensive prazosin demethylated metabolites have been synthesized by Althuis and Hess (Althuis & Hess, 1977), and the schematic representation of these synthetic pathways are given in Figure 24.

In the concept of drug metabolite synthesis, mammalian cytochrome P450 enzymes have been given great attention in terms of their usage as biocatalysts. Various systems for the heterologous expression of mammalian *cyp* genes have been developed. *Escherichia coli* strains are also used as a host for

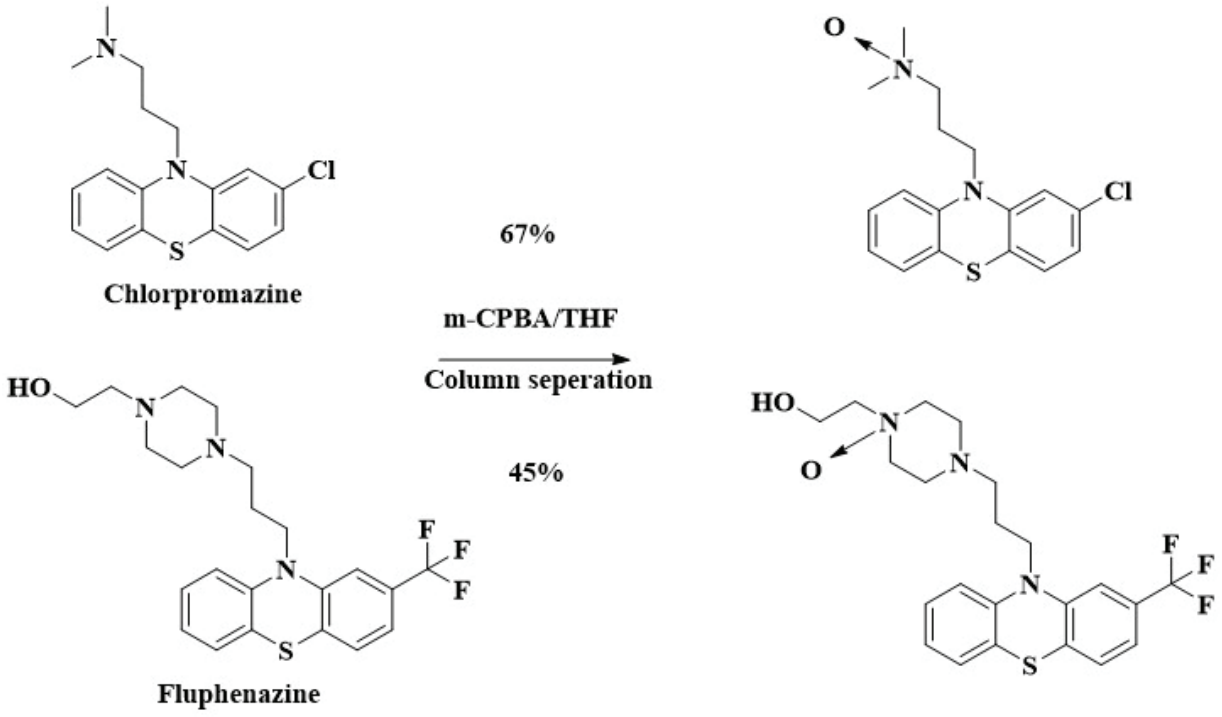

Figure 17. Chemical synthesis of N-oxide metabolites of chlorpromazine and fluphenazine.

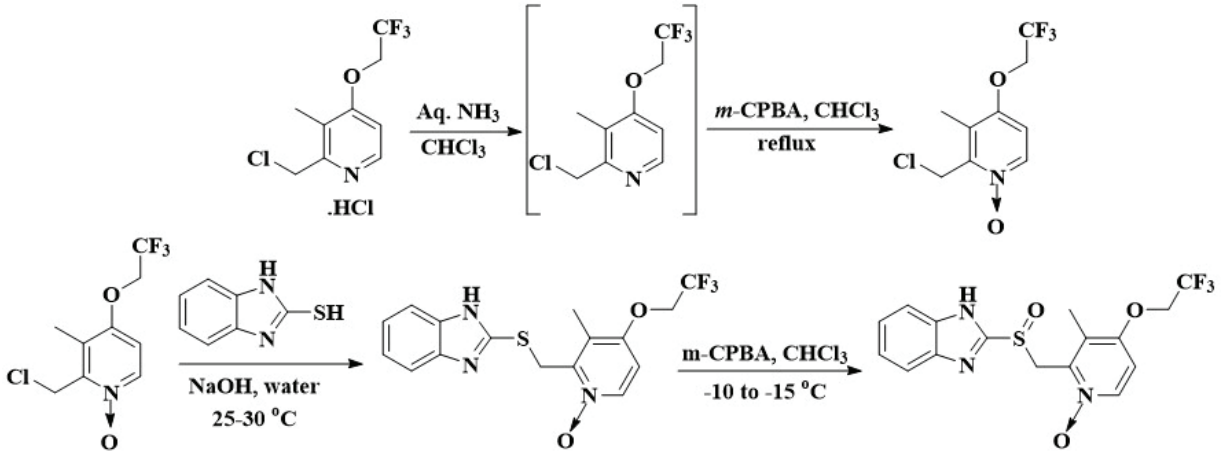

Figure 18. Synthesis protocol for lansoprazole N-oxide.

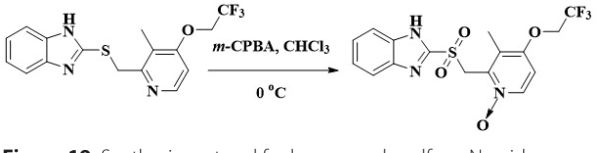

Figure 19. Synthesis protocol for lansoprazole sulfone N-oxide.

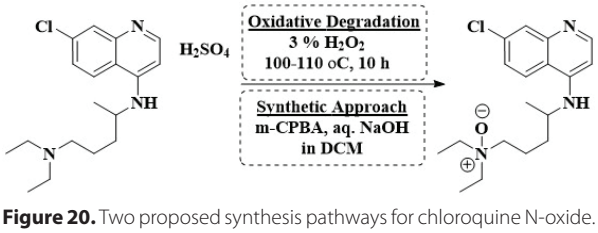

Figure 20. Two proposed synthesis pathways for chloroquine N-oxide.

drug metabolite synthesis. Particularly, the CYP1A family is an important enzyme for drug metabolite synthesis (Cornelissen, Julsing, Schmid, & Buhler, 2012; Lu et al., 2020). Verapamil (Caswell, O'Neill, Taylor, & Moody, 2013), testosterone (Fessner et al., 2020), lorcaserin (Cusack et al., 2013), NVP-AAG561 (Schroer et al., 2010), diclofenac, diazepam, ibuprofen, phenacetin and cortisol (Winkler, Geier, Hanlon, Nidetzky, & Glieder, 2018), are examples of drugs where metabolites have been synthesized by engineered cytochrome enzymes.

Extremely selective oxyfunctionalization reactions on drugs including O-dealkylation and hydroxylation can be performed by mimicking the role of the human liver cytochrome P450 monooxygenases and unspecific peroxygenases (UPOs). Gomez de Santoz et al. reported that several UPO variants for their capacity to synthesize human drug metabolites from three pharmaceutical agents: dextro-

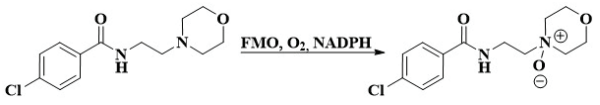

Figure 21. Flavin monooxygenase catalyzed *N*-oxidation of moclobemide.

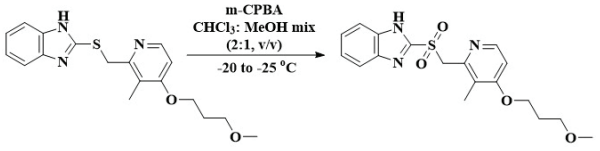

Figure 22. Preparetion of rabeprazole sulfone from rabeprazole sulfide.

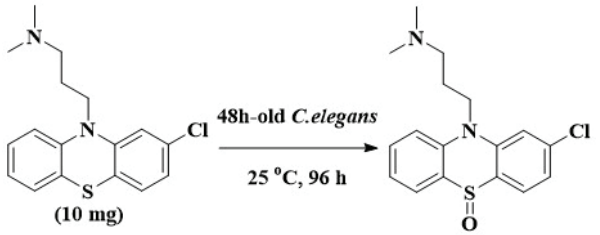

Figure 23. Obtained chlorpromazine sulfoxide metabolite of chlorpromazine after incubation with *C. elegans*.

methorphan (Figure 25), naproxen, and tolbutamide (Gomez de Santos et al., 2019).

Antiinflammatory indomethacin major metabolites are N^1-deacyl, O-desmethyl, and ester glucuronide analogs. The O-demethylated metabolite (1-p-chlorobenzoyl-5-hydroxy-2-methyl-3-indolylacetic acid) of indomethacin was synthesized by Strachan et al and the pathway is given in Figure 26 (Strachan, Meisinger, Ruyle, Hirschmann, & Shen, 1964).

Oxidative aromatization

In the literature, metabolic dehydrogenation reaction of some compounds have been reported. Indapamide and nifedipine can be given as examples that can undergo metabolic dehydrogenation. As given in Figure 27, a diuretic drug indapamide dehydrogenation metabolite was synthesized by Sun et al. (H. Sun et al., 2009). Briefly, indapamide has been oxidized with MnO_2 in acetone and then the indolin ring was aromatized to indol.

The cyclic metabolites of drugs

Some drugs can be converted to their cyclic metabolites such as hydralazine and methadone.

The main metabolic route of antihypertensive hydralazine is acetylation. The initially formed N-acetyl hydralazine is unstable and cyclizes intramolecularly to form 3-methyl-1,2,4-triazole[3,4-*a*]phtalazine as the major metabolite that was synthesized by Dutkiewicz et al (Figure 28) (Dutkiewicz, Chidan Kumar, Yathirajan, Mayekar, & Kubicki, 2009).

Pohland et al. (Pohland, Boaz, & Sullivan, 1971) synthesized 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine and 2-eth-

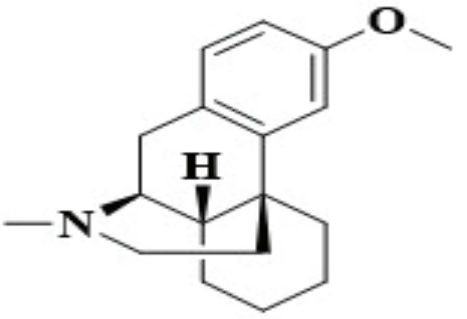

Figure 25. Chemical structure of dextromethorphan.

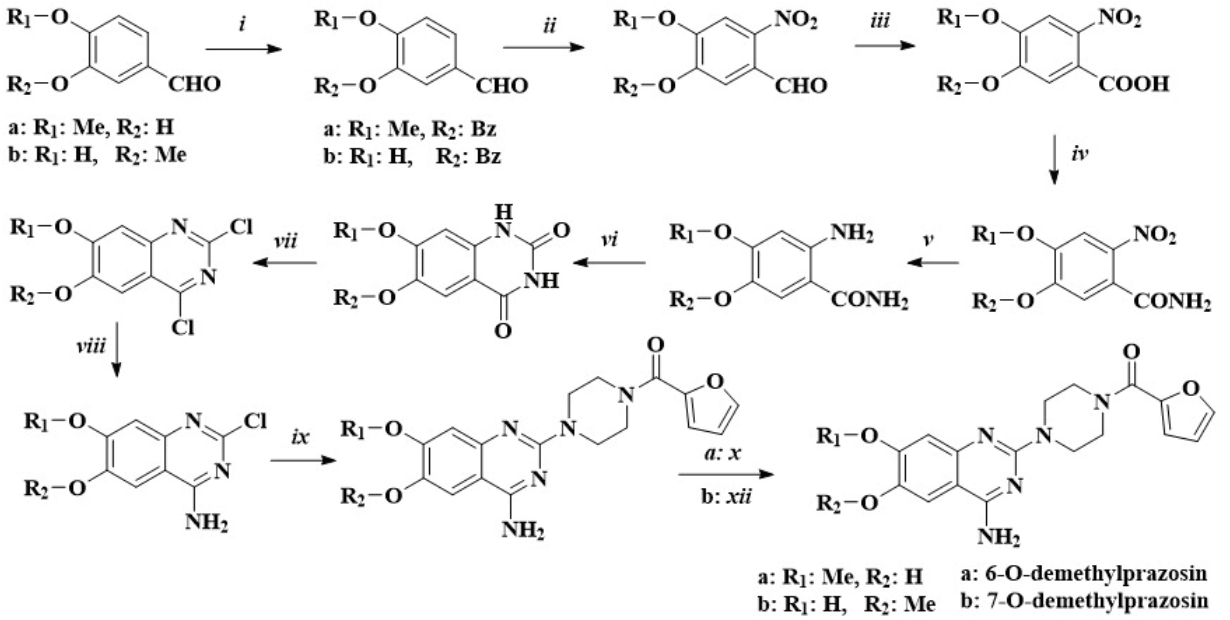

Figure 24. Synthesis of demethyl metabolites of prazosin (**i**: abs. ethanol, K_2CO_3,benzyl chloride, reflux; **ii**: 0 ˚C, HNO_3, 30 ˚C 15 min; **iii**: acetone, 10%$KMnO_4$, **iv**:$SOCl_2$ reflux, evap., dioxane, NH_3; **v**: glacial acetic acid, Fe powder, 90 ˚C, **vi**: urea, pyridine in 10%HCl, reflux; **vii**: $POCl_3$, N,N-dimethylaniline, N_2, reflux; **viii**: THF, dry NH_3, R.T.; **ix**: 1-(2-furoyl)piperazin, isoamyl alcohol, reflux; **x**: CF_3COOH, reflux 2.5h; **xi**: conc. H_2SO_4, r.t., 0.5h.

yl-5-methyl-3,3-diphenylpyrroline, cyclization metabolites of DL-methadone (Figure 29).

Reduction

Reduction reactions play an important role in the biotransformation of many drugs and other xenobiotics containing carbonyl, nitro, and azo groups. N-oxides and sulfoxides are reduced to their corresponding tertiary amines and sulfides. Ketones are reduced to secondary alcohols. Azo compounds are reduced to corresponding amines.

Nitro groups are reduced to amines by many reagents. Drugs containing a nitro group are easily converted to their amine metabolites by the treatment with a suitable reducing agent. Nimesulide is a potent antiinflammatory, antipyretic, and analgesic drug and its amine metabolites can be found in man. Küçükgüzel et al. (Kucukguzel, Kucukguzel, Oral, Sezen, & Rollas, 2005) reported the synthesis of amino nimesulide in rats (Figure 30).

Feely et al. (Feely, Kavanagh, McNamara, & O'Brien, 1999) reported the synthesis of 7-aminoflunitrazepam. 7-aminoflunitrazepam has been readily prepared from the mixture of flunitrazepam and a reducing agent tin (II) chloride dihydrate in ethanol (Figure 31).

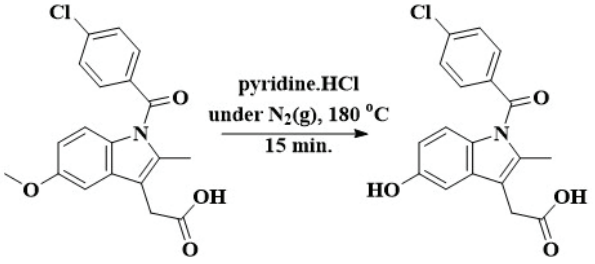

Figure 26. Synthesis of 1-p-chlorobenzoyl-5-hydroxy-2-methyl-3-indolylacetic acid.

Clonazepam is metabolized to 7-aminoclonazepam by nitro reduction via hepatic cytochrome P450. De Paula et al. (de Paula et al., 2015) demonstrated the production of 7-aminoclonazepam metabolite of clonazepam by the microbial transformation **(**Figure 32).

Azo dyes are used as colorants in the food, drug, and cosmetic industry. The azo compounds are reduced by intestinal anaerobes to toxic amine metabolites. Chung et al. (Chung, Fulk, & Egan, 1978) reported that azo dyes are reduced by intestinal anaerobes. The reduction of product metabolites of drugs and other xenobiotics are commercially available.

Rollas developed a method for the synthesis of aromatic and heteroaromatic amines by reducing azo compounds treatment with hydrazine hydrate without a catalyst (Figure 33). These reactions may be employed for the amine metabolite synthesis from azo compounds (Rollas, 2010).

Hydrolysis

Hydrolysis is a major biotransformation route for drugs containing ester and amide functionality. The metabolic products are carboxylic acids, alcohols, phenols, and amines. Esters are easily converted into their acides and alcohols in the presence of alkaline or acidic medium. The hydrolysis of amides is slower than esters. The hydrolysis product of metabolites of drugs are commercially available.

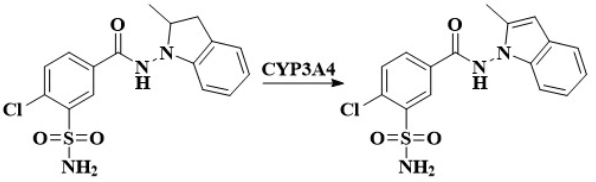

Figure 27. Dehydrogenated metabolite of indapamide.

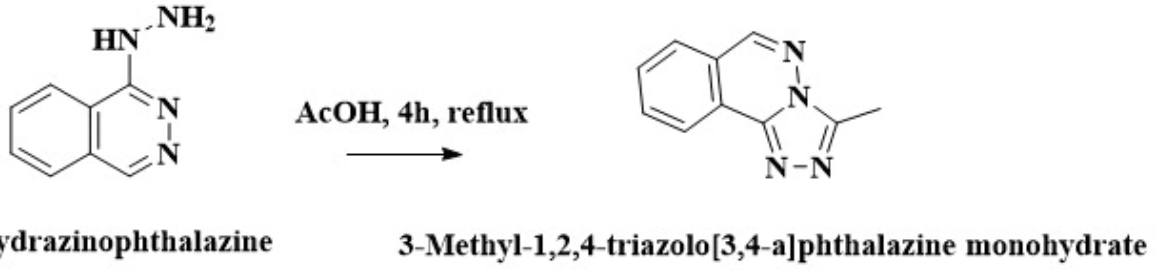

Figure 28. Synthesis of the major metabolite of hydralazine.

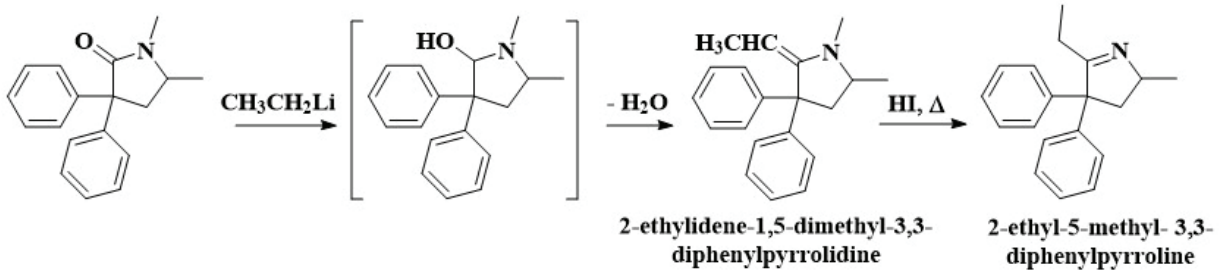

Figure 29. Synthesis of 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine and 2-ethyl-5-methyl-3,3-diphenylpyrroline.

The synthesis of Phase II metabolites

Major Phase II (conjugative) reactions of the phenolic hydroxyl group are glucuronidation, sulfation, and methylation. Acetylation, methylation, and phosphate conjugation may rarely occur. The carboxyl groups containing drugs and xenobiotics give ester type O-glucuronides (acyl glucuronides) (Baldwin, Robinson, & Williams, 1960). Glucuronide metabolites of resveratrol, flavonoids, morphine, and such phenolic compounds are achieved by silver or borane catalyzed reactions (Stachulski & Meng, 2013). Another conjugative reactions of carboxyl groups are amino acid conjugation. Acyl glucuronides are chemically unstable in an aqueous solution and undergo an intramolecular acyl migration. Therefore, unstable acyl glucuronides are capable of cellular injury such as hepatotoxicity and carcinogenesis (Bailey & Dickinson, 2003). An excellent review about reactivity of acyl glucuronide was published by Bradshaw et al (Bradshaw, Athersuch, Stachulski, & Wilson, 2020). Glutathione conjugation is a formation of a thioether bond by the nucleophilic sulfhydryl group of glutathione.

The synthesis of glucuronide metabolites

The glucuronidation reaction is the most well-known conjugative route in drug metabolism and is catalyzed by the family of uridine diphosphate (UDP) glucuronosyltransferases (UGTs). Metabolites are classified as oxygen, nitrogen, sulfur, or carbon glucuronide according to the heteroatom or carbon attached to the C_1 atom of the glucuronyl group. Conjugation of glucuronic acid occurs on nucleophilic functional groups such as alcohol, phenol, primary, secondary, and tertiary amines, and carboxylic acids, etc. (Argikar, 2012). The most systematic research was initiated in the 1930s by R.T. Williams and collaborators (Pryde & Williams, 1933; Williams, 1938). Phenolic glucuronides have been prepared using glycosyl donors and a chart is given for glucuronic acid donors in Figure 34 (Arewang, Lahmann, Oscarson, & Tiden, 2007).

Yoshimura et al. (Yoshimura, Oguri, & Tsukamoto, 1968) prepared codeine and morphine glucuronides using glycosyl donors (Figure 35).

Acyl glucuronidation is one of the major metabolic pathway of acidic drugs or acidic metabolites that produce by the hydrolysis of ester, amide, and nitrile functional groups or the oxidation of drugs and their metabolites.

The synthesis of naproxen glucuronide conjugate that is shown in Figure 36 was obtained with a 70% yield (Arewang et al., 2007).

O-Glucuronides are generally synthesized by a Koenigs-Knorr reaction (Figure 37). The aglycone reacts with methyl (2,3,4-tri-O-acethyl-1-bromo-1-deoxy-α-D-glucopyran)uronate in the presence of Ag_2CO_3) or $Hg(CN)_2$ (Kaspersen & Van Boeckel, 1987). Lou et al. (Luo, Hawes, McKay, & Midha, 1992) developed a synthetic method for the quaternary

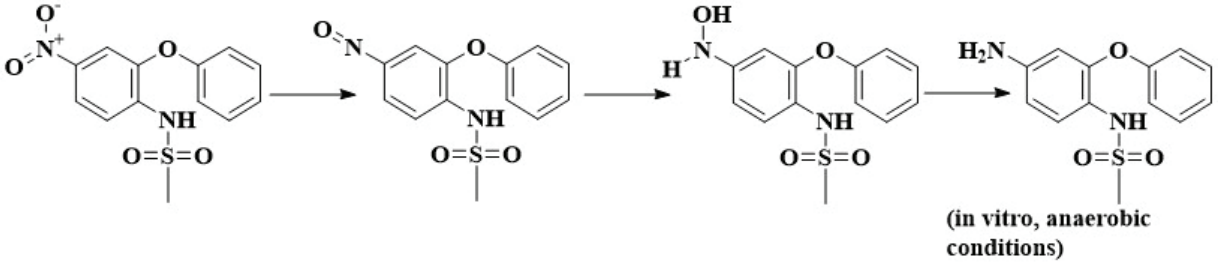

Figure 30. Proposed pathway for the aminonimesulide formation of in vitro metabolite of nimesulide.

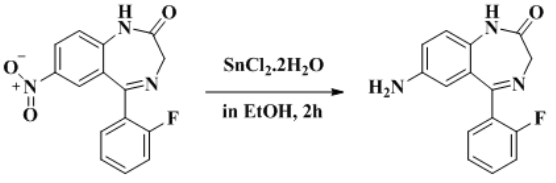

Figure 31. Synthesis of 7-aminoflunitrazepam.

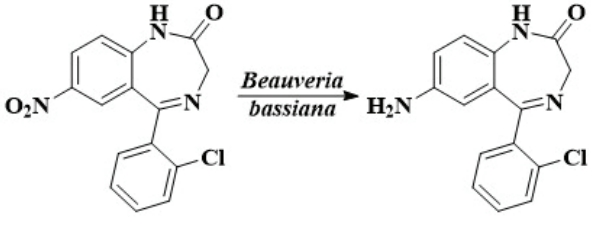

Figure 32. 7-aminoclonazepam metabolite of clonazepam produced by the microbial transformation.

amonium-linked glucuronide metabolites of the aliphatic tertiary amine group using the same reagent but with $NaHCO_3$, not silver carbonate.

The synthesis of sulfate metabolites

Sulfate metabolites were prepared using the reaction of drugs containing phenol, alcohol, or amine groups activated by sulphuric acid.

Hydroxyl derivatives and sulphamates (N-sulphates) have been sulfated with SO_3.pyridin complex, SO_3.trimethyl complex, or chlorosulphonic acid (Kaspersen & Van Boeckel, 1987).

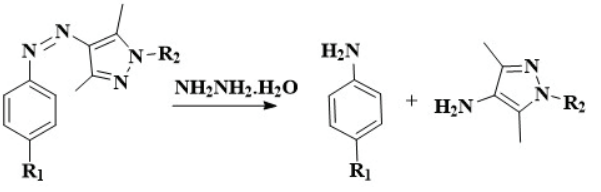

Figure 33. The reduction of azo compounds with hydrazine hydrate.

Foster et al. (B. C. Foster et al., 1991) reported sulfate conjugation of 4'-hydoxyfenazopyridine which is a metabolite of urinary tract analgesic phenazopyridine (Figure 38).

Hoshino et al. (Hoshino et al., 2010) synthesized a sulphate conjugate of resveratrol (Figure 39).

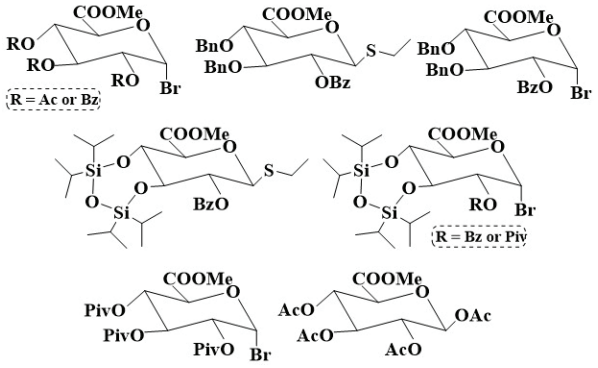

Figure 34. Charts for glucuronic acid donors.

The synthesis of amino acid conjugated metabolites

The major metabolic biotransformations of drugs and other xenobiotic carboxylic acids, with an amino acid or glucuronic acid, are established. Carboxylic acids are mainly converted to glycine conjugates and are rarely converted to glutamine and other amino acid conjugates (Hutt & Caldwell, 1990).

The amino acid conjugation of carboxylic acid was produced from acid chlorides or esters and amino acids. Sinha et al. (Sinha, Praveen, Shrivastava, & Shrivastava, 2012) synthesized amino acid conjugation of valproic acid as prodrugs using thionyl chloride and amino acid esters (Figure 40).

Rasheed et al. (Rasheed, Kumar, Shama, & Mishra, 2011) synthesized amino acid conjugations of aceclofenac as prodrugs using methyl ester of aceclofenac (Figure 41).

The synthesis of glutathione conjugated metabolites

Glutathione conjugation is an important route for the biotransformation of chemically reactive electrophilic drugs, metabolites, and other xenobiotics. Glutathione conjugation is the formation of a thioether bond between an electrophilic center and glutathione (Ketterer & Mulder, 1990).

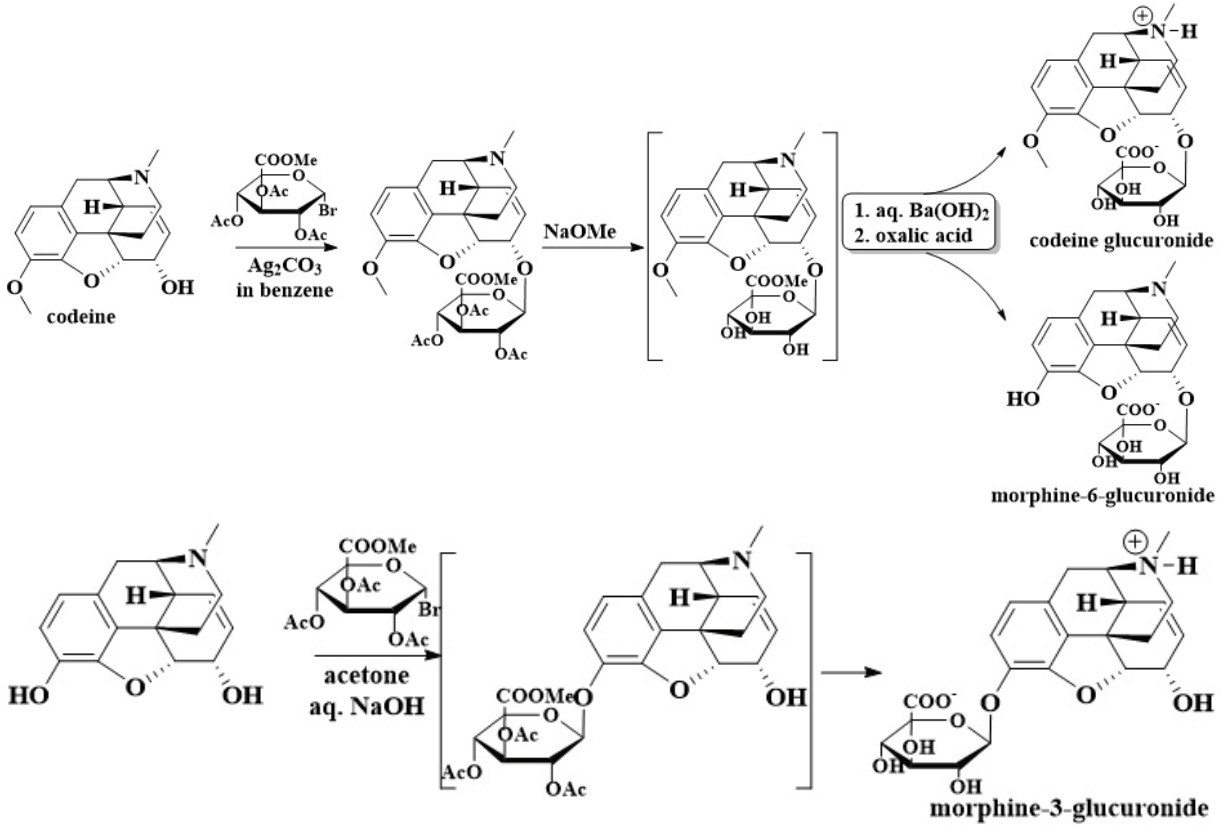

Figure 35. Synthesis of A) codeine glucuronide, morphine-6-glucuronide and B) morphine-3-glucuronide.

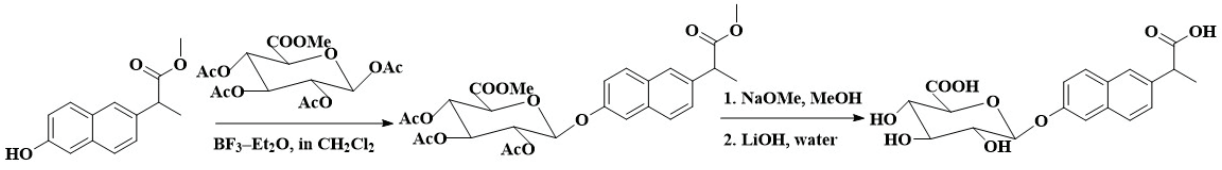

Figure 36. Synthesis pathway for naproxen glucuronide conjugate.

Huber et al. (Huber, Bartha, Harpaintner, & Schroder, 2009) reported the metabolism of acetaminophen in plant tissues using the cell culture of *Armoracia rusticana* L. as a model system. Acetaminophen glutathione conjugate obtained in the root cells of *A. rusticana* produced a 17% yield.

The synthesis of acetylated metabolites

Acetylation reactions are the metabolic pathways of drugs containing primary aromatic amine, hydrazine, hydrazide, primary aliphatic amine groups, and amine metabolites produced from aryl nitro and azo compounds, such as clonazepam, nitrazepam, and sulfasalazine. Drugs and amine metabolites are generally converted to their acetylated metabolites with acetic anhydride as an acetylating agent.

As given in Figure 42, the acetyl-desethyl metabolite of antiarrhythmic procainamide was synthesized by Adamczyk and Fino (Adamczyk & Fino, 1996).

Nobilis et al. (Nobilis et al., 2006) reported the synthesis of the N-acyl-5-aminosalicylic acid metabolite of mesalazine (Figure 43).

Major metabolites of anticancer aminoglutethimide are acetylamino-glutethimide and 5-hydroxiglutetimide. The acetylated metabolite of the N-hydroxy metabolite of aminoglutethimide was synthesized by Foster et al. (A. B. Foster et al., 1984) using pentafluorophenyl acetate as an acetylating agent (Figure 44).

The major metabolite of antitubercular isoniazid is acetyl isoniazid. Fox et al. (Fox & Gibas, 1953) synthesized acetyl isoniazid from isoniazid, acetic anhydride, and glacial acetic acid (Figure 45).

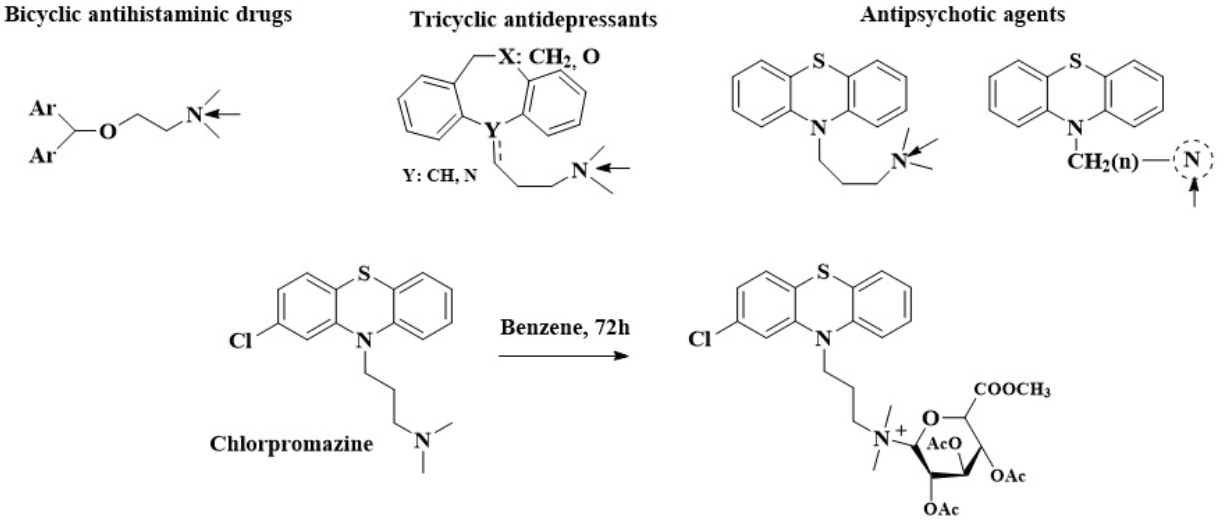

Figure 37. Synthesis of glucuronide metabolites from aliphatic tertiary amines and chlorpromazine by Koenigs-Knorr reaction.

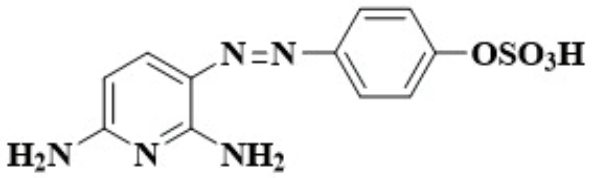

Figure 38. Chemical structure of 4'-SO_4, O-sulfate monoester of 4'-hydroxyphenazopyridine.

The synthesis of methylated metabolites

Methylation reactions are a minor pathway for conjugating drugs, xenobiotics, and dietary compounds. O-methylation occurs in the phenolic groups of a variety of endogenous and catecholic compounds (Sang, Lambert, Ho, & Yang, 2011). The methylated drugs are less polar than the substrate. As a

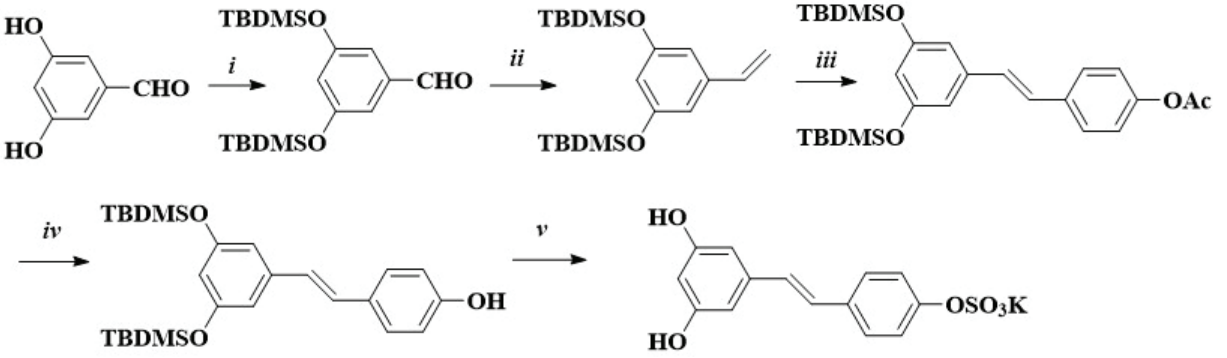

Figure 39. Synthesis of sulfate conjugates of resveratrol (**i**: TBDMSCl, DMF, 80%; **ii** $NaNH_2$, $MePPh_3Br$, Et_2O, 72%; **iii**: $Pd(OAc)_2$, Et_3N, PPh_3, MeCN, 40%; **iv**: NaOMe, MeOH, 92%; **v:** SO_3-NMe_3, MeCN, Et_3N; and KF, MeOH/H_2O).

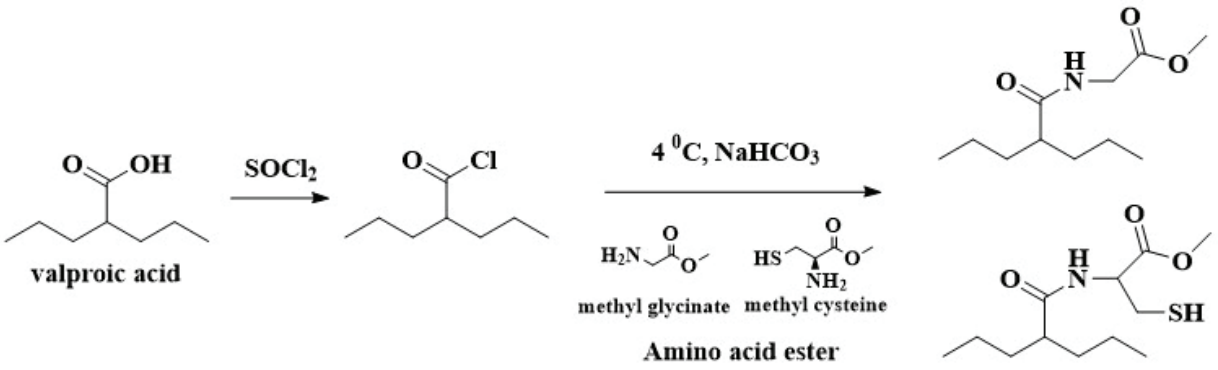

Figure 40. Amino acid conjugate synthesis of valproic acid.

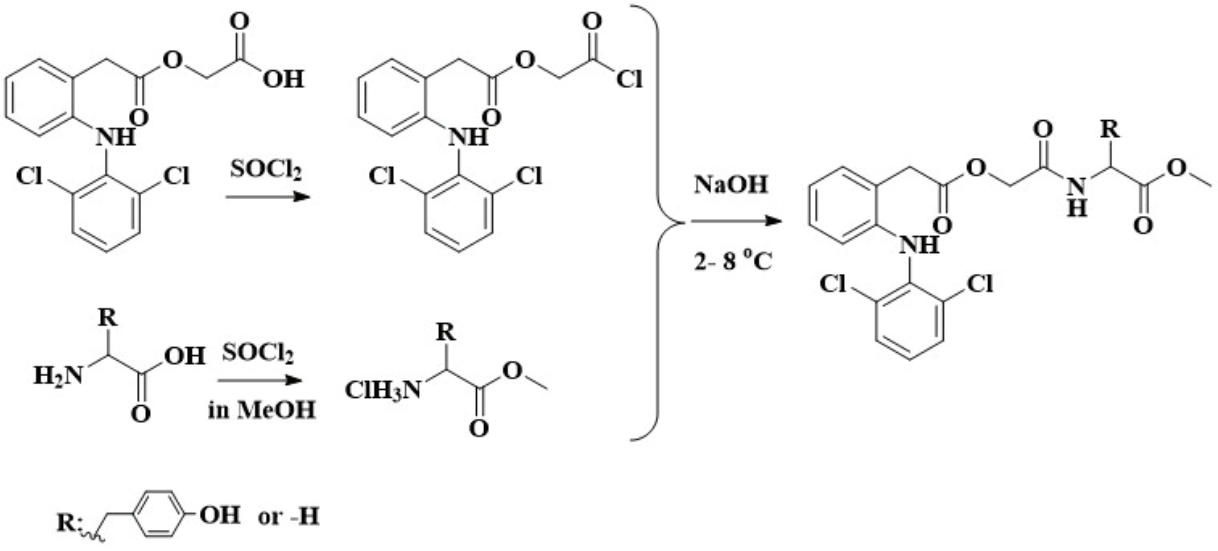

Figure 41. Synthesis of prodrugs of aceclofenac.

Figure 42. Synthesis of N-acetyl desethylprocainamide.

result of the methylation of morphine codeine was produced (Boerner, 1975). As shown in Figure 46, biogenic amines histamine and norepinephrine were converted to methylated metabolites from their amine groups (Brown, Axelrod, & Tomchick, 1959; Frère & Verly, 1971; Rice, 1977).

The analysis methods of metabolites

For reference standards, the metabolites of drugs and other xenobiotics are used *in vivo* and *in vitro* studies (Sidelmann et al., 1997; Turgeon, Pare, Lalande, Grech-Belanger, & Belanger, 1992; Williams, 1943). In preclinical drug development, the ADMET properties of the potential drug candidates were evaluated in terms of their prospectivity. The essential process in the discovery and development of new drugs is the isolation and identification of their metabolites. The instability in a small number of metabobolites in the biological matrix makes isolation and identification difficult. Therefore, one of the best methods is the synthesis of metabolites as a reference standard. The structure of metabolites has been elucidated using several spectroscopic methods, particularly NMR and MS.

The drug and metabolites analysis is important for pharmacokinetic experiments. Drugs and their metabolite levels are commonly measured using high performance liquid chromatography (HPLC). Mass spectrometry coupled with chromatography and nuclear magnetic resonance spectroscopy (NMR) are widely used techniques for metabolite analysis (Schaber et al., 2001). The mass spectrometer is an important instrument for the identification of metabolites of drugs and other xenobiotics (Constanzer, Chavez-Eng, Fu, Woolf, & Matuszewski, 2005; Nelson, Garland, Breck, & Trager, 1977). Generally, analytes are separated by the suitable Liquid Chromatography (LC) column (Gill, Law, & Gibbs, 1986; Mackichan, 1980). The mass spectrometer is used as a detector. The LC/MS or GC-MS techniques are employed for the isolation and identification of metabolites in biological fluids or tissue extract (Petsalo, Turpeinen, Pelkonen,

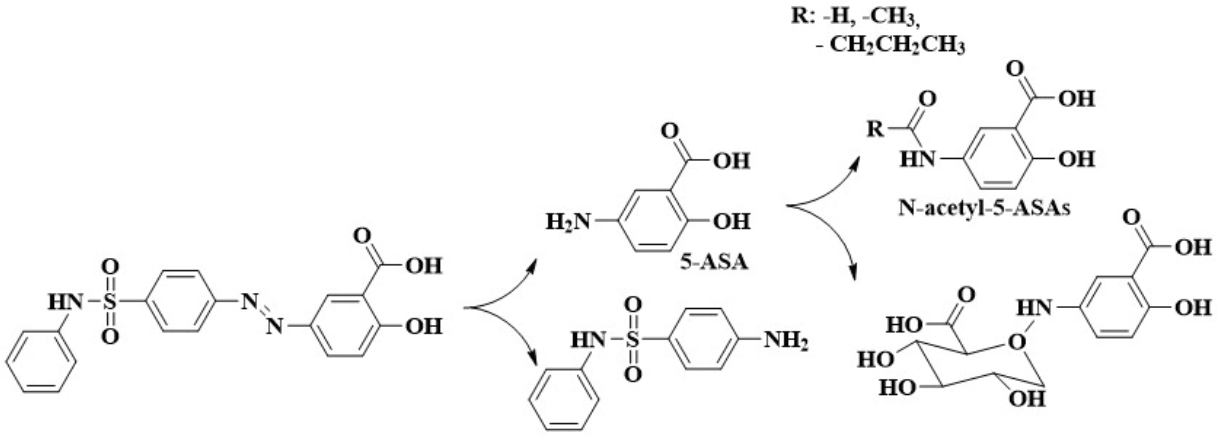

Figure 43. Synthesis of N-acyl-5-aminosalicylic acid metabolite.

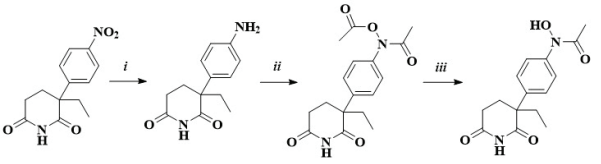

Figure 44. Synthesis of acetylated metabolites of N-hydroxy aminoglutethimide (**i:** acetone, Zn, NH_4OAc; **ii**: $(CH_3CO)_2O$; **iii**: 4M NH_3, 30 min).

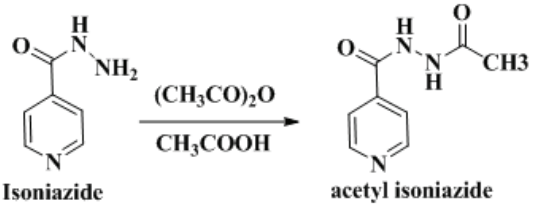

Figure 45. Synthesis of acetylisoniazide.

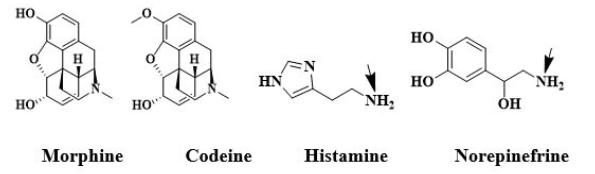

Figure 46. Drugs undergo methylation metabolism.

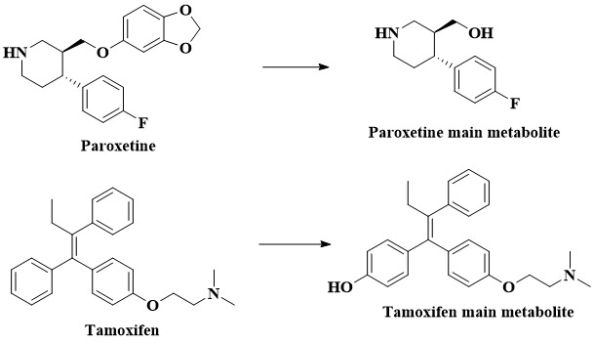

Figure 47. Paroxetine, tamoxifen, and their main metabolites.

& Tolonen, 2008; Ren, Zhang, Kong, & Wang, 2018; Wu, Wang, Yang, & Sui, 2019).

Wheals and Jane (Wheals & Jane, 1977) reviewed an analysis of drugs and their metabolites using high-performance liquid chromatography. In this review, the applications of steroids, antibiotics, oxygen-containing compounds, vitamins, alkaloids, nitrogen-containing compounds, prostaglandins, sulfur-containing compounds were demonstrated. The LC-MS/MS technique provides superior selectivity, sensitivity, and analysis for detecting plasma concentration in pharmacokinetics studies and prognosis of acute poisoning. A novel validation method to measure the amount of venlafaxine and its five metabolites by using LC-MS/MS was suggested by Gu et al. (Gu et al., 2018). Michely and Maurer (Michely & Maurer, 2018) reported a fast LC-MS/MS quantification approach for 45 drugs and their relevant metabolites (Fang et al., 2006). Correia et al. (Correia, Rao, Ballet, & Globisch, 2019) demonstrated the combination of untargeted metabolomic analysis and metabolic conversion for the selective detection of glucuronide conjugates by using the UPLC-MS/MS in human urine samples. Ishigai et al. (Ishigai, Langridge, & Bordoli, 2001) studied the dynamics of enzyme-catalyzed glutathione conjugation by electrospray quadrupole/time-of-flight (Q-TOF) mass spectrometry with a nanospray interface. The online combination of LC with the inductively coupled plasma (ICP) mass spectrometer offers an excellent method for metabolite identification and structure characterization (Kostiainen et al., 2003). Currently, a variety of techniques are used for the analysis of drug metabolites such as orbitrap technology (OT), high-resolution mass spectrometry (HRMS), high-performance liquid chromatography- inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) high-performance liquid chromatography- inductively coupled plasma tandem mass spectrometry (HPLC-ICP-MS/MS), and hydrophilic interaction liquid chromatography-mass spectrometry (HILIC -MS). (Cece-Esensencan et al., 2016; Helfer, Michely, Weber, Meyer, & Maurer, 2015; King et al., 2019; Klencsar et al., 2018; Li et al., 2018) Xing et al. (Xing, Zang, Zhang, & Zhu, 2015) presented a new application of high-resolution mass spectrometry (HRMS)- based data-mining tools in tandem to provide a fast and comprehensive metabolite profiling of combination drugs. In his research, a metronidazol-pantoprazole-clarithromycin combination was used as the model.

Sundell et al. (Sundell et al., 2019) developed a LC-MS/MS method for the qualification of rifampicin, isoniazid, pyrazinamide, ethambutol, and their metabolites 25-desacetylrifampisin, isonicotinic acid, acetyl isoniazid, and 5-hydroxy pyrazinamide.

Wang et al. (Wang et al., 2019) designed, developed, and validated a rapid, simple, and sensitive method for simultaneous quantitation of four CYP450 probe drugs; phenacetin, omeprazole, metoprolol, midazolam, and their metabolites (acetaminophen, 5'-hydroxyomeprazole, α-hydroxymetoprolol, 1'-hydroxymidazolam) using an ultra high-performance liquid chromatography- tandem mass (UHPLC-MS/MS) spectrometry.

Therapeutic drug monitoring is an important tool for correlating the drug dose to drug and metabolite concentrations in the body and the therapeutic and adverse effects. Protti el al. (Protti et al., 2020) reported an analysis method, a capillary volumetric blood micro sampling, for the selective serotonin reuptake inhibitors fluoxetine and its metabolite, norfluoxetine; sertraline and its metabolite, desmethyl sertraline.

In some cases, the structure of the formed metabolites was not elucidated by the LC/MS technique. An alternative possibility was to isolate the metabolite from the incubation matrix and to elucidate the exact structure using a nuclear magnetic resonance (NMR) method (H. Sun et al., 2009; Zhang et al., 1996).

The non-aqueous capillary zone electrophoresis (NACE) method was used for the analysis of some drugs and their metabolites. Flores et al. (Flores, Nevado, Salcedo, & Diaz, 2004) reported the analysis of paroxetine, tamoxifen, and their main metabolites in urine by NACE (Figure 47).

CONCLUSION

In recent years, discovery and development of new drugs are prerequisite for the evaluation of drug safety and risk assessment. The metabolic profile, metabolite toxicity, metabolite stability, active metabolites pharmacological testing, and pharmacokinetics of a new drug should be defined. Therefore, the synthesis of metabolites is an important area of research for metabolites. Several analytical methods are used for the isolation and detection of metabolites. The LC-MS/MS and LC-MS-NMR systems enable a routine analysis of metabolites.

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