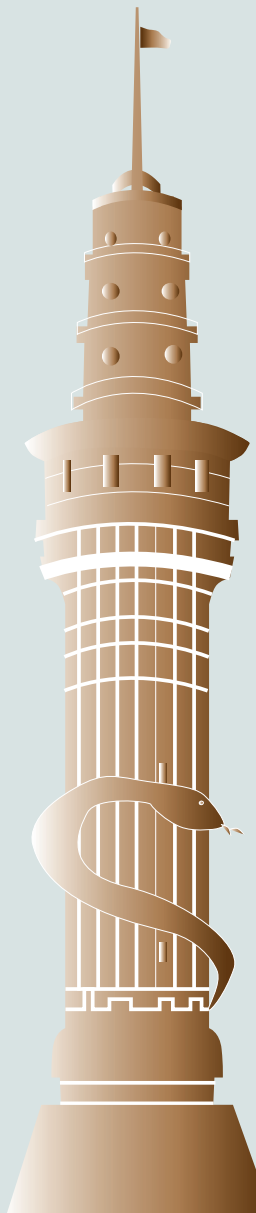
ISTANBUL UNIVERSITY PRESS

Indexed in Web of Science

İstanbul Journal of Pharmacy

VOLUME 51 • ISSUE 3 • DECEMBER 2021



Original Articles

Review Articles

ijp.istanbul.edu.tr

EISSN 2587-2087

İstanbul Journal of Pharmacy

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Web of Science - Emerging Sources Citation Index (ESCI)

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PUBLISHER

İstanbul University Press

İstanbul University Central Campus,
34452 Beyazit, Fatih / İstanbul, Turkey
Phone: +90 212 440 00 00

The publication languages of the journal is English.

This is a scholarly, international, peer-reviewed and open-access journal published triannually in April, August and December.

Publication Type: Periodical

İstanbul Journal of Pharmacy

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INSTRUCTIONS TO AUTHORS

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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.

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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).

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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.

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PEER REVIEW PROCESS

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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?
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Reviewers must ensure that all the information related to submitted manuscripts is kept as confidential and

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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.

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- Author Form
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- 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.

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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

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Original Article	3500	250 (Structured)	6	7 or total of 15 images
Review Article	5000	250 (Unstructured)	6	10 or total of 20 images
Short Paper	1000	200	No tables	10 or total of 20 images
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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.
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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 (;).

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(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;

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Citations in the Reference

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

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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

c) Dissertation/Thesis from Web

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

d) Dissertation/Thesis abstracted in Dissertations Abstracts International

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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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Editor in Chief: Emine AKALIN
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Publisher: İstanbul University Press
Address: İstanbul University Central Campus, 34452 Beyazit, Fatih / İstanbul - Turkey
Phone: +90 212 440 00 00

İstanbul Journal of Pharmacy

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ORIGINAL ARTICLES

İstanbul Journal of Pharmacy

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Istanbul J Pharm 51 (3): 291-298
DOI: 10.26650/IstanbulJPharm.2021.835751
Original Article

Clinical pharmacist directed anticoagulation monitoring services: A prospective interventional study

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Cite this article as: Sarika, A., Reghu, A., Karattuthodi, M. S., & Sreelatha, A. R. P. (2021). Clinical pharmacist directed anticoagulation monitoring services: A prospective interventional study. *İstanbul Journal of Pharmacy, 51*(3), 291-298.

ABSTRACT

Background and Aims: Antithrombotic therapies are established as cornerstones of treatment for a wide variety of ischemic vascular diseases. The study aimed to develop clinical pharmacist directed anticoagulation monitoring services in the cardiology department at tertiary care referral super specialty hospital.
Method: Prospective interventional study was conducted for 12 months in the cardiology inpatient setting of a private tertiary care referral hospital in the Malabar region of Kerala. Constituted to be in three phases, they are: assessment of the present anticoagulation related practices, the development and sharing of anticoagulation protocol and finally the intervention and implementation of the system.
Results and Discussion: Monitoring of the treatment could enhance the drug selection approaches, improved adherence to clinical guidelines and health outcomes. Heparin was the commonly prescribed drug in the cardiology department which had the risk of thrombocytopenia and major bleed, whereas this risk was lesser with fondaparinux. Early detection of bleeding could prevent complications from happening to the patients. The collaboration of the clinical pharmacist into anticoagulation therapy influenced the physician to comply with the ACCP guideline.
Conclusion: The clinical pharmacist directed anticoagulation monitoring services improved the overall medical status of the patient.

Keywords: ACCP, Anticoagulation, Clinical Pharmacist, Guideline Compliance, Management service, Medication Error

INTRODUCTION

Cardiovascular diseases are the leading cause of death worldwide. Both anticoagulants and antiplatelet agents are the landmark accomplishments and the established cornerstone of therapy for a wide range of ischemic vascular abnormalities, including acute coronary syndrome (ACS), stroke, peripheral vascular disease, atrial fibrillation, deep vein thrombosis, and pulmonary embolism (Kollias et al., 2020).

Anticoagulants are one of the most sensitive drug classes that are liable to errors and adverse events. Several studies suggested the influence of anticoagulation management clinics and services to revamp the patients' therapy. Pharmacists and nurses have a pivotal role in the establishment and maintenance of such clinics.

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Submitted: 05.12.2020
Revision Requested: 23.03.2021
Last Revision Received: 08.05.2021
Accepted: 19.05.2021
Published Online: 09.12.2021

Monitoring is a prospective supervision, observation, and testing of an on-going process (Pranckeviciene, Kadusevicius, & Putniene, 2013). Hence, therapeutic drug monitoring should focus on the efficacy, and safety of drugs. The cooperation of patients in bequeathing the transmute in disease or treatment to the physician is also vital. However, very little consideration has been given to develop effective schemes for monitoring the contingency of adverse drug reactions concerning biochemical or haematological disturbances. Forecasting the probable adverse reactions that impart reversible or irreversible damage to the patient is another important aspect. Worldwide heparin utilization trended from 10% to 15% yearly growth in the past decade. Even though the medicine is primarily prescribed in the inpatient setting, heparin is consumed up to 10% of the total medication costs among hospitals (Kolhatkar, Cheng, Chan, Harrison, & Law, 2016). Pharmacist-directed anticoagulation management services (AMSs) have been shown to improve patient outcomes and reduce their length of hospital stay. Anticoagulation stewardship programs are mostly limited to inpatient populations and can also be expanded to outpatients. To achieve such an intrinsic multisystem process, a multidisciplinary collaborative effort is essential (Schumock et al., 2003).

American College of Clinical Pharmacy (ACCP) noted a decrease in health care cost due to clinical pharmacy services (Roberts, Patel, & Arya, 2009; Talon et al., 2020). Thus, to cut down the financial and heath crisis, we established clinical pharmacist directed anticoagulation monitoring services in the cardiology department at tertiary care referral super specialty hospital.

METHODOLOGY

The prospective interventional study was carried out for 12 months in the cardiology inpatient department of 350 bedded private tertiary care referral hospital in the Malabar region of Kerala. The Institutional Ethics Committee met on 03/02/2015, approved the proposal for the dissertation as per letter No. IEC/ASH/2015/PD/9. Informed consent was also obtained from each patient.

N-Master software was used to estimate the sample size as 45 in both the pre-interventional (control) and post-interventional groups. The expected proportion was set at 0.20 based on the pilot study conducted on 15 patients. The level of significance was set at 0.05 and precision at 15%.

Selection Criteria

Patients with age >18 years, and those who had received at least one prescription order for heparin, made up the study population. Patients who had voluntarily discharged themselves, lactating mothers, those with missing data, patients of non-Indian origin and pregnant patients were excluded.

Assessment of current prophylactic practices and compliance to the regimen

The clinical pharmacist followed the patients from their hospital admission to discharge. Laboratory parameters and INR value of patients were documented. The collected data were compiled into graphs and tables. Calculation of the mean and standard deviation was done by using statistical calculators.

A data collection form was developed to tabulate the information relevant to the study. The form consists of the following details:

1. Patient demographics such as name, age, sex, body weight,
2. Medication Record Department number,
3. Date of admission,
4. Date of discharge,
5. Diagnosis and all other relevant information.

Phases of the study

The current anticoagulant practices in the study site were compared to the standard treatment recommended by the ACCP in its 9th antithrombotic guidelines, which was meant to assess the extent of compliance.

The 3 phases were as follows:

Phase 1- Assessment of earlier practice (Pre-interventional /Control Phase)

In the first 5 months, data associated with the earlier treatment practice (no interventions) were retrieved from the study population. A total of 45 subjects were enrolled. The recommendations of ACCP 9th antithrombotic guideline were the standard to determine the appropriateness of therapy. The cases that do not comply with ACCP regimen either in dose or INR value would be marked as non-compliant.

Phase 2- Development of anticoagulant protocol and dissemination of information (Passive Intervention phase)

The phase covered over 2 months, and it involved passive interventional strategies. Initially, we presented the data to the cardiology specialists and also conveyed the standard practices recommended by ACCP 9th guidelines. And further, the information obtained from the initial audit was disseminated within the hospital. The phase also involved the preparation of the hospital specific anticoagulant protocol based on data obtained from the control phase.

Phase 3- Active Intervention and Reassessment (Intervention phase)

This phase was carried out for the next 5 months. The patient interviews were performed, and case files were decoded for interpreting the INR values, medication errors and adverse drug reactions. bleeding risk factors of the patient were also monitored and periodically reported to the physician. The data from the intervention phase and control phase were compared to finalize the success of the program.

Clinical Pharmacist led anticoagulation service

All patients and their caregivers were counseled on anticoagulation therapy and its importance, common ADRs (Adverse Drug Reactions) and management, the importance of patient compliance, dose titration,and dietary modifications. Patients or their caregivers in the intervention group were given the contact numbers (on-call phone number) of the clinical pharmacist to report INR test results and get their anticoagulant dose titrations.

Statistical Tool

SPSS 18.0 Windows version was used to perform the statistical analysis. The tests used were one-sample and two-sample chi-square test, one sample binomial test and unpaired t-test. The power of the study was estimated using OpenEpi version 3.03 for Windows OS.

RESULTS AND DISCUSSION

A total of 90 patients were enrolled, consisting of equal samples in pre-intervention and post-intervention phases.

Demographics of evaluated population

The gender distribution portrayed 71 males and 19 females. Among them, males were more prone to cardiovascular diseases. The study found 37 (82%) and 34 (75.6%) male patients in the pre- and post- intervention phases, respectively. The prescription rate of anticoagulants is also higher among male (Sharma, Krishnamurthy, Snyder, & Mauro, 2017; Thompson et al., 2017). Numerous evidences state the prevalence of anticoagulant administration among geriatric population (Reddy, Prasad TS, Swetha, Nirmala, & Ram, 2018). We also attained similar results with mean age between 60 to 65 years. The demographic details of the patients are represented in Table 1.

Cardiovascular disease conditions of the patients

The myocardial infarction was observed to predominate in our study, with Non ST-segment elevation Myocardial Infarction (NSTEMI) being more prevalent with ≥20 percent in both pre- and post interventional phases and ST-segment elevation Myocardial Infarction (STEMI) along with 20% in post-interventional phase and 11% in pre-interventional phase (Table 2).

Cardiovascular diseases are the chief fatal lifestyle disease estimated to take away the lives of 17.9 million every year. Four

Table 1. The demographic characteristics of the subjects enrolled in the study.

Demographic characters	Pre-intervention phase	Post-intervention phase
Gender	Male=82.2% (n=37)	Male=75.6% (n=34)
	Female=17.8% (n=8)	Female=24.4% (n=11)
Age	Range=33 to 85 years	Range=39 to 85 years
	Mean=60.49 years (S.D=11.69)	Mean=62.60 years (S.D=11.762).

S.D Standard Deviation

Table 2. Diagnosis distribution in pre-interventional group and post-interventional groups.

Diagnosis	Pre-intervention phase No.(%)	Post-intervention phase No.(%)
Non ST Segment Elevation Myocardial infarction (NSTEMI)	10 (22.2)	9(20.0)
Inferior Wall Myocardial Infarction (IWMI)	6 (13.3)	4(8.9)
Cerebrovascular Accident	2(4.4)	0(0)
Steroid Abuse	1(2.2)	(0)0
Pulmonary Arterial Hypertension	1(2.2)	0(0)
ST Segment Elevation Myocardial Infarction	5(11.1)	9(20.0)
Anterior Wall Myocardial Infarction (AWMI)	4(8.9)	5(11.1)
Transient Ischemic Attack	1(2.2)	0(0)
Coronary Artery Disease	5(11.1)	2(4.4)
Unstable Angina	1(2.2)	0(0)
Ischemic Heart Disease	2(4.4)	0(0)
Acute Coronary Syndrome	1(2.2)	5(11.1)
Not Specified	1(2.2)	0(0)
IWMI/STEMI	1(2.2)	5(11.1)
IWMI/NSTEMI	2(4.4)	1(2.2)
AWMI/STEMI	1(2.2)	2(4.4)
AWMI/NSTEMI	1(2.2)	2(4.4)

STEMI: ST Segment Elevation Myocardial Infarction, NSTEMI: Non ST Segment Elevation Myocardial Infarction, IWMI: Inferior Wall Myocardial Infarction, AWMI: Anterior Wall Myocardial Infarction

out of five deaths related to cardiovascular disease are due to heart attacks and strokes, and one-third of these deaths occur among people under 70 years of age. The mean age of both the control and intervention groups was greater than 60 years, which indicated the age to be one of the major risk factors for cardiovascular diseases. Changes in the diet pattern and sedentary lifestyle are the major confounders for precipitating and worsening the disease (Akesson, Larsson, Discacciati, & Wolk, 2014; Mosca, Barrett-Connor, & Wenger, 2011). Large proportions of people in the Perinthalmanna locality are financially overwhelmed, which tempt them towards junk foods and improper exercise. This had a colossal negative impact on the health status of the people.

Anticoagulants

Heparin, enoxaparin and fondaparinux are commonly prescribed agents. The mechanism of action of heparin showed to inhibits multiple clotting factors (i.e. Xa, IXa,Xia,XIIa) and antithrombin III, whereas enoxaparin act on factor Xa with little effect against thrombin. This change in the pharmacodynamics of unfractionated heparin was stated to trigger excess hemorrhage among patients (Heparin induced Thrombocytopenia) comparing to its derivative (Bauer, 2001). Fondaparinux is a factor Xa inhibitor similar to low molecular weight heparin that does not inhibit thrombin (Yau et al., 2011). The PENTALYSE study demonstrated fondaparinux to be safer compared to unfractionated heparin (Coussement et al., 2001). Table 3 portrays the prescription pattern of anticoagulant therapy among patients.

Table 3. The categorization of patients based on the anticoagulant therapy at pre and post-intervention phases.

Anticoagulant used	Pre-intervention phase	Post-intervention phase
Heparin	27	31
Enoxaparin	9	9
Fondaparinux	1	1
Heparin +Fondaparinux	1	0
Nil	7	4

Adverse reactions associated with anticoagulant use

The incidence rate of ADRs or medication errors is evident in patients treated with anticoagulants. In the pre-intervention phase, 48.9% (n=22) of patients came across adverse drug reactions (ADR). When we tested the observation with one sample Binomial test, it was statistically significant with p-value=0.037. In the post-intervention phase, 26.7% (n=12) patients had ADRs. Similarly, one sample Binomial test yielded highly significant findings (p-value=0.007). Hence, we erected a slight reduction in the frequency of ADR due to the intervention.

Hematuria and major bleeding was prominently observed in the study, apart from them, gastrointestinal bleeding and postmenopausal bleeding do exist in a slight lower proportion (Ahmed, Majeed, & Powell, 2007). Other side effects identified were ecchymosis, epistaxis, oral bleeding, conjunctival hemorrhage, middle ear bleeding, and hematospermia. The Naranjo scale defined all the adverse events to be definite. The mortality rate and healthcare expense were higher in the patients who had been reported with adverse events (Piazza et al., 2011). Assessment of risk factors for bleeding should become an integral part of the drug monitoring system, and it is represented in figure 4 A and 4 B for pre- and post-interventional phases, respectively.

Medication errors associated with anticoagulant use

In the pre-intervention phase, the medication errors of the patients were identified 65% related to heparin, 14% of fondaparinux, and about 25% because of enoxaparin administration. Out of 22 (48.9%) medication errors; 15 were prescribing errors, 2 dispensing errors, 4 drug administration errors and a patient error (Figure 1). However, the post-intervention phase showed 63%, 23% and 8% medication errors among patients on heparin, enoxaparin and fondaparinux therapy, respectively. Of the total 12 (26.66%) medication errors, 7 had prescribing errors, 2 dispensing errors, 2 drug administration errors and a patient error. Table 4 and 5 represent the prescribing errors reported in our study population. A study reported 8.3% anticoagulant medication error; most were encountered associated with low molecular weight heparin. They emphasized the necessity to peer on the prescribing phase of error. The difference between our pre- and post- intervention groups regarding medication error was statistically significant (with chi-square value=4.727 and p-value=0.0297). Within a short period of time, the adherence to the treatment protocol slightly decreased the fallacy. Every quarter of the year, compliance to the treatment guideline should be measured, and this periodical reviewing would enhance patient safety. The pharmacist should support the physician for patient follow-up by collecting and interpreting their previous laboratory investigations or encouraging discontinuation of drugs, when indicated. There is evidence that justified and proved the ex-

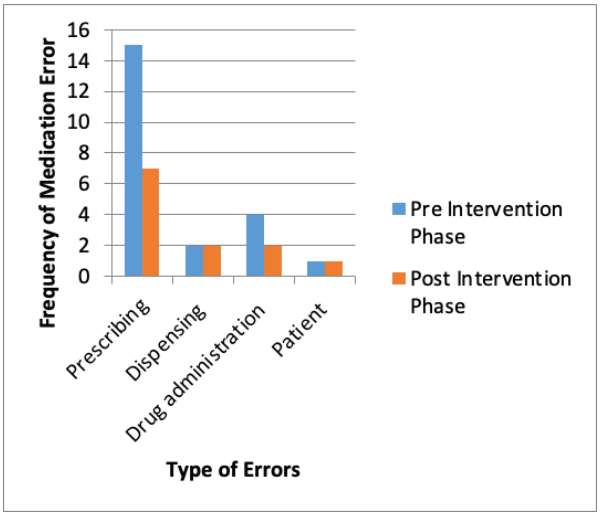

Figure 1. The frequency and type of medication error in Pre and Post intervention groups.

Table 4. The prescription errors concerning Heparin and Enoxaparin.

	Pre-intervention phase (rate of infusion)		Post-intervention phase (rate of infusion)	
Anticoagulants used	**Specified in prescription**	**Not Specified in prescription**	**Specified in prescription**	**Not Specified in prescription**
Heparin	7	19	21	10
	Pre-intervention phase(dose)		**Post-intervention phase(dose)**	
Anticoagulant used	**Specified in prescription**	**Not Specified in prescription**	**Specified in prescription**	**Not Specified in prescription**
Enoxaparin	3	7	9	0

Table 5. Categorization of medication errors with respect to the drug at pre and post-intervention phases.

Anticoagulants	Percentage of errors in pre-intervention phase	Percentage of errors in post-intervention phase
Heparin	63%	67%
Enoxaparin	23%	25%
Fondaparinux	14%	8%

traordinary contribution of clinical pharmacists in attenuating medication errors in hospital settings (Tariq, Vashisht, Sinha, & Scherbak, 2021).

Researchers observed profound incidences of fatal and severe medication issues and claimed preponderance of them to be preventable. Anticoagulants, being a class with aloft utilization, warrant close monitoring of blood parameters (Lakshminarayan, Solid, Collins, Anderson, & Herzog, 2006). Excess or insufficient dosing would impart significant detrimental consequences to the patients (Henriksen, Nielsen, Hellebek, & Poulsen, 2017). In our study, within a 6-month period, there was a drastic improvement (48.9% to 26.66%) in medication error, and compliance to treatment guidelines was ameliorated from 46.7% to 62.22%. This positive shift reflected the physician's acceptance for our interventions. Meanwhile, most of the errors we noticed resulted from lack of written guidelines/policies (i.e. standardized heparin dosing nomogram) and from the limitation in implementing the weight-based heparin dose adjustment in the Cardiology Department. Figure 2 A and 2 B depict the prime complications of anticoagulant therapy identified in pre-intervention and post-intervention phases, respectively.

Compliance to treatment guideline

The anticoagulant compliance rate with the ACCP guideline was nominal (Hajj et al., 2021; Yu, Dylan, Lin, & Dubois, 2007). Our adherence assessment of the pre-intervention therapy to American College of Chest Physicians Antithrombotic guidelines 9th edition (Table 6) revealed that 46.7% (n=21) of the anticoagu-

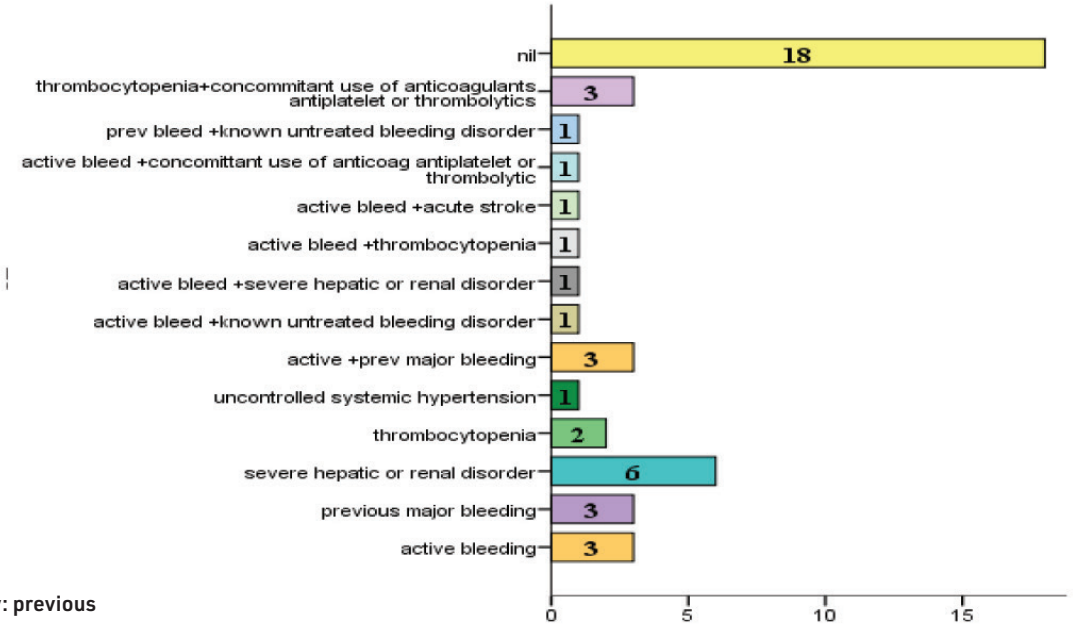

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Figure 2A. Bar graph representing bleeding risk factors among pre-intervention subjects.

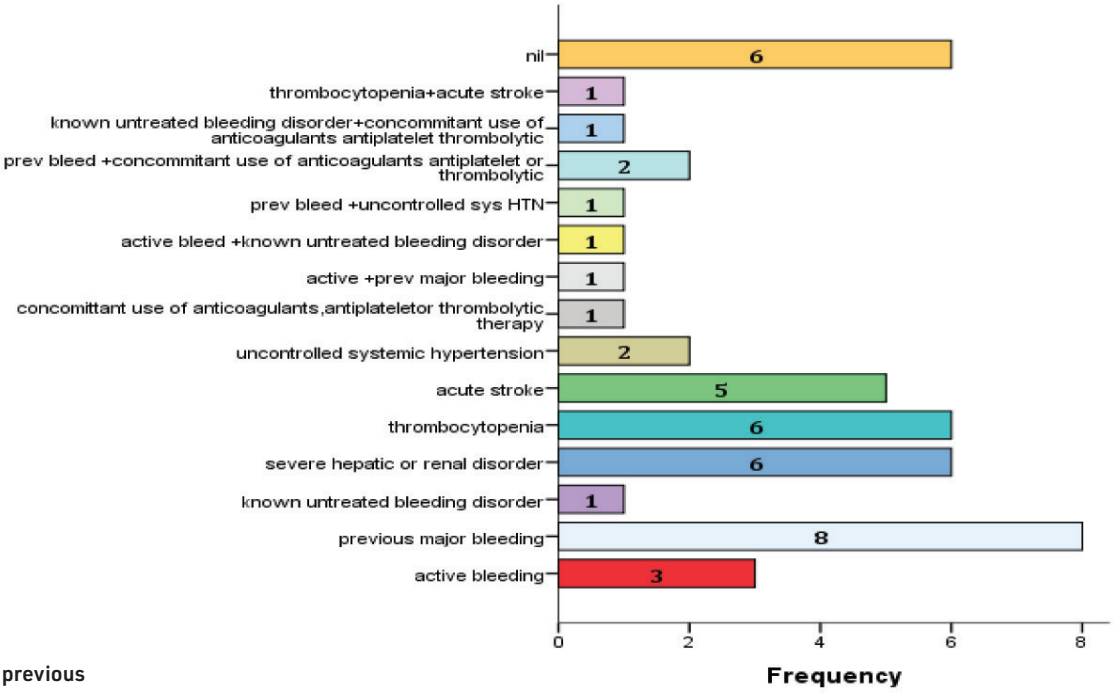

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Figure 2B. Bar graph representing bleeding risk factors among post-intervention subjects..

lation therapy was non-compliant with the regimen (Table 7). Partial compliance to the standard treatment was demonstrated by 24.4% (n=11). On the other side, our clinical pharmacist led anticoagulation monitoring service incremented (62.22%) the physician's compliance to ACCP guideline treatment, and partial compliance was also escalated (26.67%, n=12).

Table 6. The monitoring parameters requisite to the assessment of compliance to anticoagulant protocol.

Parameters for Full compliance	Parameters for Partial compliance	Parameters for Non-compliance
Baseline values APTT, PT, INR, serum creatinine, AST, ALT, ALP **Other orders;** Blood urea, serum creatinine, PCV, Total RBC Platelet count, Hb, TLC, serum ALP, SGOT, SGPT **Order of heparin;** Bolus dose according to specific diagnosis **Infusion rate;** Specified	**Baseline values** APTT, PT, INR, serum creatinine, AST, ALT, ALP ONLY other orders like blood urea, serum creatinine, total RBC, platelet count, Hb **Order of heparin;** Bolus dose according to specific diagnosis **Infusion rate;** Not specified	No baseline values obtained OR only APTT/PT, INR are obtained NO VALUES obtained OR only APTT/ PT, INR are obtained Not specified Not specified

APTT: Activated platelet thromboplastin time, INR: International normalized ratio, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, RBC: Red blood Cell, Hb: Hemoglobin, PT: Prothrombin Time, SGOT: Serum glutamic oxaloacetic transaminase, SGPT: Serum glutamic pyruvic transaminase

Table 7. ACCP Compliance in Pre and Post interventional phases.

ACCP Compliance	Pre-intervention	Post-intervention
Yes	13(28.9%)	28(62.22%)
No	21(46.7%)	5(11.11%)
Partial	11(24.4%)	12(26.67%)

A study of 500 patients for two years concluded that implementation of pharmacist directed anticoagulation monitoring services provides 73% transition of care metric compliance occurred in pharmacist led group; also, there was a 32% reduction in the composite safety end point in the pharmacist led group (Schillig et al., 2011). Here, we could not consider transition care metrics due to limited availability of patients on warfarin therapy. Instead, compared two groups of people (before

and after intervention) with equal demography, there was no significant difference in gender, age, admission setting, days of hospitalization, and route of administration. Pharmacist-managed group of patients were found to have no major risk of bleeding (Mamdani et al., 1999).

CONCLUSION

Inappropriate anticoagulant dosing would increase the risk of developing significant bleeding diathesis with failure to produce a therapeutic response. The pharmacist directed anticoagulation monitoring system took the edge of medication error and also was able to anticipate the ADRs. The involvement of the clinical pharmacist and his/her cooperation with physicians prompted their prescription orders in line with the ACCP guideline.

Abbreviations

a. AMS - Pharmacist-directed Anticoagulant Monitoring Services

b. INR – International Normalized Ratio

c. CVD – Cardiovascular Diseases

Peer-review: Externally peer-reviewed.

Ethics Committee Approval: This study was approved by the Institutional Ethics Committee (03/02/2015, No. IEC/ASH/2015/PD/9).

Informed Consent: Written consent was obtained from the participants.

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

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

Financial Disclosure: Authors declared no financial support.

Acknowledgment: We extend our sincere thanks to the department of pharmacy practice for providing all kind of assistance for the completion of the work.

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Istanbul J Pharm 51 (3): 299-306
DOI: 10.26650/IstanbulJPharm.2021.933139
Original Article

The inverse association between ANGPTL8 and PI3K-mTOR-PPARγ expressions in adipose tissue of high-fructose-fed rats: The modulatory effect of kefir

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Cite this article as: Akar, F., Guney, C., Ozer, H. B., Pektas, M. B., Koca, H. B., Kocabas, A., & Sadi, G. (2021). The inverse association between ANGPTL8 and PI3K-mTOR-PPARγ expressions in adipose tissue of high-fructose-fed rats: The modulatory effect of kefir. *İstanbul Journal of Pharmacy, 51*(3), 299-306.

ABSTRACT

Background and Aims: The dietary high-fructose intake might be a risk factor for several metabolic diseases. Kefir, a fermented milk product, has been proposed to have beneficial health effects. In this study, we aimed to investigate the effects of fructose consumption and kefir supplementation on the lipogenesis-related genes including angiopoietin-like protein 8 (*angptl8*), phosphoinositide 3-kinase (*pi3k*), mammalian target of rapamycin (*mtor*), and peroxisome proliferator-activated receptor γ (*pparγ*) as well as inflammatory factors in the adipose tissue to provide new mechanistic insights into lipogenesis.
Methods: Fructose was given to the rats as a 20% solution in drinking water for 15 weeks. Kefir was administered by gastric gavage once a day during the final six weeks.
Results: There was an upregulation of *angptl8* mRNA expression in adipose tissue of rats given fructose. However, expressions of *pi3k, mtor,* and *pparγ* mRNAs were impaired in the adipose tissue. The increased interleukin (IL)-1β levels, but decreased IL-10, were also measured. There was no change in expressions of sirtuin1 (*sirt1*) and nuclear factor erythroid 2-related factor 2 (*nrf2*). Kefir supplementation suppressed expression of *angptl8*, but increased *pi3k* and *mtor* in the adipose tissue of high-fructose-fed rats.
Conclusion: Activation of gene expression of *angptl8*, together with the suppression of *pi3k*, *mtor*, and *pparγ*, showed that there was an inverse association between these lipogenic genes in the adipose tissue of rats fed with high-fructose. Kefir supplementation has modulatory effects on fructose-induced changes except for *pparγ* expression. These findings showed that dietary fructose and kefir might reciprocally affect the lipogenesis-related genes in the adipose tissue.

Keywords: Dietary fructose, Kefir, ANGPTL8, PI3K-mTOR-PPARγ pathway, Lipogenesis

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Submitted: 05.05.2021
Revision Requested: 21.08.2021
Last Revision Received: 24.08.2021
Accepted: 03.09.2021
Published Online: 06.12.2021

INTRODUCTION

The metabolic syndrome is a cluster of conditions including hyperlipidemia, hyperinsulinemia, hypertension, central adiposity, fatty liver disease, and chronic low-grade inflammation. The increased prevalence of metabolic syndrome in the world might be due to high consumption of fructose, particularly in the form of soft drinks, in the current human diet (Hannou, Haslam, McKeown, & Herman, 2018; Jensen et al., 2018). We have previously shown that dietary high-fructose leads to a change in adipose tissue function with an alteration in insulin signaling, activation in inflammatory cytokines, and upregulation in lipogenic genes (Pektas, Koca, Sadi, & Akar, 2016; Akar, Sumlu, Alçığır, Bostancı, & Sadi, 2021). Besides, we have determined that high-fructose-induced metabolic disturbance is more likely related to abdominal fat accumulation, but independent from general obesity (Pektaş, Sadi, & Akar, 2015; Akar et al., 2021). Previously, it was shown that the expansion of white adipose tissue was related to insulin resistance and low-grade inflammation (Bastard et al., 2006). Adipose tissue has endocrine and metabolic functions by secreting several hormones and factors that influence lipid and glucose metabolism as well as the effectiveness of insulin (Scherer, 2006). Therefore, dysfunction of adipose tissue might be associated with metabolic diseases.

Angiopoietin-like proteins (ANGPTLs) have been proposed to play essential roles in lipoprotein metabolism, adipogenesis, and inflammation (Carbone et al., 2018). ANGPTL8, also known as lipasin, refeeding induced fat and liver (RIFL) and betatrophin, is mainly expressed in the liver and adipose tissue (Ren, Kim, & Smas, 2012; Zhang, 2012; Wang et al., 2013). Previously, ANGPTL8 was found to regulate plasma triglyceride levels by inhibiting lipoprotein lipase, a key enzyme in the lipoprotein lipolysis pathway (Quagliarini et al., 2012). Deletion of the *angptl8* gene in mice was shown to reduce plasma triglyceride levels (Ren et al., 2012; Quagliarini et al., 2012). On the contrary, overexpression of ANGPTL8 causes an increase in plasma triglyceride levels in mice (Quagliarini et al., 2012; Zhang, 2012). Moreover, in type 2 diabetic patients with insulin resistance, ANGPTL8 levels were positively correlated with triglyceride levels (Chen, Susanto, Chuang, Liu, & Wang, 2016). Metabolic effects of insulin including adipogenesis and lipid accumulation are mediated by phosphoinositide 3-kinase (PI3K)- protein kinase B (Akt) pathway (Wang & Sul 1998; Sakaue et al., 1998). PI3K-Akt pathway was damaged in obesity and type 2 diabetes resulting in insulin resistance (Huang, Liu, Guo, & Su, 2018). Additionally, the stimulatory effect of insulin on lipid metabolism and deposition was mediated by PI3K-Akt-mammalian target of rapamycin (mTOR) (Han et al., 2015). Indeed, mTOR is involved in the regulation of adipogenesis, lipid, and glucose metabolism, as well as insulin resistance (Laplante & Sabatini, 2012). The mTOR complex, mTORC1, increases the expression of peroxisome proliferator-activated receptor γ (PPARγ), which is the master transcriptional regulator of adipocyte differentiation and lipid storage (Zhang et al., 2009). Moreover, mTOR deficiency was found to cause insulin resistance and downregulation of PPARγ expression in white adipose tissue of mice (Shan et al., 2016). The relationship between ANGPTL8 and PI3K-mTOR-PPARγ pathway in adipose tissue is not understood. Therefore, in this study, the association between these lipogenic genes was examined for the first time in the fat tissue of high-fructose-fed rats.

Kefir, a fermented milk product, contains several lactic acid and acetic acid bacteria in a polysaccharide matrix (Rosa et al., 2017). Kefir consumption was reported to have beneficial effects in several disease models (Kim, Jeong, Kim, & Seo, 2019). Kefir peptides were shown to reduce hepatic lipid accumulation and inflammation in high-fructose-fed mice (Chen et al., 2016). Besides, *Lactobacillus kefiri (L. kefiri)*, a kefir bacterium, reduced epididymal adipose tissue expansion and inflammatory factors in fructose-rich diet-fed mice (Zubiría et al., 2017). In another study, kefir grain powder was shown to suppress the lipid synthesis and inflammatory cytokines in adipose tissue and liver of diet-induced obese mice (Choi et al., 2017). In a very recent study, we showed that kefir supplementation improved the level of plasma triglyceride, hepatic weight, triglyceride content, and fatty degeneration as well as omental fat accumulation in high-fructose-fed rats. Moreover, kefir supplementation reduced expression of lipogenic genes, sterol regulatory element-binding protein (*srebp*)*-1c* and fatty acid synthase (*fasn*), as well as produced a marked downregulation in tumor necrosis factor-alpha (TNF-α) and nuclear factor-kappa B (NF-κB) expressions in the liver, but not in adipose tissue of high-fructose-fed rats (Akar et al., 2021). Tibet kefir milk administration reduced serum triglyceride and abdominal fat mass together with an upregulation of the *angptl4* gene in fat tissue of high-fat diet-fed rats (Gao et al., 2019). Modulation of ANGPTL8 and PI3K-mTOR-PPARγ pathway in adipose tissue by kefir remains to be investigated. In the present study, we have examined the impacts of dietary fructose and kefir supplementation on gene expressions of *angptl8* and PI3K-mTOR-PPARγ pathway elements, as well as the levels of inflammatory cytokines in the adipose tissue of rats in order to provide new mechanistic insights into lipogenesis.

MATERIALS AND METHODS

Animals and diets

The Ethical Animal Research Committee of Gazi University (G.Ü.ET-18.018) approved the protocol for animal usage. Three-week-old male *Wistar* rats were housed in temperature- and humidity-controlled rooms (at 20-22°C and 40-60% humidity), with a 12-h light-dark cycle. The rats were fed with a standard rodent chow diet composed of 62% starch, 23% protein, 4% fat, 7% cellulose, standard vitamins, and salt mixture. At the end of the acclimation of one week, the rats were randomly divided into three groups: as control, fructose, and fructose+kefir. Fructose (Danisco Sweeteners OY, Kotka, Finland) was given to the rats as a 20% solution (w/v) in drinking water ad libitum for 15 weeks. Kefir, which was fermented in our laboratory, was given to the rats once a day as 1 ml per 100 g of body weight of animal by gastric gavage for the final 6 weeks. The same volume of water was also given to the control and fructose groups by gavage in the same period for sham operation. At the end of the feeding periods, the rats were anesthetized with a mixture of ketamine-xylazine (100 and 10 mg/kg, respectively, i.p.). The

omental adipose tissues were immediately dissected, blotted dry, and frozen in liquid nitrogen and stored at -85°C for the measurements.

Preparation of kefir

The kefir grains were obtained from Ankara University, Faculty of Agriculture and used (5% w/v) to inoculate the pasteurized cow's whole milk. The fermentation was carried out at 22°C for 24 h. Afterward, the mixture of kefir grain and fermented milk were separated by filtering through a sieve. Kefir was freshly prepared every other day. Kefir grains were stored at 4°C until further use. It contained 8.74±0.46 log CFU/ml of lactic acid bacteria and 4.12±0.78 log CFU/ml of yeast, not containing acetic acid bacteria. Microbial combination of kefir was determined with a metagenomic approach based on next-generation sequencing technology, as we stated in a very recent study (Akar et al., 2021).

Determination of gene expressions with quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were isolated from adipose tissue using RNeasy total RNA isolation kit (Qiagen, Venlo, Netherlands), as described according to the manufacturer protocol. After isolation, the amount and the quality of the total RNAs were determined by spectrophotometry and agarose gel electrophoresis. Then, one µg of total RNA was reverse transcribed to cDNA using a commercial first-strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). Expression levels of *angptl8*, *pi3k, mtor, ppary,* sirtuin1 (*sirt1*) and nuclear factor erythroid 2-related factor 2 (*nrf2*) were determined with a real-time quantitative polymerase chain reaction (qRT-PCR, LightCycler480 II, Roche, Basel, Switzerland). To do this, 1 µl cDNA, 5 µl 2X SYBR Green Master mix (Roche FastStart Universal SYBR Green Master Mix, Roche, Basel, Switzerland), and 2 µl primer pairs of each (Table 1) at 0.5 µM concentrations in a final volume of 10 µl were mixed. qRT-PCR was performed as follows: initial denaturation at 95°C for 10 min, denaturation at 95°C for 10 s, annealing at 58°C for 15 s and extension at 72°C for 15 s with 40 repeated thermal cycles measuring the green fluorescence at the end of each extension step. All reactions were performed in triplicate and the specificity of PCR products was confirmed by melt analysis. The relative expression of genes to internal control glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) was calculated with the quantification tool provided by LightCycler® 480 SW 1.5.1 software.

Measurement of inflammatory parameters in the adipose tissue

The adipose tissue samples were homogenized in 0.1 M phosphate buffer 1:10 (w/v), pH 7.4 and 24,000 cycles/min (Ultra Turrax, IKA Works Inc., USA), and then ultrasonicated at 20,000 cycles/s for 1 min (Dr. Hielscher, Germany). Homogenates were centrifuged at 10,000 x g and 4°C for 15 min, and the supernatants were collected. All the samples were stored at -85°C until analysis. Interleukin (IL)-1β, IL-6, and IL-10 levels in the adipose tissue of rats were measured by using ELISA kits (Cusabio, Houston, TX, USA) according to the manufacturer's instructions.

Statistical analysis

The results are given as the mean ± standard error of the mean (SEM); n is the number of rats. Statistical analyses were performed using one-way ANOVA followed by the Bonferroni *post hoc* test. Data were evaluated with GraphPad Prism (version 6.0, GraphPad Software, La Jolla, CA, USA). *P* values smaller than 0.05 were considered as statistically significant.

RESULTS

Metabolic parameters

Firstly, we used adipose tissue of the rats from our very recent study (Akar et al., 2021), where we have presented the data with metabolic parameters, including body weight, omental fat mass, plasma levels of glucose, insulin, and triglyceride in rats subjected to high-fructose diet as well as kefir supplementation. Briefly, in that paper (Akar et al., 2021), we reported that the major bacteria strains found in the kefir were *L. kefiranofaciens* (85.5%) and *L. helveticus* (12.5%), while the most abundant yeast species were *Kluyveromyces marxianus* (70.4%) and *Saccharomyces mikatae* (29.2%). Regarding metabolic parameters, dietary high-fructose or kefir supplementation did not change the body weight of rats. Importantly, high-fructose intake increased omental fat mass of rats which was markedly reduced by kefir supplementation. High-fructose-induced elevation in plasma glucose was not changed by kefir, but the increase in the insulin level was diminished with kefir. However, dietary fructose-induced augmentation in plasma triglyc-

Table 1. Primer sequences *angptl8*, *pi3k, mtor, ppary, sirt1, nrf2* and internal standard *gapdh* used for the mRNA expression determination of qRT-PCR.

Gene	Forward primer sequence (5'→3')	Reverse primer sequence (3'→5')	Genbank accession number
angptl8	CCTTTTTGACCAAGCACTGG	AAGTGTCCTCTTCTGCCTGA	NM_001271710.1
pi3k	ATGCAACTGCCTTGCACATT	CGCCTGAAGCTGAGCAACAT	NM_053481.2
mtor	GCAATGGGCACGAGTTTGTT	AGTGTGTTCACCAGGCCAAA	NM_019906.1
ppary	CTCAGGTCAGAGTCGCCCC	GAGAGAGACCTCGTCAGGCT	NM_001145367.1
sirt1	CGGTCTGTCAGCATCATCTTCC	CGCCTTATCCTCTAGTTCCTGTG	XM_008772947.1
nrf2	GATTCGTGCACAGCAGCA	GCCAGCTGAACTCCTTAGAC	XM_006234397.2
gapdh	TCCTTGGAGGCCATGTGGGCCAT	TGATGACATCAAGAAGGTGGTGAAG	NM_017008.4

eride was reduced by supplementation of kefir. To investigate possible mechanisms of omental fat accumulation due to the dietary high-fructose and modulatory effect of kefir, we tested the adipose tissue of the same animals in the current study.

High-intake of fructose augmented the gene expression level of *angptl8* in the adipose tissue (2.68 fold, p<0.05 versus control group). The supplementation of kefir significantly decreased the expression of *angptl8* in the adipose tissue of rats (2.29 fold, p<0.05 versus fructose group; Figure 1a). However, high-fructose intake reduced the expression level of *pi3k* (5.49 fold, p<0.05 versus control group), which was significantly increased by kefir supplementation (2.40 fold, p<0.05 versus fructose group), in the adipose tissue (Figure 1b). In the adipose tissue, high-fructose intake decreased *mtor* mRNA expression (2.75 fold, p<0.05 versus control group), while kefir treatment improved the expression of this gene (1.43 fold, p<0.05 versus fructose group; Figure 1c). Excess fructose intake suppressed *pparγ* gene expression (1.55 fold, p<0.05 versus control group), and kefir supplementation did not show any marked change of *pparγ* expression in the adipose tissue of rats (Figure 1d). On the other hand, mRNA expression levels of *sirt1* and *nrf2,* which are cytoprotective factors, were not altered by fructose or kefir treatment in the adipose tissue of rats (Figure 2a, b).

High-fructose intake increased the level of proinflammatory cytokine IL-1β (1.66 fold, p<0.05 versus control group), which was not improved by kefir supplementation in the adipose tissue of rats (Figure 3a). Also, dietary high-fructose reduced the level of antiinflammatory cytokine IL-10 (1.64 fold, p<0.05

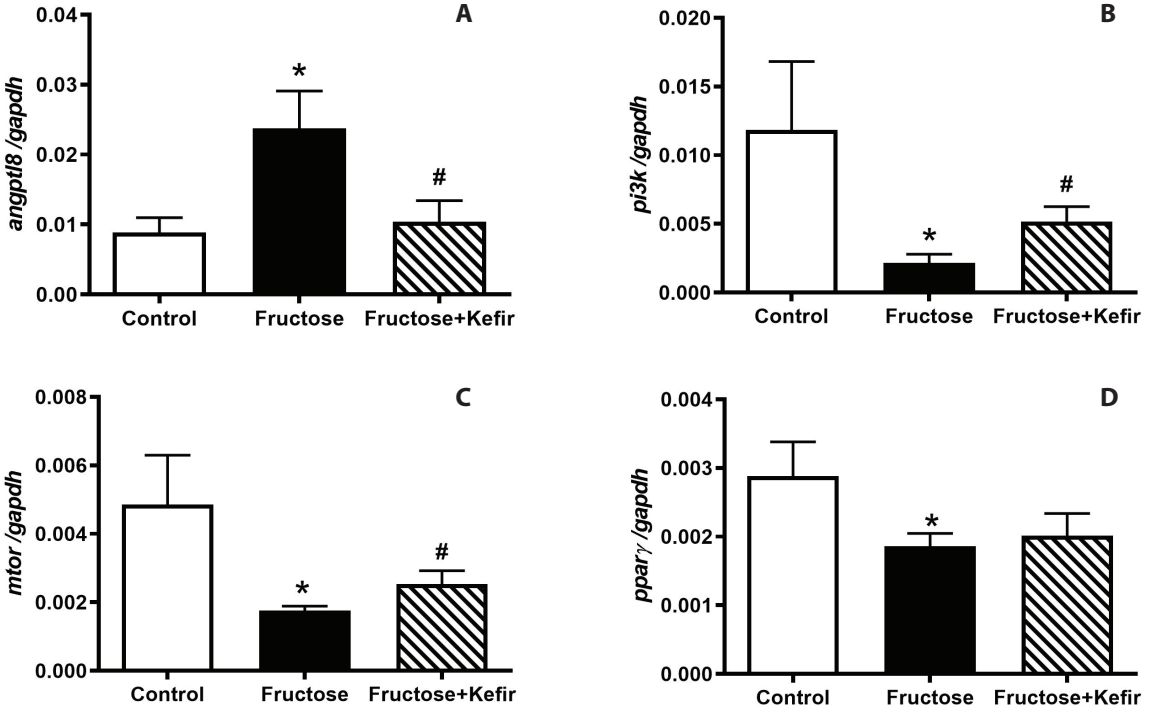

Figure 1. The mRNA expression levels of *angptl8* (A), *pi3k* (B), *mtor* (C), and *ppar*γ (D) in the adipose tissue of control, fructose, and fructose+kefir groups. Data were normalized using *gapdh*. Each bar represents the means from at least six rats. *P<0.05, significantly different from the control; #p<0.05, significantly different from the fructose group.

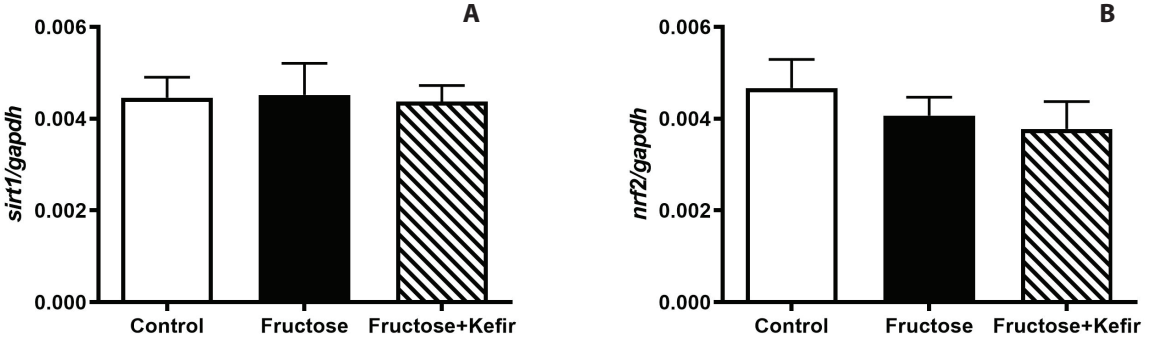

Figure 2. The mRNA expression levels of *sirt1* (A) and *nrf2* (B) in the adipose tissue of control, fructose, and fructose+kefir groups. Data were normalized using *gapdh*. Each bar represents the means from at least six rats.

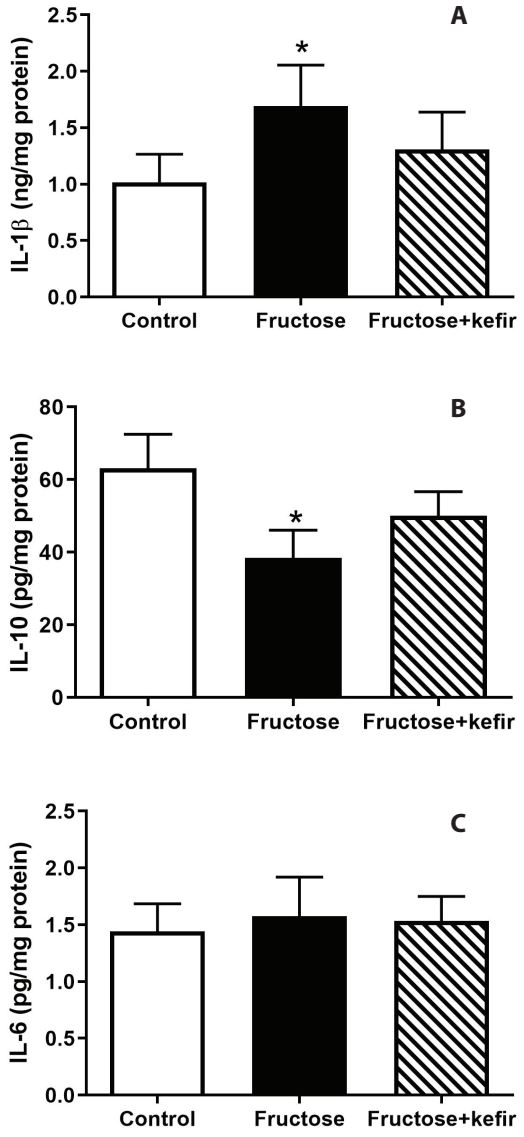

Figure 3. The levels of IL-1β (A), IL-10 (B), and IL-6 (C) in the adipose tissue of control, fructose, and fructose+kefir groups. Each bar represents the means from at least six rats. $^{*}p<0.05$, significantly different from the control.

versus control group) in the adipose tissue, but kefir treatment did not show any regulatory effect on this factor (Figure 3b). Another cytokine IL-6 was not affected by fructose or kefir treatment in the adipose tissue of rats (Figure 3c).

DISCUSSION

The worldwide high frequency of metabolic syndrome is generally accepted due to excess consumption of sugar and fat in the human diet. Accumulating evidence indicates that high intake of fructose provokes metabolic syndrome manifesting dysfunction of the liver, adipose, and vascular tissue (Hannou et al., 2018; Jensen et al., 2018). In our previous studies, we have reported that oral administration of 20% (w/v) fructose causes metabolic syndrome characterized by hypertriglyceridemia, hyperglycemia, hyperinsulinemia, and abdominal fat accumulation leading to dysregulation in vascular, hepatic, renal, intestinal, and testicular functions (Akar et al., 2012, Yildirim et al., 2019; Korkmaz et al., 2019a; Korkmaz et al., 2019b; Sumlu, Bostancı, Sadi, Alçığır, & Akar, 2020; Akar et al., 2021). In the current study, we have shown that dietary fructose increased expression of *angptl8*, but suppressed *pi3k*, *mtor*, and *pparγ* mRNAs together with a change in the levels of inflammatory cytokines in the adipose tissue of rats. Kefir supplementation exerted an improving effect on the expression of these genes, except *pparγ*. Thus, we propose that dietary fructose and kefir may reciprocally affect the lipogenesis-related genes in the adipose tissue.

Insulin plays a vital role in the regulation of energy metabolism by inhibiting gluconeogenesis and promoting lipogenesis (Brown & Goldstein, 2008). In the insulin-resistant state, the hormone loses its ability to reduce glucose production, but paradoxically maintains the lipid synthesis (Brown & Goldstein, 2008; Li, Brown, & Goldstein, 2010). In this context, we showed that fructose intake in the long-term period increased abdominal fat mass, expression of genes involved in insulin signaling, despite induction of proinflammatory markers in the adipose tissues from rats (Pektas et al., 2015; Pektas et al., 2016). Therefore, we propose that the upregulation of insulin signaling pathway in adipose tissue leads to increased visceral adiposity together with an inflammatory status (Pektas et al., 2016). We have also demonstrated that abdominal fat accumulation is accompanied by upregulation of lipogenic genes such as *srebp-1c* and *fasn* expression in the adipose tissue of high-fructose-fed rats (Akar et al., 2021). Indeed, fructose-induced metabolic disturbance is more likely related to increase in abdominal fat mass, independent of general obesity (Pektas et al., 2015; Akar et al., 2021). The contribution of the other lipogenesis-related genes such as *angptl8*, *pi3k*, *mtor* and *pparγ* to abdominal fat accumulation in high-fructose-fed rats was not identified.

The studies showed that overexpression of ANGPTL8 in the liver increases circulating triglyceride concentration and causes activation of insulin secretion (Quagliarini et al., 2012; Zhang, 2012; Wang et al., 2013). However, lacking hepatic ANGPTL8 causes low plasma triglyceride levels and decreases delivery of dietary lipids to adipose tissue (Oldoni et al., 2020; Izumi et al., 2018). Basically, insulin decreases circulating triglycerides by also inhibiting adipose tissue lipolysis (Nidhina Haridas et al., 2015). It was also reported that insulin significantly increases ANGPTL8 expression in the 3T3-L1 adipocytes cell line (Ren et al., 2012). Besides, the circulating ANGPTL8 levels were found to be increased in certain metabolic diseases, including type 2 diabetes mellitus (Abu-Farha et al., 2015; Hu et al., 2014; Chen et al., 2016), non-alcoholic fatty liver disease (Lee et al., 2016; García-Monzón et al., 2018), obesity (Fu et al., 2014), and metabolic syndrome (Abu-Farha et al., 2016; Wang et al., 2016). Herein, we demonstrated an upregulation of *angptl8* mRNA expression in samples of abdominal adipose tissue in high-fructose-fed rats. In the same protocol, we have very recently reported an increase in plasma insulin and triglyceride levels also in abdominal fat mass alongside upregulation of lipogenic genes *srebp-1c* and *fasn* in the adipose tissue (Akar et al., 2021). Overall, it can be evident that abdominal fat accumulation together with hyperinsulinemia and hypertriglyceridemia due to

dietary high-fructose is associated with increased expression of *angptl8* mRNA and other lipogenic genes, namely *srebp-1c* and *fasn* in the adipose tissue.

The gene and protein expression of ANGPTL8 is stimulated by different proinflammatory cytokines such as TNFα and IL-1β in various cell lines. Also, it was observed that there was a correlation between ANGPTL8 level and lipopolysaccharide-induced acute inflammatory response in the different tissues of mice (Zhang et al., 2017). In this study, proinflammatory cytokine IL-1β was increased in the adipose tissues of rats fed with high-fructose consistent with the results of previous studies (Ma et al., 2013; Pektas et al., 2016). Besides, we also showed that dietary high-fructose leads to low level of IL-10, which is known as an antiinflammatory cytokine, in fat tissue, in agreement with an earlier observation (Barroso et al., 2015). Herein, for the first time, we established that upregulation of *angptl8* is associated with an increase in inflammatory factor, but a decrease in counter regulatory response in adipose tissue. Thus, it can be suggested that there was a correlation between ANGPTL8 and inflammatory factors in adipose tissue in dietary intervention with high-fructose. These findings may be valuable to better understand the role of ANGPTL8 in metabolic syndrome.

In adipose tissue, insulin suppresses lipolysis activating PI3K formation (Okada, Kawano, Sakakibara, Hazeki, & Ui, 1994). Adipocytes obtained from type 2 diabetic patients showed diminished PI3K activation (Rondinone et al., 1997). Also, expression levels of *pi3k* and *pparγ* mRNAs were reduced in adipose tissue of mice with high-fat diet-induced insulin resistance (Li, Yu, & Zhao, 2019). PPARγ activation improves hyperglycemia by increasing sensitivity to peripheral insulin, also decreases triglyceride and adipocyte hypertrophy (Yamauchi et al., 2001). Deletion of PPARγ in the adipose tissue of mice resulted in marked adipocyte hypertrophy and elevation in plasma triglyceride together with insulin resistance (He et al., 2003). It has been shown that the PPARγ signaling through mTOR regulates the differentiation of pre-adipocytes. mTOR deficiency was reported to cause insulin resistance and downregulation of PPARγ expression in white adipose tissue of mice (Shan et al., 2016). All these indicated that PI3K, PPARγ, and mTOR work together as insulin downstream effectors in the regulation of adipogenesis. In a previous study, we surprisingly detected an upregulation in insulin downstream effectors, including *pi3k*, *mtor*, and *pparγ* mRNAs in adipose tissue of rats subjected to fructose intake (10% fructose in drinking water) in the long-term period of 24 weeks (Pektas et al., 2016). In the present study, expressions of *pi3k*, *mtor*, and *pparγ* mRNAs were impaired in the adipose tissue of rats given 20% fructose solution for 15 weeks. These discrepancies can be attributed to the differences in concentration and duration of fructose given in diet reflecting adaptive and compensatory changes. Taken all together, the increases in *angptl8* expression and inflammatory factor IL-1β level were associated with the impairment in *pi3k*, *mtor*, and *pparγ* mRNAs, also anti inflammatory factor IL-10 levels, in the adipose tissue of rats fed with high-fructose. Regarding cytoprotective factors such as *sirt1* and *nrf2*, which could be activated as a compensatory mechanism to counteract inflammation in adipose tissues, we did not measure any change in their genes expression by dietary fructose indicating these factors are not closely related to the process in adipocytes during fructose feeding (Yoshizaki et al., 2009; Schneider & Chan, 2013).

Recently we and others showed that, kefir supplementation improved the metabolic parameters including plasma triglyceride, and insulin levels as well as omental fat mass in high-fructose-fed rats and metabolic disorders induced by monosodium glutamate (Akar et al., 2021; Rosa et al., 2016). Furthermore, supplementation with *L. kefiri*, which is one of the probiotic bacteria of kefir, suppressed body weight gain and epididymal adipose tissue expansion in high-fructose-fed mice (Zubiría et al., 2017). Herein, kefir supplementation suppressed the increased *angptl8* mRNA expression in adipose tissue of high-fructose-fed rats. In this line, Tibet kefir intake was shown to reduce plasma triglyceride levels and abdominal fat mass as well as to normalize gene expression of *angptl4* which is an activator of triacylglycerol metabolism (Janssen et al., 2018) in the adipose tissue of high-fat diet-fed rats (Gao et al., 2019). In our previous study, kefir has not normalized the upregulation of lipogenic genes *srebp-1c* and *fasn* in the adipose tissue but reduced abdominal fat mass (Akar et al., 2021). Given, improving effect of kefir on abdominal fat accumulation may depend on downregulation of *angptl8* mRNA in adipose tissue of high-fructose-fed rats. Additionally, for the first time, we have found a decrease in *pi3k* and *mtor* mRNAs, which are downstream effectors of insulin, in the adipose tissue after kefir supplementation to fructose feeding rats. However, kefir supplementation did not change the decreased *pparγ* mRNA expression in this tissue. Treatment with kefir powder obtained from households in Russia reduced epididymal fat pad weight and PPARγ in high-fat diet-induced obese mice (Choi et al., 2017). Notably, lactic acid bacteria strains possessed PPARα/γ agonist activity and ameliorated dyslipidemia in obese mice (Nakamura et al., 2016). More studies are required to elucidate the modulatory effect of kefir on lipogenesis-related genes, including *pi3k*, *mtor*, and *pparγ* in adipose tissue.

In conclusion, dietary fructose-activated gene expression of *angptl8*, together with the suppression of *pi3k*, *mtor*, and *pparγ* mRNAs, showed that there was an inverse association between these two classes of lipogenic genes in adipose tissue of rats fed with high-fructose. These were accompanied by changed inflammatory factors. Kefir supplementation has modulatory effects on fructose-induced changes except for the levels of inflammatory factors and *pparγ* expression. Our findings revealed that dietary fructose and kefir might reciprocally affect the lipogenesis-related genes in the adipose tissue. Further studies are necessary to clarify dietary regulation of lipogenesis in adipose tissue.

Peer-review: Externally peer-reviewed.

Ethics Committee Approval: This study was approved by the Ethical Animal Research Committee of Gazi University (G.Ü.ET-18.018).

Author contributions: Conception/Design of Study- F.A.; Data Acquisition- F.A., C.G., H.B.Ö., M.B.P., H.B.K., A.K., G.S.; Data Analysis/Interpretation-F.A., G.S., C.G., H.B.K.; Drafting Manuscript- F.A., C.G.; Critical Revision of Manuscript-F.A., G.S.; Final Approval and Accountability- F.A., C.G., H.B.Ö., M.B.P., H.B.K., A.K., G.S.

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

Financial Disclosure: This study was supported by Gazi University Research Fund with the grant number BAP 02/2018-14.

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Istanbul J Pharm 51 (3): 307-312
DOI: 10.26650/IstanbulJPharm.2021.870571
Original Article

Evaluation of the adherence to antidiabetic medications among Iraqi patients with T2DM using the Iraqi antidiabetic medication adherence scale (IADMAS)

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Cite this article as: : Hussain, S. A., Al-Tukmagi, H. F., Abdulrahman, Z. S., Sadeq, M. M., Hashim, D. N., Kahdhim, R. S. ... Al-Tugmagi, D. H. (2021). Evaluation of the adherence to antidiabetic medications among Iraqi patients with T2DM using the Iraqi antidiabetic medication adherence scale (IADMAS). *İstanbul Journal of Pharmacy, 51*(3), 307-312.

ABSTRACT

Background and Aims: Management of diabetes is a multifactorial process, and adherence to treatment programs plays a role in glycemic control. The present study evaluates the adherence rate to the antidiabetic medications with a newly developed scale and patients' HbA1c levels among Iraqi patients with type-2 diabetes.

Methods: A cross-sectional study among adult patients receiving diabetic care in public and private healthcare settings within Baghdad City was conducted. The medication adherence was evaluated using the Iraqi Antidiabetic Medication Adherence Scale (IADMAS) to determine the factors associated with non-adherence to anti-diabetic medications. Glycated hemoglobin (HbA1c) levels of the participants were used as an indicator of glycemic status.

Results: The outcome measures include the comparison between glycemic status and the claims of antidiabetic medication adherence of patients with T2DM, in addition to the expected benefits to clinical practice. A total of 442 patients with type 2 diabetes participated in the study and responded by completing the given questionnaire (response rate: 96.1%). The prevalence of non-adherence to medication was reported to be no more than 30%. Adherence based on HbA1c values reflected a high false-positive value of adherence with an extremely high true negative value. The adherence of Iraqi patients to their medications demonstrated a high negative predictive value (0.905) and a high sensitivity (0.928) with low specificity (0.156) values.

Conclusion: The use of IADMAS in this study failed to confirm the consistency between the apparent claims of adherence to anti-diabetic medications and the HbA1c value as a marker of glycemic control.

Keywords: T2DM, antidiabetic medications, patient adherence, IADMAS

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Submitted: 31.01.2021
Revision Requested: 15.09.2021
Last Revision Received: 18.09.2021
Accepted: 30.09.2021
Published Online: 00.00.0000

INTRODUCTION

Diabetes mellitus (DM) is a common chronic metabolic condition that has been on the rise in recent years (Ogurtsova et al, 2017). Based on its pathophysiology, diabetes causes a variety of acute and chronic health problems that, if not adequately managed, can result in severe morbidity and mortality (Stolar, 2010; Bertoni, Krop, Anderson, & Brancat, 2002). In terms of epidemiology, the number of adults diagnosed with diabetes has risen rapidly, from 108 million cases in 2000 to 422 million cases in 2014. Most patients are unconcerned about their health issues, and many do not undergo treatment (Chan, 2017). Deaths from diabetes are anticipated to rise, and diabetes may become one of the top ten causes of death by 2030 (Sarwar et al., 2010). Type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) are the two main kinds of diabetes mellitus (DM). T1DM is caused by a lack of insulin production and is usually linked to an autoimmune illness, whereas T2DM is caused by insufficient insulin production and/or resistance of target tissues to its action and is the most prevalent type of diabetes worldwide (ADA, 2020; Kaiser, Zhang, & Van der Pluijm et al, 2018). T2DM is caused by a number of risk factors, including a lack of physical activity, poor dietary habits, and smoking (among many others), all of which raise the risk of getting the disease (Bi et al., 2012). As a result, the treatment strategies for T2DM include both medication and lifestyle changes. Poor adherence to T2DM treatment guidelines might result in an adverse response and serious comorbidities such as gangrene, nephropathy, retinopathy, neuropathy, and stroke (Forbes & Cooper, 2013), while proper adherence to the treatment program is crucial and required to get the best possible treatment-related outcomes (Chaudhury et al., 2017). Unfortunately, poor adherence is a widespread issue among patients with T2DM around the world (Polonsky & Henry, 2016), which may be linked to poor glycemic control and higher treatment costs as a result of greater use of healthcare resources (Egede, Gebregziabher, Echols, & Lynch, 2014; Egede et al., 2012). Currently, a variety of practical and accurate patient-reported measures are frequently used to assess treatment adherence among diabetic patients (Stirratt et al, 2015; Capoccia, Odegard, & Letassy, 2016). The vast majority of currently valid and widely used tools to assess antidiabetic medication adherence, such as the Medication Adherence Questionnaire (MAQ), the Morisky Medication Adherence Scale-eight items (MMAS-8), the Medication Adherence Rating Scale, and the Adherence to Refills and Medication Scale, are not specific enough to achieve the ideal targets because they are not primarily used for assessing antidiabetic medication adherence (Nguyen, Caze, & Cottrell, 2014). Although most of the adopted instruments are designed to assess medication adherence in patients in affluent nations, only a few scales, such as the Malaysian Medication Adherence Scale (MALMAS), are designed to assess adherence to anti-diabetic drugs in developing countries (Chung, Chua, Lai, & Morisky, 2015). Because of the significant differences in cultures and beliefs in this regard, it is acknowledged that community-related personal characteristics and cultural beliefs can significantly influence the adherence of patients with T2DM to their treatment protocols (Chia, Schlenk, & Dunbar-Jacob, 2006). This will not ensure proper implication of an empirical formula for evaluating medication adherence among Iraqi patients.

The aim of this study is to examine adherence to antidiabetic drugs in Iraqi patients with T2DM using a locally designed modified scale produced by Mikhael et al in 2019 (Mikhael, Hussain, Shawky, & Hassali, 2019) and HbA1c values.

METHODS

Patient selection and study design

In this cross-sectional study, a sample of 460 patients (>18 years of age) previously diagnosed with T2DM were randomly recruited from public and private clinical settings within the Baghdad City area from August 2019 to April 2020. They had previously been on antidiabetic medications for at least 6 months before inclusion in this study. Only 442 patients successfully filled the questionnaire, and their responses were included in the study. The exclusion criteria included T2DM patients with associated comorbidities such as impaired renal function, cognitive disorder, depression, and pregnancy. Before inclusion, all patients were clinically assessed to exclude anyone with suspected but undiagnosed depression (Whooley, Avins, Miranda, & Browner, 1997).

Ethical approval

The research protocol was evaluated and ethically approved by the Local Research Ethics Committee for Medical Studies, Faculty of Pharmacy, Al-Rafidain University College (REC: 4/2019). All the enrolled patients were required to provide verbal consent before participation in the study.

Assessment and outcome evaluation

All the recruited patients were interviewed by the researchers to thoroughly explain the purpose of the study and details of the protocol. Only those who provided their informed consent were requested to complete a self-administered questionnaire based on the locally designed and validated form, and the Iraqi Antidiabetic Medication Adherence 5 Scale (IADMAS) (Mikhael et al, 2019). The IADMAS has eight items in total, three of which were used to assess medication-taking behavior directly by providing five responses: (1) always (daily), (2) often, (3) occasionally, (4) seldom, and (5) never. The remaining five items were utilized to determine the cause of non-adherence by providing a binary response of "Yes" or "No." The questionnaire was presented to patients with unsatisfactory educational levels and those with visual impairment *via* a face-to-face interview with the researchers. The participants needed approximately 5-10 min to complete the questionnaire. HbA1c values less than 7 were considered to have good glycemic control (Grant & Kirkman, 2015).The association between glycemic control and adherence to antidiabetic medications was utilized to measure true positive (TP) results (patients who are non-adherent to their treatment and have poor glycemic control), false positive (FP) results (patients with poor glycemic control despite being adherent to their treatment), true negative (TN) results (patients with good glycemic control and good medication adherence), and false-negative (FN) results (patients with good glycemic control despite being non-adherent to their treatment). Specificity and sensitivity, as well as positive

and negative predictive values of the IADMAS outcomes, were measured using the following equations:

Positive predictive value = 100% TP/(TP+FP)

Negative predictive value=TN/(TN+FN)×100%

Sensitivity=TP/(TP+FN)×100%

Specificity=TN/(TN+FP)×100%

The reliability of the scale was evaluated by test-retest on 40 patients with a Cronbach-α value of 0.87.

Statistical analysis

Data input and analysis were done using the Statistical Package for the Social Sciences (SPSS) V.24. Categorical variables were presented as percentages and frequencies, while mean and SD was used to present continuous variables. The Kolmogorov-Smirnov test was used to test the normal distribution of continuous variables. The Mann-Whitney U test was used to test the mean difference between continuous variables when applicable. The P values below 0.05 were considered for significant differences between variables.

RESULTS

A total of 460 participants were recruited to participate in the present study and 442 of them responded by completing the given questionnaire (response rate: 96.1%). Demographic and clinical details of the participants are presented in Table 1. The male gender represents 56% of the participants. The other characteristics of the participants show that 76.6% of them are positive smokers and 71.3% of them have a positive family history of T2DM and only 22% of them are physically active. However, 63.3% of the participants followed a dietary restriction program. Table 1 also shows that 82% of the participants followed a treatment program that included oral antidiabetic medications only. In Table 2, many participants stated they never missed or changed the doses of their medication, and never changed the timing of their doses (56%-75%) but only a small percentage actually practiced such actions (2%-15%). Regarding of the adherence of participants to their medications in different conditions, Table 3 indicates that 99.3% take their medications with them when they are away from home; and 97% did not stop taking their medications without medical consultation about the awareness of adverse effects. Ninety-seven percent did not

Table 1. Demographic and characteristic data of the participants (n=442).

Parameter	Value
Age (years), mean±SD (range)	52.55±12.31 (24-80)
Gender n(%)	
Male	247 (56)
Female	195 (44)
Body mass index (kg/m^2), mean±SD (range)	28.47±4.81 (17-44)
Positive cigarette smoking, n(%)	339 (76.6)
Positive family history, n(%)	315 (71.33)
Positive physical activity, n(%)	97 (22)
Positive Dietary restriction, n(%)	280 (63.33)
Duration of DM (year), mean±SD (range)	8.62±4.66 (1-23)
Number of prescribed medications, mean±SD (range)	1.81±0.94 (1-5)
Type of medication, n(%)	
Oral	362 (82)
Injectable	50 (11.3)
Combination	30 (6.7)
Duration on treatment (year), mean±SD (range)	6.67±3.97 (1-20)
Glycosylated hemoglobin (%), mean±SD (range)	7.83±1.1 (5-10)

SD, Standard deviation; BMI, Body mass index; n, number of patients.

Table 2. Adherence of patients with T2DM to the dosing of anti-diabetic medications (n=442).

Self-Action n(%)	Rarely	Sometimes	Never	Always
Missing drug doses	171 (38.7)	19 (4)	248 (56)	6 (1.3)
Change drug doses	71 (16)	21 (4.7)	332 (75.3)	18 (4)
Change the timing of drug doses	88 (20)	45 (10)	297 (67.3)	12 (2.7)

Table 3. Diabetic patients' adherence to their medications in various conditions (n=442).

Question	Yes	No
Did you take your medication(s) with you when you are away from home?	419 (99.3)	3 (0.7)
Did you stop taking your medication(s) without consulting your physician because of medication side effects?	12 (2.7)	430 (97.3)
Did you take less of your medication(s) without consulting a physician because you feel better?	13 (3)	429 (97)
During sick days, did you take less of your medication(s) without consulting a physician due to reduced appetite?	23 (5.3)	419 (94.7)
Did you take less of your medication(s) without consulting a physician because of a high medication cost?	97 (22)	345 (78)

Table 4. TP, TN, FP, and FN values for IADMAS calculated based on HbA1c values (n=442).

Parameter	Low Adherence n(%)	Moderate and High Adherence n(%)
Poor glycemic control, HbA1c ≥7%	77 (20.2) TP	303 (79.8) FP
Good glycemic control, HbA1c <7%	56 (90.5) TN	6 (9.5) FN

FN, false negative; FP, false positive; IADMAS, Iraqi Anti-Diabetic Medication Adherence Scale; MAQ, TN, true negative; TP, true positive.

Table 5. Specificity, sensitivity, as well as positive and negative predictive values for the IADMAS in the included patients with T2DM (n=442).

Parameter	Value
Positive Predictive Value	0.202
Negative Predictive Value	0.905
Sensitivity	0.928
Specificity	0.156

consume fewer medications because they felt better, and 94% did not consume fewer medications due to a reduced appetite during sick days. Additionally, only 22% of the participants showed the tendency to consume fewer antidiabetic medications than indicated by their physician due to the high-cost burden. Table 4 indicates that the calculation of adherence based on the HbA1c values reflected a high false-positive value (79.8%) of adherence with an extremely high true negative value (90.5%). Table 5 shows that using IADMAS to screen the adherence of Iraqi T2DM patients to their medications demonstrated a high negative predictive value (0.905) and high sensitivity (0.928) with low specificity (0.156) values.

DISCUSSION

In addition to the outcomes of adequate cure and disease control, antidiabetic medications can be used to improve the quality of life and limit the progression of the disease. Accordingly, patient compliance with the treatment protocols supports the success of the treatment plan (Wilke et al., 2013). Poor adherence to prescribed diabetes medications has been identified as a major source of suboptimal T2DM management and its associated complications (Ho et al., 2006). The pattern of changes in the scores that evaluate adherence to antidiabetic medications from variations in parameter values was highly expected based on theoretical considerations. For instance, for an acceptable rate of adherence, the variation to label patients as "adherent" can significantly influence the scores of adherence measures (Sodihardjo-Yuen, van Dijk, Wensing, De Smet, & Teichert, 2017). Recently, a modified formula (IADMAS) was designed and validated for the evaluation of T2DM patients' adherence to their medications in the Iraqi community (Mikhael et al., 2019). Although IADMAS shows acceptable internal consistency, stable reliability, and good concurrent validity, sensitivity, and specificity compared with other internationally developed scales, its local large-scale application to evaluate adherence of Iraqi patients to their antidiabetic medications is not tried and may reflect conflicting results, especially when the expected outcomes are not compatible with the biochemical evidence of glycemic control (HbA1c) (de Vries McClintock, Morales, Small, & Bogner, 2016). In the present study, more than one-third of the participants can be ranked as poor in relation to their adherence in taking anti-diabetic medications. Although this finding could indicate that diabetic patients pay insufficient attention to their health, it could also indicate limitations in the diabetes care program or healthcare services provided in healthcare settings, as well as poor patient counseling on the importance of strict adherence to anti-diabetic medications. Moreover, the reported level of non-adherence seems to be an underestimate since it is based on patient self-reports which usually overestimate patient adherence levels. Similar findings were reported by Ahmad et al. who showed that 53% of their respondents were non-adherent to medication, in addition to the finding of Abebe et al. (2014) in Ethiopia which indicated the prevalence of 54.1% (Ahmad, Ramli, Islahudin, & Paraidathathu, 2013).

However, lower rates of non-adherence have been reported in Uganda (16.7%) (Bagonza, Rutebemberwa, & Bazeyo, 2015; Abebe, Berhane, & Worku, 2014). This difference in adherence rates could be due to the differences in the healthcare services, socio-economic status, and scales used for the evaluation of adherence across the study settings. The present study indicated a weak but significant inverse correlation between HbA1c values and the total IADMAS scores of the patients; meanwhile, all subscales of IADMAS had a significant inverse correlation with HbA1c. Furthermore, adherent patients were presented with significantly higher mean HbA1c levels than those who were non-adherent. Based on this variable (HbA1c), this finding did not confirm the concurrent validity of the IADMAS, and it conflicted with other studies that reported a positive association between medication adherence and glycemic control (Krapek et al., 2004; Schectman, Nadkarni, & Voss, 2002). Furthermore, the current study found that the IADMAS had acceptable sensitivity but low specificity; this result was nearly universal with most self-reporting approaches used to assess patient adherence to prescribed medications (Lingam & Scott, 2002). This may be attributed to the bias associated with social desirability which may result from the face-to-face interview method of administering the questionnaire to many patients (Del Brutto, & Mera, 2016). Besides, social desirability is highly different among cultures and appears to be significantly involved in developing countries such as Iraq (Heissam, Abuamer, & El-Dahshan, 2015). This study had some limitations, including the use of convenience consecutive sampling, which may lead to selection bias since not all type T2DM patients recruited from the out-patient settings will have had clinical follow-up during the recruitment process. Accordingly, the interpretation of outcomes in terms of generalizability should be cautiously performed. Additionally, the predicted low specificity of the IADMAS in the current study can be due to the limitations of consistent sampling during recruitment (Aminde et al., 2019; Musenge, Michelo, Mudenda, & Manankov, 2016). Meanwhile, inadequate sample size and recruitment from inconsistent healthcare institutions could be one of the major limitations of the present study. Furthermore, the IADMAS was only validated for patients with T2DM, and further studies are needed to confirm its validity among patients with other types of diabetes.

CONCLUSION

Although IADMAS is a reliable and valid tool for assessment of adherence to antidiabetic medications, its application in this study failed to confirm the consistency between the apparent claims of adherence to antidiabetic medications and the HbA1c value as a marker of glycemic control.

Peer-review: Externally peer-reviewed.

Ethics Committee Approval: The study was approved by the Local Research Ethics Committee for Medical Studies, Faculty of Pharmacy, Al-Rafidain University College (REC: 4/2019).

Informed Consent: Written consent was obtained from the participants.

Author Contributions: Conception/Design of Study- S.A.H., H.F.A., Z.S.A.; Data Acquisition- M.M.S., D.N.H., R.S.K., M.M.M.K., D.H.A.; Data Analysis/Interpretation- S.A.H., Z.S.A.; Drafting Manuscript- H.F.A., M.M.S.; Critical Revision of Manuscript- S.A.H.; Final Approval and Accountability- S.A.H., H.F.A., Z.S.A., M.M.S., D.N.H., R.S.K., M.M.M.K., D.H.A.

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

Financial Disclosure: Authors declared no financial support.

Acknowledgement: The authors thank Al-Rafidain University College for supporting the project.

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Istanbul J Pharm 51 (3): 313-318
DOI: 10.26650/IstanbulJPharm.2021.932945
Original Article

Thyrotropine releasing hormone accelerates wound healing in hyperglycemia inducted mouse fibroblast cell line

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Cite this article as: Denizalti, M., Kazkayasi, I., & Telli, G. (2021). Thyrotropine releasing hormone accelerates wound healing in hyperglycemia inducted mouse fibroblast cell line. *İstanbul Journal of Pharmacy, 51*(3), 313-318.

ABSTRACT

Background and Aims: It has been suggested that thyrotropin-releasing hormone (TRH) may have functions beyond its fundamental regulating function. Previous studies have demonstrated that TRH promotes wound healing. We aimed to perform an in vitro study in fibroblasts to assess the role of TRH in wounds that frequently occur in diabetes. Thus, we investigated the effects of TRH in wound healing both under normoglycemic and hyperglycemic-conditions.
Methods: L929 mouse fibroblast cell line was used in the experiments. The cell viability was confirmed with XTT. Then the scratch migration assay was used for assessing the wound healing. TRH was added to both control and hyperglycemia groups at 100 nM after the scratch was created. The wound areas were measured after 24 and 48 hours after the scratch.
Results: TRH and/or hyperglycemia did not affect the cellular activity after 48 hours. TRH reduced the wound areas (42.6%) compared to the control (52.2%) after 24 hours. In the hyperglycemia group the wound area was 64.3% and 61.0% of initial area at the 24th and 48th hours respectively. TRH incubation reduced these wound areas to 55.2% and 47.1% of initial areas.
Conclusion: TRH treatment accelerated wound healing in hyperglycemia, which indicates the positive effects of TRH in wounds, may occur in diabetes.

Keywords: Hyperglycemia, wound healing, scratch migration assay, fibroblast

INTRODUCTION

Skin protects the body like a barrier against external factors such as chemical substances, UV rays and infections. It also has an important role in homeostasis by regulating body temperature and fluid balance. Wound healing is a crucial process for the body. This process is extremely complex; many molecules and cells such as cytokines, chemokines, growth factors and inflammatory cells work together for healing (Hahm, Glaser, & Elster, 2011). As a result of the problems that may develop during this process, wounds may become chronic. C hronic, non-healing wounds lead to pain, restlessness, recurrent infections and even amputation in progressive situations. Non-healing wounds have been shown to be one of the major causes of gangrene and septic deaths. Furthermore, industrialized countries spend more than 3% of their health-care budgets on non-healing wounds (Ohnstedt, Lofton Tomenius, Vagesjo, & Phillipson, 2019). Diabetes is one of the most common causes of non-healing wounds. In diabetic patients, even minor wounds are difficult to heal while susceptibility to infection increases. Amputations are performed in many diabetic patients, especially due to diabetic foot syndrome. Current symptomatic treatments are often insufficient for wound healing. Furthermore, more efficient treatments are expensive and long-term treatments reduce the quality of life. Therefore, new, efficient and inexpensive treatment choices are needed for non-healing wounds in diabetes.

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Submitted: 19.11.2020
Revision Requested: 06.09.2021
Last Revision Received: 10.10.2021
Accepted: 11.10.2021
Published Online: 00.00.0000

The interaction between thyroid hormone and wound healing has been known and studied for many years (Zamick & Mehregan, 1973). In euthyroid rats, thyroid treatment accelerated wound healing and, with supraphysiological doses of T4, increased the tensile strength of the skin (Lennox & Johnston, 1973; Zamick & Mehregan, 1973; Mehregan & Zamick, 1974). However, after the expression of the hypothalamic–pituitary–thyroid axis components on the skin was reported (Slominski et al., 2002), studies about thyroid-wound healing issues started to increase again. Topical application of thyroid hormones accelerated wound healing for both normal and diabetic animals (Safer, Crawford, & Holick, 2005; Kassem, Liberty, Babaev, Trau, & Cohen, 2012; Tarameshloo, Norouzian, Zarein-Dolab, Dadpay, & Gazor, 2012; Kaykhaei, 2016).

High levels of another important element of the thyroid axis, TRH, were also found in the skin (Slominski et al., 2002; Gaspar et al., 2010). Studies have shown that TRH has a remarkable role in hair growth and keratinocyte proliferation (Gaspar et al., 2010; Vidali et al., 2014). Meier et al. demonstrated that TRH promoted wound healing in frog and human skin in organ culture assays (Meier et al., 2013). TRH and its analogue taltirelin provided important wound healing in a primary rat fibroblast culture scratch test and also in an in vivo skin wound (Nie et al., 2014). Despite these remarkable results, the studies investigating the role of TRH on wound healing are very limited and the role of TRH on wound healing in diabetic conditions has not been studied. In this study; we investigated the possible effects of TRH on in vitro wound healing assays in both normoglycemic and hyperglycemic conditions.

MATERIAL AND METHODS

Chemicals

Low Glucose Dulbecco's Modified Eagle Medium (DMEM) was purchased from Wisent Bioproducts (Quebec, Canada). Fetal bovine serum (FBS) and trypsin -EDTA was purchased from Cegrogen-Biotech (Germany). L-glutamine and penicillin-streptomycin were purchased from Biochrom (Cambridge, UK). Sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT) was purchased from PanReac Applichem (Germany). Phenazine methosulfate (PMS), glucose and mannitol were purchased from Sigma Aldrich (St Louis, USA). TRH Ferring 0.2 mg/ml intravenous (Ferring Pharmaceuticals) was used in the experiments.

Cell culture

L929 mouse fibroblast cell line was used in the experiments. DMEM supplemented with 10% FBS, 2% L-glutamine and 1% penicillin-streptomycin was used in all groups. In hyperglycemic groups, fibroblasts were incubated in DMEM medium containing 25 mM glucose for 48 hours (Ueck et al., 2017). Untreated fibroblasts which were incubated with only DMEM medium were the control group. In the hyperglycemia+TRH group, fibroblasts were incubated in DMEM containing 25 mM glucose, and after 48 hours 100 nM concentration of TRH was added, in line with a previous study performed in fibroblasts (Nie et al., 2014). In the TRH treatment group, fibroblasts were incubated in DMEM along with TRH (100 nM). In order to investigate the effects of osmolarity we used a mannitol treatment group with the same molarity as the hyperglycemia group. In the mannitol group, fibroblasts were incubated in DMEM with 20 mM mannitol.

XTT assay

L929 cells were seeded into 96-well plates and incubated for 24 hours (5% CO_2, 37°C) to form a semi-confluent monolayer. After 24 hours of incubation, the medium was aspirated from the cells. All incubation conditions of different groups were mimicked. Cells were incubated for 48 hours (5% CO_2, 37°C). After 48 hours of treatment, 50 µl of the XTT/PMS solution was added to each well and the plates were further incubated for 3 to 5 hours in the incubator at 37°C. Then the plates were swayed carefully and an aliquot of 100 µl was transferred from each well into the corresponding well of a new microplate and read at 450 nm. (reference wavelength 630 nm). The results were normalized to the control group.

Scratch migration assay

The migration of fibroblasts was analyzed by the scratch migration assay as previously described (Liang, Park, & Guan, 2007; Nie et al., 2014). L929 cells were seeded in 24-well plates and maintained at 37°C and 5% CO_2 to permit cell adhesion and the formation of a confluent monolayer. After 48 hours, when 90% confluence was achieved, an artificial gap called a "scratch" approximately 0.2-0.4 mm in width, was created with a 200 µL sterile pipette tip. The culture medium was then immediately removed (along with any detached cells and debris).

Cells were then incubated at the experiment conditions at 37°C and 5% CO_2 for 48 hours. The cell migration and morphological changes of cells were observed in the images taken by an inverted microscope, equipped with a digital camera. According to previous in vitro studies showing that TRH was effective at the 72nd hour (Nie et al., 2014) and studies demonstrated that TRH provided faster wound healing (Meier et al., 2013) images were captured at three different time points (at the 0, 24th and 48th hours). The width of the scratch and wound closure was analyzed by ImageJ 1.53e software (National Institute of Health). Wound closure was quantified as wound area relative to the initial wound area.

Statistical analysis

Statistical analysis was performed with one-way ANOVA *post hoc* Tukey test. Data were represented as mean ± standard error mean (SEM). The width of the scratch and wound closure was analyzed by ImageJ 1.53e software. GraphPad Prism 5.0 software was used for statistical analysis (San Diego, USA).

RESULTS

Firstly, the cell viability and proliferation were assessed with XTT. TRH incubation and/or hyperglycemia did not affect the cell after 48 hours (Figure 1).

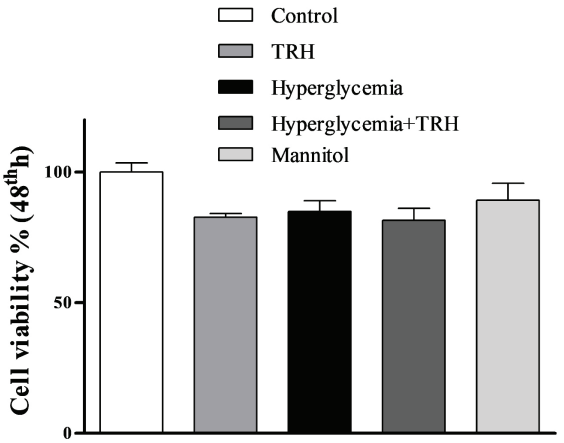

Figure 1. Cell viability % of each group after 48 hours. Data were presented as mean±SEM. Statistical analysis was performed with one-way ANOVA, post hoc Tukey test. n=8.

The scratch migration assay was used for assessing the wound healing. At the end of the 24th hour, the TRH incubation to the normoglycemic cells reduced the wound area statistically significantly compared to that of the control group. The wound area was the greatest in the hyperglycemia group, which indicated that the hyperglycemia, as in diabetes mellitus, delayed wound healing in the L929 cell line. 100 nM TRH incubation accelerated the wound healing also in the hyperglycemia group and provided a significantly smaller wound area compared to the hyperglycemia group. The wound area in the mannitol group was not different from that of the control group, which showed that the differences in the wound areas between the groups were not related with hyperosmolarity (Figure 2).

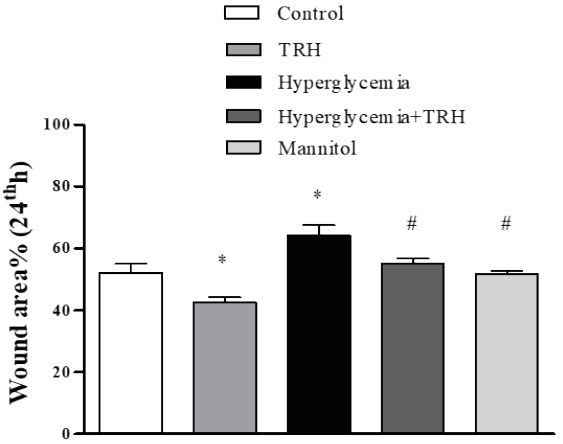

Figure 2. The wound areas of the groups at the end of the 24 hours. Data were presented as mean±SEM. Statistical analysis was performed with one-way ANOVA, post hoc Tukey test, *p<0.05 vs control and #p<0.05 vs hyperglycemia group. n=8.

The 48-hour TRH incubation continued to reduce the wound area; however, this reduction was not different from that of the control group. On the other hand, in the hyperglycemic condition TRH incubation provided statistically significant healing in the wound area compared to the hyperglycemia group (Figure 3).

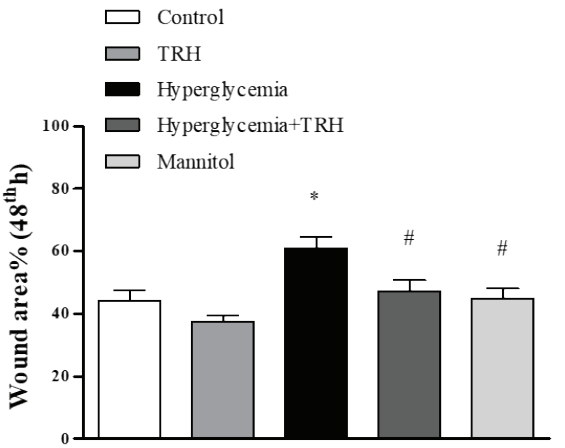

Figure 3. The wound areas of the groups at the end of the 48 hours. Data were presented as mean±SEM. Statistical analysis was performed with one-way ANOVA, post hoc Tukey test, *p<0.05 vs control and #p<0.05 vs hyperglycemia group. n=8.

DISCUSSION

Recurrent, non-healing wounds are a serious problem in diabetic patients. Approximately 15% of these patients suffer from recurrent foot ulcers (Chun et al., 2019). The wounds that do not heal and become chronic affect quality of life and require high patient compliance. About 20% to 33% of costs related to diabetes mellitus are used for treatments of diabetic foot ulcers (Schreml & Berneburg, 2017). This situation affects the individuals and communities not only emotionally but also financially (Gouin & Kiecolt-Glaser, 2011). The importance of more efficient choices in the treatment of wound healing will increase as indicated by the report notifying the increasing numbers of diabetic patients year by year (WHO, 2020).

Hyperglycemia affects many significant factors in the wound healing process (Pan et al., 2015; Leguina-Ruzzi & Valderas, 2017). The fibroblasts under hyperglycemic conditions exhibited impaired function in previous studies (Lerman, Galiano, Armour, Levine, & Gurtner, 2003). In wound healing, fibroblasts are critical cells that are responsible for the breakdown of fibrin clot, creating the collagen structures and extracellular matrix (Bainbridge, 2013). Therefore, fibroblast cells are very suitable targets for investigating the effects of new drugs or therapies in wound healing. Firstly, we showed that 100 nM TRH, or the hyperglycemic condition created in cells, was not cytotoxic to the L929 cell line until the end of the experiments. However, the proliferation of the cells was only slightly reduced with hyperglycemia after 48 hours and TRH treatment did not provide an increase in the proliferation. There are conflicting results in the literature about this issue. In some of the experiments performed on various cells, a significant decrease was observed with high glucose administration (Gupta & Tikoo, 2013), while high glucose did not change the proliferation in other cells (Morais, Westhuyzen, Pat, Gobe, & Healy, 2005). In our study also there was no significant reduction in our experiments at the 48th hour. However, considering the study that indicated the significant increase in proliferation of the primary fibro-

blasts of rats with TRH after 72 hours, longer use of TRH may be required to increase the proliferation.

The scratch migration assay is a well-established, inexpensive and reproducible method that is used frequently in initial wound healing studies (Pitz Hdaet al., 2016). For these reasons, our study was designed in fibroblasts and the scratch migration assay was used as a wound healing test. Consistent with previous studies, hyperglycemia caused a delay in wound healing at both the 24th and 48th hours independent from hyperosmolarity in our study.

Today, once a wound occurs in the diabetic patient, off-landing, bandages and dressing that provides a moiety environment is used first (Everett & Mathioudakis, 2018). However, the appropriate pharmacological interventions are essential for optimum healing. Many different effective antiseptics and topical antibiotics are used in the treatment in the first stage. However, with the recurrence of the wound these drugs can be inadequate. As a result, new and more powerful antibiotics, matrix metalloproteinase inhibitors, protease inhibitors, recombinant human platelet derived growth factor, and other new therapies such as negative pressure wound therapy and surgical processes are needed. Although more efficient results are obtained from these treatments, the very high cost of treatment and increased use of antibiotics are considered to be an important problem (Qi et al., 2020). Therefore, new, safer and cheaper treatments will be preferred for this challenging clinical problem. The repurposing of drugs has been frequently preferred to in recent years due to their reliability and more rapid usage in treatment (Abels & Soeberdt, 2019). TRH has safely been used in thyroid diseases for many years. The efficient roles of TRH in skin elements such as keratinocytes, hair follicles and fibroblasts (Werner, Krieg, & Smola, 2007) have directed investigators to wound healing. In an ex vivo study on frog skin that were showed that TRH provided significant reduction in the wound size 72 hours after the application and reached maximum at the 7th day(Meier et al., 2013). The promising results were also obtained in the wound healing within days with TRH in the mice (Nie et al., 2014). Our study has taken it one step further and has demonstrated the efficiency of TRH on wound healing in hyperglycemic conditions. TRH treatment accelerated wound healing both 24 and 48 hours after the creation of the wound in hyperglycemic conditions, w hich may indicate that TRH may be beneficial for wound healing in diabetes mellitus.

There are many factors that delay wound healing in diabetes mellitus. It is thought that the increase in blood glucose level directly affects components in the wound healing process. Furthermore, neuropathic and biochemical abnormalities, vascular pathologies, reduced angiogenesis and impaired immune function may have effects on wound healing (Qi et al., 2020). It has been shown that TRH stimulated proliferation of immune cells and inhibited monocyte activity in nature killer cells in vitro (Frohlich & Wahl, 2019). TRH injection reduced the elevated blood glucose levels in mice. In another study, thyroxine promoted angiogenesis of ex vivo wounded human skin cells (Zhang et al., 2019). Thus, it may be suggested that TRH can both directly and indirectly affect impaired functions due to hyperglycemia, and also provide accelerated wound healing in hyperglycemia.

The present study is the first that investigated the effect of TRH in wound healing under hyperglycemic conditions We also assessed the role of TRH under the normoglycemic conditions of L929 fibroblast cell line for the first time. Our results supported the previous studies investigating the effects of TRH on wound healing. Nie et. al reported direct effects of TRH on the alpha smooth muscle actin in fibroblasts and showed TRH increased proliferation and migration capacity of them (Nie et al., 2014). Similarly, in our study, after 24 hours of TRH treatment the wound area got smaller compared to the control group. However, after 48 hours there was no statistically significant difference between the control and TRH groups, even though TRH continued to be used to close the wound area. It could be related with the high proliferation capacity of cell lines that was used (Pitz Hda et al., 2016). Because of the fast migration of the cells in the control group, the healing that TRH provided could be masked. The earlier observation time can be preferred in future experiments. Wound healing is a very complex process, and it is known that keratinocytes are involved in this process as well as fibroblasts (Pastar, Stojadinovic, & Tomic-Canic, 2008). Another limitation of our study is that evaluations were made only in fibroblasts, and future studies plan to evaluate the efficacy of TRH in keratinocytes. Thus, the mechanism of action of the role of TRH in wound healing will also be evaluated.

CONCLUSIONS

TRH indirectly regulates the thyroid hormone by stimulating TSH. Thyroid hormone has important physiological effects and affects many systems in the body. Therefore, our results demonstrate the direct effects of TRH and are promising for further studies and will guide in vivo experiments. TRH is an inexpensive and relatively safe drug that has been used for many years. The possible efficiency of TRH in impaired wound healing in diabetes will reduce health expenses caused by ulcers in diabetes. Because it is a drug that patients can easily access, the compliance of patients will increase. If our study will be confirmed by further in vivo studies a new treatment choice may be available.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- M.D., İ.K., G.T.; Data Acquisition- M.D., İ.K., G.T.; Data Analysis/Interpretation- M.D., İ.K., G.T.; Drafting Manuscript- M.D., İ.K., G.T.; Critical Revision of Manuscript- M.D., İ.K., G.T.; Final Approval and Accountability- M.D., İ.K., G.T.

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

Financial Disclosure: Authors declared no financial support.

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Istanbul J Pharm 51 (3): 319-325
DOI: 10.26650/IstanbulJPharm.2021.892746
Original Article

Bongardia chrysogonum (L.) Spach as a potential medicinal plant against cancer and Alzheimer's disease management

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Cite this article as: Gezici, S., & Sekeroglu, N. (2021). *Bongardia chrysogonum* (L.) spach as a potential medicinal plant against cancer and Alzheimer's disease management. *İstanbul Journal of Pharmacy, 51*(3), 319-325.

ABSTRACT

Background and Aims: *Bongardia chrysogonum* (L.) Spach (Berberidaceae), an ancient medicinal plant in Eastern Mediterranean countries, is traditionally used for the treatment of epilepsy, hemorrhoids, urinary system infections and gastrointestinal disorders. This study was designed to evaluate potential neuroprotective and anticancer effects of different parts from *B. chrysogonum*.

Material and Methods: Leaf, stem, and tuber parts of the plant were extracted and subjected to determine cholinesterase enzyme inhibitory potentials and anticancer activities. Enzyme inhibition assays were carried out to screen neuroprotective potentials, while the MTT assay was performed for evaluating anticancer activities of the extracts towards human lung carcinoma (A549), breast adenocarcinoma (MCF-7), brain glioma (C6), and non-tumorous human umbilical vein endothelial cell (HUVEC) lines.

Results: Among the tested extracts, the highest enzyme inhibitory activity was exhibited by tuber-water extract (83.81±0.33% and 62.14±0.60% inhibition on AChE and BChE at 400 µg/mL $p<0.01$, respectively), whilst the lowest enzyme inhibition was exerted by the tuber-chloroform extract. Moreover, the tuber part of the plant was found to have the most cytotoxic activity against all the cancer cells, and the best anticancer activity was determined in tuber ethanol extract against MCF-7 cells (IC_{50}= 28.36±0.04, $p<0.01$).

Conclusion: To the best of our knowledge, this research is the first study that assessed the *in vitro* neuroprotective effect of the aerial parts and tuber extracts of the plant through inhibition of cholinesterase enzymes alongside an anticancer capacity towards human cancer cells. The results revealed that this plant is a good candidate for performing further clinical studies in cancer and neurodegenerative diseases.

Keywords: *Bongardia chrysogonum* (L.) Spach, cancer, enzyme inhibition, medicinal plant, neuroprotection

INTRODUCTION

Medicinal plants used in traditional medicine are well-known to have numerous pharmacological actions and extraordinary therapeutic potentials in the prevention and cure of several diseases, particularly oxidative stress related-diseases including diabetes, hypertension, atherosclerosis, cancer, ischemia, multiple sclerosis, cardiac hypertrophy, amyotrophic lateral sclerosis, Alzheimer's, and Parkinson's diseases (Fang *et al.,* 2017; Ismail, Amanat, Iqbal & Mirza, 2018; Gezici & Sekeroglu 2019a; Ahmad, Waheed, Rozeen, Mahmood & Kamal, 2019. Moreover, medicinal plant-derived natural compounds serve as a therapeutic alter-

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Submitted: 07.03.2021
Revision Requested: 22.09.2021
Last Revision Received: 05.10.2021
Accepted: 06.10.2021
Published Online: 00.00.0000

native for combating cancer and neurodegenerative disorders, owing to rich phytochemical contents and natural antioxidant potentials (Safarzadeh, Shotorbani & Baradaran, 2014; Chen, Lin, Dong, Zhang & Du, 2018; Sahoo, Dandapat, Dash & Kanhar, 2018; Gezici & Sekeroglu, 2019b). According to statistical data, there has been a great increase in Alzheimer's disease (AD) incidence around the world, from this point of view, finding effective and safe therapy approaches have an accountable role to manage the high incidence of this disease. On the other hand, cholinergic-based therapy that means inhibition of the level of some enzymes including acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) seems to be as one of the most effective treatment strategies for the AD pathogenesis (Ismail *et al.,* 2018; Harris, 2019; Gezici, 2018). In this concern, medicinal plants and natural products have been gaining interest among the researchers almost all over the world. Thus, the researchers have focused on the development of novel plant-derived natural products with less or no side effects as well as unlimited availability.

The genus *Bongardia* L. (Berberidaceae), native to the Eastern Mediterranean region, is represented by only one species (*Bongardia chrysogonum*) in the flora of Turkey. This species is generally known as 'Uruf-el-Deek' and locally known as 'catlak otu' in Anatolia, which is distributed in the Eastern Mediterranean region, North America, Jordan, Afghanistan, Syria, Iran, Iraq, Azerbaijan, and Turkey. Tubers of this plant have been mainly used in folk remedy since ancient times (Karl & Strid, 2009; Abuhamdah *et al.,* 2017a; Abuhamdah *et al.,* 2017b). From the past to today, scientific records have stated that the tubers of *B. chrysogonum* have been utilized for the treatment of epilepsy and hematological malignancies in the form of decoction (2-3%) around Afghanistan, Iran, Jordan, Syria, and Turkey by the local rural communities. The tubers have also been reported to be used in order to treat gastrointestinal disorders, urinary tract infections, prostate hypertrophy, hypercholesterolemia, hemorrhoids, and diabetes, because of their antidiarrheal, antimicrobial, antiulcerogenic, anticonvulsant, and antioxidative activities (Alfatafta, Abu Zarga, Sabri, Freyer & Shamma, 1989; Baydoun, Chalak, Dalleh & Arnold, 2015; Dokuyucu *et al.,* 2016; Abuhamdah *et al.,* 2017a; Abuhamdah *et al.,* 2017b).

Recent studies reported the antioxidant and anti-convulsant activities of the tuber extracts of *B. chrysogonum* (Abuhamdah *et al.,* 2017a; Abuhamdah *et al.,* 2017b). However, the other biological activities have not been identified using each part of this plant, yet. In this context, extracts of the aerial parts (leaf and stem, individually) and tuber of *B. chrysogonum* extracted by using different solvents including ethanol, water, and chloroform were screened for revealing potential anticancer and neuroprotective capacities in the presented work. The neuroprotective potential of the extracts was evaluated through AChE and BChE inhibitory activities. The MTT assay was performed for determining cytotoxic and anticancer activities of the extracts against cells; A549 (human lung carcinoma), MCF-7 (human breast adenocarcinoma), C6 (human brain glioma), and non-tumorous HUVEC (human umbilical vein endothelial cells), in a concentration and time dependently.

MATERIALS AND METHODS

Chemicals and equipment

5,5-dithio-bis-2-nitrobenzoic acid (DTNB), ethanol (EtOH), methanol (MeOH), chloroform ($CHCl_3$), dimethyl sulfoxide (DMSO), neocuproine, ammonium acetate (NH_4Ac), AChE (electric eel acetylcholinesterase, Type-VI-S, EC 3.1.1.7), BChE (horse serum butyrylcholinesterase, EC 3.1.1.8), acetylthiocholine iodide (ATCI), butyrylthiocholine chloride (BTCI), sodium dihydrogen phosphate (NaH_2PO_4), sodium bicarbonate ($NaHCO_3$), and reference standards were purchased from Sigma (St. Louis, MO, USA). Cell culture mediums and chemicals were purchased from ThermoFisher Scientific, Gibco®. A Microplate reader spectrophotometer from Thermo Lab systems (408 Multiskan) was used to measure the absorbance.

Collection of plant material

The plant sample used in this study was collected from its natural habitat from the Gaziantep province (Turkey) in April, 2018. The plant species was identified by Res. Assist. Fatih Yayla, a taxonomist from the Department of Biology, Gaziantep University, Gaziantep (Turkey). A voucher specimen (number: GAUN1603) was deposited at the Herbarium of Department of Biology, Gaziantep University, Turkey (Figure 1).

Figure 1. *Bongardia chrysogonum* a) aerial parts, b) tuber.

Preparation of the extracts

The air-dried plant material including the tuber and aerial parts (leaf and stem) of *B. chrysogonum* was extracted with 70% ethanol (EtOH), distilled water (dH_2O), and chloroform ($CHCl_3$) for two days at room temperature as described in the previous publications (Gezici & Sekeroglu, 2019a; Gezici, 2019). After the extraction procedures, the extracts were deposited at -20°C until further analysis.

AChE and BChE enzyme inhibitory studies

The Neuroprotective potential of the extracts expressed as their inhibitory activity against cholinesterase enzymes (AChE and BChE) was determined by as described by Ellman, Courtney, Andres & Featherstone, (1961) and all conditions were similar to that described previously (Senol, Sekeroglu, Gezici, Kilic & Orhan, 2018; Sekeroglu & Gezici, 2019). The reaction was observed at 412 nm utilizing a microplate reader spectrophotometer, using galanthamine as the reference drug.

The percent inhibition of the enzymes was calculated by a comparison of reaction rates in the extract with a blank sample using the formula as given below:

Percent enzyme inhibition (%) = (Aenzyme-Asample)/Aenzyme × 100

[Where A_{enzyme} is the activity of enzyme without the extract, and A_{sample} is the activity of enzyme with the extract].

Anticancer activity studies

Cell lines and cultures

A549 (lung carcinoma), MCF-7 (breast adenocarcinoma), C6 (brain glioma), and non-tumorous HUVEC (human umbilical vein endothelial cells) cell lines, obtained from the American Type Culture Collection (ATCC, USA) were used to determine the *in vitro* anticancer effects of *B. chrysogonum* extracts. The lung carcinoma cell was cultured on Roswell Park Memorial Institute Medium (RPMI), supplemented with 10% (v/v) fetal bovine serum (FBS), antibiotics (1%, 100 U/ml penicillin and 100 µg/ml streptomycin), and L-glutamine (1%) in the flasks at 37°C in a humidified CO_2 (5%) incubator. MCF-7, C6, and HUVEC cells were grown in Dulbecco's modified Eagle medium (DMEM): Ham's F12 nutrient medium (1:1) with the same additives. All the assays were carried out in triplicate using the cell lines from passage 24 or less.

MTT anticancer activity assay

To evaluate the cytotoxic potentials of the extracts, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed as described by Mosmann (1983) with a minor modification (Gezici, Sekeroglu & Kijjoa, 2017; Gezici, 2019). Briefly, densities of 5×10^3 cells were seeded in 200 µl medium into 96-well plates for 24 hrs and after seeding, the extracts with concentrations from 50 µg/mL to 400 µg/mL were added into all the wells, and then they incubated at 37°C for 48 hrs. After incubation, the medium was discarded and 50 µL/well of MTT (Sigma-Aldrich) solution (5 mg/mL) was added into each well and incubated for 4 hrs at 37°C. The medium-containing MTT was discarded and 200 µL of DMSO added for both lysis the cells and solubilization formazone. Then, the absorbance was measured at 570 nm with a Thermo Lab system 408 Multiskan multiplate spectrophotometer. The dose-response curve was used to generate the IC_{50} (µg/mL) values, and doxorubicin used as the reference.

Statistical analysis

The experiments were carried out in triplicate and data were presented as mean±S.D. values (n=3). A linear regression analysis was conducted for generating IC_{50} values. ANOVA (one way) was used for the evaluation of statistical differences between the control and the sample groups. A p value of <0.05 was examined to be significant, and p <0.01 was considered to be very significant statistically (*$p < 0.05$, **$p < 0.01$).

RESULTS

AChE and BChE enzyme inhibitory results

All the extracts from the leaf, stem, and tuber of *B. chrysogonum* were evaluated for their inhibitory activities against AChE and BChE enzymes at 50, 100, 200, and 400 µg/mL concentrations, and galanthamine was used as the reference drug (Table 1). As

Table 1. Inhibitory activity of the extracts against AChE and BChE enzymes.

Plant parts	Extract type	(% Inhibition±S.D.[a])							
		AChE				BChE			
		50 µg/mL	100 µg/mL	200 µg/mL	400 µg/mL[b]	50 µg/mL	100 µg/mL	200 µg/mL	400 µg/mL[b]
Leaf	Ethanol	45.01±0.56*	52.28±1.15**	60.14±0.52**	68.93±0.22**	14.08±0.66*	19.52±0.84**	27.97±1.01*	33.06±1.02**
	Water	36.32±0.48**	41.27±0.62*	51.96±0.71**	62.10±0.45**	12.35±0.92*	20.91±0.52*	26.02±0.12**	31.48±0.83*
	Chloroform	---[c]	---[c]	---[c]	---[c]	---[c]	---[c]	---[c]	---[c]
Stem	Ethanol	47.85±1.10*	59.16±0.91**	65.08±0.36**	72.16±1.02**	22.02±0.14**	34.77±0.60*	41.68±0.63**	49.85±0.15**
	Water	38.20±0.21**	44.36±0.13*	49.71±1.19*	65.04±1.16**	17.54±0.67**	23.45±0.42**	30.06±0.82*	36.11±0.78*
	Chloroform	---[c]	---[c]	---[c]	---[c]	---[c]	---[c]	---[c]	---[c]
Tuber	Ethanol	50.25±1.05**	63.04±1.20**	70.16±0.65*	81.45±0.42**	27.61±1.01**	35.69±0.76*	44.12±0.16**	51.20±1.17**
	Water	54.21±1.23**	66.05±1.42*	74.92±0.82**	83.81±0.33**	32.98±0.78**	43.01±1.20**	50.16±0.47*	62.14±0.60**
	Chloroform	23.45±0.14**	27.61±0.65**	31.04±0.93**	43.86±0.90*	9.96±0.08**	17.26±0.18**	23.18±0.59**	27.63±1.09*
Galanthamine[d]		82.64±1.08* at 100 µg/mL				86.04±0.45* at 100 µg/mL			

[a]S.D.: Standard deviation (n=3). [b]The final concentration in the well. [c]No inhibition. [d]Galanthamine; reference for AChE and BChE enzymes. *p value of < 0.05; **p value of < 0.01

summarized in Table 1, the extracts tested herein demonstrated from moderate to a high level of inhibition against the above-mentioned enzymes. The extracts inhibited AChE at high levels, whilst they inhibited BChE at moderate levels (below 50%). Although a significant variation was not assessed among the tested extracts, the tuber water extract showed a significant inhibition against AChE and BChE (83.81±0.33% and 62.14±0.60%, respectively) at 400 µg/mL. The tuber chloroform extract was exerted the lowest enzyme inhibition through AChE and BChE (43.86±0.90% and 27.63±1.09%, respectively), and the chloroform extracts from leaves and stems were also observed to be ineffective in inhibiting both cholinesterase. In the assay, the water extracts from the leaves and stems exhibited moderate inhibitory activities on both AChE and BChE enzymes in the range of 31.48±0.83% and 65.04±1.16% as compared to those of the ethanol extracts (Table 1).

Anticancer activity results against human cancer cells

The cytotoxic effects of the mentioned plant parts of *B. chrysogonum* were assessed against A-549, MCF-7, and C6 cancer cells, in comparison to non-tumour HUVECs. The tested extracts exerted a noteworthy cancer prevention potential against the cancer cells in a dose and time dependent manner; however, the IC_{50} values were varied depending on the cancer cells and plant parts. The results were expressed as IC_{50} (µg/mL) values after 72 hrs treatment period at 200 µg/mL concentration (Table 2).

As concluded from the IC_{50} values given in Table 2, the tuber part of the plant was found to possess higher cancer prevention potentials, when compared to those of the aerial parts. In all cases, all plant parts caused a much more cytotoxicity on MCF-7 cells, while they exhibited lower effects against C6 cells. On the other hand, the tuber EtOH extract showed the best

Table 2. Anticancer activities of the extracts against A549, MCF-7, and C6 human cancer cells.

Human cancer cells	Plant parts	Extract type	IC_{50} values[a] (µg/mL)
A549	Leaf	Ethanol	190.66±1.47*
		Water	145.02±0.96**
		Chloroform	228.16±1.03*
	Stem	Ethanol	235.16±1.92**
		Water	201.80±2.07*
		Chloroform	280.94±0.39*
	Tuber	Ethanol	147.01±0.82**
		Water	80.06±1.25**
		Chloroform	178.12±1.94**
MCF-7	Leaf	Ethanol	74.48±1.01*
		Water	98.07±0.19*
		Chloroform	129.35±0.43**
	Stem	Ethanol	174.27±0.92**
		Water	191.95±2.06**
		Chloroform	237.03±2.54*
	Tuber	Ethanol	28.36±0.04**
		Water	61.23±1.10**
		Chloroform	69.72±0.38**
C6	Leaf	Ethanol	285.24±0.68**
		Water	259.09±2.06*
		Chloroform	306.75±1.94**
	Stem	Ethanol	286.10±0.71*
		Water	262.55±1.32**
		Chloroform	321.68±2.14*
	Tuber	Ethanol	240.92±1.19**
		Water	214.50±0.25**
		Chloroform	287.01±0.52**
Doxorubicin[b]			7.96±0.18
DMSO (dimethyl sulfoxide)[c]			0

[a]Values were expressed as IC_{50}±S.D. from three independent experiment (n=3). [b]Doxorubicin, positive control. [c]DMSO; negative control.
*p value of < 0.05; **p value of < 0.01.

anticancer activity (IC_{50} = 28.36±0.04 µg/mL, $p< 0.01$) towards MCF-7 human cancer cells, when the stem chloroform extract was found to have the weakest anticancer activity against C6 cells (IC_{50} = 321.68±2.14 µg/mL, $p< 0.05$). As for the extraction type, the water extracts obtained from any part of the plant demonstrated the highest anticancer activity towards A549 and C6 cancer cells. However, the EtOH extracts owned the highest cancer prevention capacities against MCF-7 cells, whereas the chloroform extracts showed the lowest ones in all cases.

DISCUSSION

Acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and tyrosinase (TYR) enzymes involved in neurodegenerative mechanisms have been reported to be associated with pathogenesis of neurodegeneration and neurodevelopmental diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), prion diseases, attention deficit hyperactivity disorder (ADHD), and autism spectrum disorder (ASD). Of which, AD is known as the most common type of neurodegenerative disorder leading to a loss of memory and behavior skills in the elderly (Alamgir, 2017; Ismail *et al.,* 2018; Ahmad *et al.,* 2019. A number of medicinal plants and plant-derived compounds have been reported to inhibit AChE and BChE, involved in the acetylcholine and butyrylcholine hydrolysis, respectively, which is the most accepted drug treatment strategy in patients with AD (Grutzendler & Morris, 2001; Sekeroglu *et al.,* 2012; Gezici & Sekeroglu, 2019a). From this point, the inhibition of key enzymes linked to the pathogenesis of the AD and PD diseases used medicinal plant and plant-derived compounds are suggested as one of the most effective strategies. For example, the inhibition of AChE and BChE, involved in the acetylcholine and butyrylcholine hydrolysis, respectively is the most accepted treatment strategy in AD. Additionally, the inhibition of TYR alongside cholinesterase (ChE) is of great importance for the treatment of these diseases as multi-target drug approaches (Sekeroglu et al., 2012; Santos, Gomes, Pinto, Camara & Paes, 2018; Gezici & Sekeroglu, 2019a; Gezici & Sekeroglu, 2021).

In our ongoing research on finding natural enzyme inhibitors, neuroprotective potentials of *B. chrysogonum* extracts through AChE and BChE enzyme inhibition were aimed to screen in this work. Based on the results of cholinesterase enzyme inhibition, the tested extracts of the plant were found to exhibit a promising enzyme inhibitory effect towards both AChE and BChE, which are closely associated with AD development. Probably, these strong enzyme inhibition potentials of this plant may be due to the presence of rich secondary metabolites including bongardine, N-acetyl bongardine, bongardol, bongardol acetate, and bongardamine, as reported by previous studies (Alfatafta *et al.,* 1989; Rahman *et al.,* 1998; Shahwar, Choudhary, Sener, Toker & Baser, 1999; Rahman *et al.,* 2000; Karl & Strid, 2009). In addition, alkaloids are important secondary metabolites for cholinesterase inhibition. Previous studies showed that this plant is also rich for isoquinoline alkaloids like retuculine, coclaurine etc. (Alfatafta *et al.,* 1989; Abuhamdah *et al.,* 2017a; Abuhamdah *et al.,* 2017b).

The increasing cancer burden worldwide requires an alternative treatment solution. Plants have been used for medicinal purposes since times immemorial, and these plants and natural compounds isolated or extracted from the medicinally important plants offer a very viable alternative in the treatment and management of several cancer types (Roleira *et al.,* 2015; Gezici & Sekeroglu, 2019b; Khan *et al.,* 2020). Based on their traditional uses and experimental evidences, cancer prevention potentials of *B. chrysogonum* were analyzed in the presented research. Particularly, the tuber parts of *B. chrysogonum* extracts prepared using different solvents demonstrated a good level of anticancer effects against the tested cancer cells. According to the anticancer activity results, increasing concentration of the extracts resulted in a decrease in the number of cancer cells, likewise, an increasing exposure time resulted in a significant inhibition of cell growth in the cancer cells. It is clearly proven that the plant could have a noteworthy potential as an anticancer agent for the management of reducing the number of cancer cells and suppressing the cell growth in cancer. In the light of the literature, the notable cancer prevention effects of *B. chrysogonum* are most likely due to the fact that it has rich phytochemical contents such as isoquinoline alkaloids and triterpenoid saponins as revealed previously (Alfatafta *et al.,* 1989; Rahman *et al.,* 1998; Shahwar *et al.,* 1999; Rahman *et al.,* 2000; Karl & Strid, 2009).

Although, 2,2- diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl (•OH) free radical scavenging activities of the tuber part of *B. chrysogonum* have been demonstrated by a few previous studies until now (Dokuyucu *et al.,* 2016; Abuhamdah *et al.,* 2017a; Abuhamdah *et al.,* 2017b), there has been no research conducted for the evaluation of anti-cholinesterase and anticancer effects using individually each part of *B. chrysogonum* in the literature. Therefore, to the best of our knowledge, the assessment of *in vitro* neuroprotective and cancer preventive potentials of different plant parts of *B. chrysogonum* were performed for the first time in the current research.

CONCLUSION

To summarize, the probable *in vitro* neuroprotective capacities and anticancer effects of the ethanol, water, and chloroform extracts from different parts of *B. chrysogonum* (L.) Spach were evaluated the first time in the current study. The results obtained exhibited that the tuber part of the plant has the greatest biomedical potential through its significant inhibitory activities on the tested enzymes related to AD and also a remarkable cancer prevention activity against A549, MCF-7, and C6 cancer cells. These findings could be of valuable scientific evidence to contribute to the pharmaceutical industry in the prevention and treatment of AD and cancer; however, detailed mechanisms based *in vitro* and *in vivo* studies are necessary in future. Besides, in order to specify the phytochemicals responsible for notable neuroprotective effect and cancer prevention capacity of the plant-extracts, phytochemistry of the extracts should be analyzed as further research.

Peer-review: Externally peer-reviewed.

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

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

Financial Disclosure: This study was financially supported by the Scientific Research Project Unit of Kilis 7 Aralik University, Kilis – Turkey (Project code no: 11911/1828MAP1).

Acknowledgements: The authors would like to thank Genetics Laboratory, Advanced Technology Application and Research Center, Kilis 7 Aralik University for technical support. The authors are also grateful to Res. Assist. Fatih Yayla for identifying and Biologist Didem Kocum for providing the plant material.

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Istanbul J Pharm 51 (3): 326-332
DOI: 10.26650/IstanbulJPharm.2021.828280
Original Article

Antiinflammatory and antidiabetic activity of *Ballota* L. species grown in Turkey

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Cite this article as: Agca, A. A., Yilmaz Sarialtın, S., Yazgan Ekici, A. N., Coban, T., Saltan Iscan, G., & Sever Yilmaz, B. (2021). Antiinflammatory and antidiabetic activity of *Ballota* L. species grown in Turkey. *İstanbul Journal of Pharmacy, 51*(3), 326-332.

ABSTRACT

Background and Aims: *Ballota* is represented by 12 species and 16 taxa in Turkey. Eleven of the 16 taxa are endemic to Turkey. *Ballota* have been used in different treatments in folk medicine. Antiinflammatory drugs act by protecting lysosomal membrane integrity or inhibiting enzymes of prostaglandins and thromboxane inflammatory mediators synthesized by deactivating of cyclooxygenase (COX), COX-1 and COX-2 enzymes. Besides, degradation of lysosomal membrane brings on the release inflammatory mediators so human red blood cells (HRBC) can be used for evaluating of antiinflammatory activity. One of the treatment approaches of diabetes mellitus is α-glucosidase enzyme inhibition. These enzymes play an important role in glucose releasing from carbohydrates. Due to the adverse effects of synthetic drugs, natural α-glucosidase inhibitors have been discussed as therapeutic agents to manage diabetes mellitus.
In the light of the traditional use of *Ballota* species, we aimed to investigate the antiinflammatory and antidiabetic activities of the ethanol and aqueous extracts from the aerial parts of 14 *Ballota* taxa.
Methods: HRBC membrane stabilizing and α-glucosidase inhibitory methods are used for determining antiinflammatory and antidiabetic activities of 14 *Ballota* taxa, respectively.
Results: According to our results, the ethanolic extracts showed higher membrane stabilization profile than aqueous extracts generally. And for antidiabetic activity, it is concluded that aqueous and ethanol extracts of *Ballota glandulosissima* exhibited the maximum α-glucosidase inhibitory activity.
Conclusion: According to our results, IC_{50} values of antiinflammatory effect of ethanolic and aqueous extracts of 14 *Ballota* samples ranged from 4,30-12,88 and 3,18-20,25 mg/ml, respectively. It is observed that aqueous extracts of *Ballota nigra* subsp. *anatolica* exhibited the maximum antiinflammatory effect. Both aqueous and ethanol extracts of *Ballota glandulosissima* are found to exhibit the maximum α-glucosidase inhibitory activity with IC_{50} values of 2,18 and 2,30 µg/ml, respectively. Among the ethanolic extracts of *Ballota* species, the strongest α-glucosidase inhibitory activities are found as *B. glandulosissima*>*B. cristata*>*B. saxatilis subsp. brachyodonta* in descending order.

Keywords: α-glucosidase, antiinflammatory activity, antidiabetic activity, *Ballota* species

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Submitted: 19.11.2020
Revision Requested: 12.12.2020
Last Revision Received: 07.05.2021
Accepted: 25.05.2021
Published Online: 22.11.2021

INTRODUCTION

Ballota L. is represented by 12 species and 16 taxa in Turkey (Davis 1982). Eleven of the 16 taxa are endemic to Turkey. *Ballota* species belong to the Lamiaceae family, commonly distributed in the mild climate condition locations of the world. Morteza-Semnani and Ghanbarimasir reviewed traditional use of *Ballota* species and mentioned commercial products of *B. nigra* sold in Europe for sedative activity (Morteza-Semnani & Ghanbarimasir 2019). In Turkey, some species of *Ballota* have been used in folk medicine as antibacterial, antiulcer, antispasmotic, diuretic, choleretic, antihemorrhoidal and sedative agents (Çitoğlu et al., 1998; Baytop, 1999). Besides, *B. acetabulosa* was mentioned in the treatment of inflammation internally and to have external use for wounds and burns (Morteza-Semnani & Ghanbarimasir, 2019; Dulger & Sener, 2010). *Ballota hispanica*, endemic of Central Mediterranean region is reported to be used to treat skin problems and for its antidiabetic activity (Riccobono, Ben Jemia, Senatore, & Bruno, 2016).

The main components of the *Ballota* species are flavonoids, labdane diterpenoids, and phenylpropanoids (Sever Yılmaz, Özbek & Saltan Çitoğlu, 2006).

Secondary metabolites, such as tannins, terpenoids, alkaloids and flavonoids, which have been found in plants have extensively different bioactivity properties. Antibiotics are commonly used in fighting bacterial infections and have widely beneficial effects for the health and quality of human life since their invention. However, occurrence of drug-resistant bacteria causes antibiotics to be less effective against certain illnesses. Antiinflammatory agents that derived from natural sources play a significant role in the prevention and treatment of infection diseases (Bhalodia & Shukla, 2011).

Inflammation is a protective mechanism of living organisms against abnormal stimulation and covers complex series of biochemical activities performed by the body in response to injury or abnormal stimulation caused by a physical, chemical, or biological agent. In general, the generation of cytokines is accepted to play a major role inducing inflammatory process and free radicals can propagate inflammation by stimulating release of proinflammatory cytokines such as interleukin-1β, interleukin-6 and tumor necrosis factor-α (Libby, 2007). Drugs that are currently used for treatment of inflammatory conditions are non-steroidal antiinflammatory drugs (NSAIDs) and corticosteroids. Antiinflammatory drugs act by protecting lysosomal membrane integrity or inhibiting enzymes of prostaglandins and thromboxane inflammatory mediators synthesized by deactivating of cyclooxygenase (COX), COX-1 and COX-2 enzymes. Besides, degradation of lysosomal membrane brings on the release inflammatory mediators so human red blood cells (HRBC) can be used for evaluating of antiinflammatory activity. Some of these drugs such as aspirin, diclofenac, ketorolac, naproxen and piroxicam have toxic effects such as risk of gastrointestinal bleeding (Dinarello, 2010; Singh, Patil &Pal, 2012; Chowdhury, Azam & Jainul, 2014).

Diabetes mellitus is a major endocrine disorder, affecting approximately 5% of the world's population. According to WHO report, the global prevalence of diabetes has been rising from 4.7% to 8.5% since 1980 and direct medical costs, loss of works force both health services and national economies (WHO, 2016). Diabetes is characterized by abnormalities in carbohydrate, lipid and lipoprotein metabolisms, which not only lead to hyperglycemia but also cause many complications such as hyperlipidemia, hyperinsulinemia, hypertension and atherosclerosis (Ozkan, Kamiloğlu & Ozdal, 2016). One of the treatment approach of diabetes mellitus is α-glucosidase enzyme inhibition. These enzymes play an important role in glucose releasing from carbohydrates. A-glucosidase inhibitors lower the blood glucose level by delaying carbohydrate absorption in digestive tract. Recently, there are α-glucosidase inhibitory preparations used in handling hyperglycemia but these synthetic derivatives of α-glucosidase inhibitors have been reported as exhibiting adverse effects (Tundis, Loizzo & Menichini, 2010; Şöhretoğlu, Sari & Barut, 2018). So natural α-glucosidase inhibitors have been discussed as a therapeutic agents to manage diabetes mellitus.

Diseases associated with both inflammation and high blood glucose level have tended to increase and cause severe complications. In the light of traditional use of *Ballota* species, we aimed to examine 14 *Ballota* taxa from Turkey for their antiinflammatory activities and antidiabetic by using membrane stabilization method and *in-vitro* α-glucosidase inhibitory activity respectively as well as their therapeutic potentials. Two assays were carried out on both aqueous and alcoholic plant extracts in order to determine which could be a potential as a natural source for the treatment of diabetes and inflammation. We established *in-vivo* antiinflammatory activity of two species of *Ballota* called *B. glandulosissima* and *B.inaequidens* in our earlier reports but to the best of our knowledge, the present study is the first report on comparison of antiinflammatory and antidiabetic activity of 14 *Ballota* species (Özbek, Saltan Çitoğlu & Dülger 2004; Sever Yılmaz et al., 2006).

MATERIALS AND METHODS

Plant Material

Fourteen *Ballota* taxa were collected in different locations of Turkey. The species are listed in Table 1. Voucher specimens were deposited at the Herbarium belonging to the Ankara University Faculty of Pharmacy (AEF).

Preparation of extracts

From the air-dried and powdered materials of aerial parts of 14 *Ballota* taxa, 2 different extracts were prepared by using ethanol (%75) and water separately.

The air dried and powdered materials of the aerial parts of each *Ballota* taxa used in this study were weighed accurately and then, extracted with ethanol (75%). It was prepared by maceration 50 g of each plant powder in 300 ml of ethanol for 8 hours, in 3 days. The macerates obtained with ethanol were evaporated until dryness.

Additionally, the air dried and powdered materials of the aerial parts of the *Ballota* taxa used in this work were weighed accurately and then, extracted with water. It was prepared by

Table 1. Scientific names and collection places of Turkish *Ballota* species.

1.	*Ballota acetabulosa* (L.) Benth.	B1 İzmir: Yenifoça, 10 m, AEF 21602
2	*Ballota antalyense* F. Tezcan & H. Duman	C3 Antalya: Turunçova, 150 m, F. Tezcan & H.Duman 1701 (holo.: GAZI)
3	*Ballota cristata* P.H. Davis	C3 Isparta: Eğridir, 910 m, AEF19899
4	*Ballota glandulosissima* Hub.-Mor & Patzak	C3 Antalya: Kumluca, 500 m, AEF 19900
5	*Ballota inaequidens* Hub.-Mor & Patzak	C3 Antalya: Alanya, 200 m, AEF 19901
6	*Ballota larendana* Boiss. & Heldr.	A4 Ankara: Kızılcahamam, 830 m, AEF 21604
7	*Ballota latibracteolata* P.H. Davis & Doroszenko	C3 Antalya: Gazipaşa, 425 m, AEF 19902
8	*Ballota macrodonta* Boiss. & Bal.	B5 Kayseri: Yahyalı, 1150 m, AEF 19907
9	*Ballota nigra* L. subsp. *anatolica* P.H. Davis	B4 Ankara: Gölbaşı, 800 m, AEF 21601
10	*Ballota nigra* L. subsp. *uncinata* (Fiori & Beg.) Patzak	B1 Izmir: Gökçealan, 250 m, AEF 21607
11	*Ballota pseudodictamnus* (L.) Benth. subsp. *lycia* Hub.-Mor.	C2Muğla: Fethiye, 20m, AEF 21603
12	*Ballota rotundifolia* C. Koch	A8 Erzurum: Tortum Lake, 1200 m, AEF 21606
13	*Ballota saxatilis* Sieber ex. J & C. Presl subsp. *brachyodonta* (Boiss.) P.H. Davis & Doroszenko	C4 İçel: Silifke, 1400 m, AEF 21505
14	*Ballota saxatilis* Sieber ex. J & C.Presl subsp. *saxatilis*	C4 İçel: Anamur, 1530 m, AEF 19904

maceration 50 g of each plant powder in 300 ml of water for 8 hours, in 3 days. The macerates obtained with water were lyophilized.

Antiinflammatory activity

The study protocol was approved by the ethics committees of the Faculty of Medicine of Ankara University, Ankara-Turkey (26.10.2015/16-695-15).

Preparation of Human Red Blood Cells (HRBC) Suspension

Fresh whole human blood was collected from healthy human volunteer who had not taken any antiinflammatory or steroidal drug for 2 weeks prior the experiment and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min. The packed cells were washed three times with equal volume of isosaline (0.85%, pH 7.2). The volume of the blood was measured and reconstituted as 10% *v/v* RBC's suspension with isosaline.

Heat Induced Hemolysis

As HRBC membrane is similar to lysosomal membrane structure, stabilization of membrane is taken as a measure of antiinflammatory activity.

Membrane stabilizing activity of the extracts was assessed using heat-induced human erythrocyte hemolysis (Shinde et al., 1999; Yalçın, Yılmaz Sarıaltın & Çiçek Polat, 2020). Inhibition of hemolysis was implied as stabilization of the HRBC membrane. The reaction mixture consisted of extracts (0,5-20 mg/ml) or acetylsalicylic acid (0.05-0.5 mg/ml) and 10% HRBC suspension. Instead of test sample, solvent of the samples was used as a control and acetylsalicylic acid was a standard drug. All the centrifuge tubes containing reaction mixture were incubated at 56ºC for 30min. At the end of the incubation the tubes were cooled under running tap water. Another set of prepared samples were placed at 0°C in an ice bath. The reaction mixture was centrifuged at 2500 rpm for 5 min. Then the absorbance of the supernatant was measured on 96-well microplate spectrophotometer at 560 nm. The experiment was performed in triplicates for all the test samples. The percentage of inhibition of hemolysis was calculated then by the formula as given below. The concentration against the percentage of inhibition of hemolysis was plotted and the half maximal inhibitory concentration (IC_{50}) was calculated using this plot for each sample.

$$\% \text{ inhibition of hemolysis} = 100 \times \left[1 - \frac{(A_2 - A_1)}{(A_3 - A_1)}\right]$$

A_1: test sample unheated, A_2: test sample heated, A_3: control sample heated

Antidiabetic Activity

***In-vitro* α-glucosidase inhibitory activity**

The α-glucosidase inhibitory activities of the ethanol and aqueous extracts were determined according to the method with slight modifications (Liu et al., 2014). The substrate solution pNPG was prepared with 0.2 M of Na-phosphate buffer (pH:6.8). The reaction mixture contained 10 µL of 0.02 U/µL α-glucosidase solution in 0.2 M Na-phosphate buffer (pH=6.8), 10 µL of sample (1-50µg/ml), 50 µL of Na-phosphate buffer, which were mixed and incubated at 37°C for 20 min. Then, 50 µL of 0.02 M PNPG was added, and the mixture was incubated at 37°C for another 30 min. Finally, the reaction was stopped by the addition of 100 µL 0.2 M Na_2CO_3 solutions. Acarbose (0.5-25 µg/ml) was used as a standard drug. The amount of the p-nitrophenol released from PNP-glycoside was quantified on a 96-well microplate spectrophotometer at 405 nm. The inhibitory activities of the samples on α-glucosidase were calculated by the following formula. The IC 50 value for each sample was determined graphically by plotting the percentage of inhibition and inhibitory concentration.

Inhibition (%) = [(Absorbance of control- Absorbance of test sample)/ Absorbance of control)] x 100

Statistical Analysis

All the tests were run in triplicate. SPSS 20.0 and 23.0 were used to examine the effect of different compounds and concentrations on antiinflammatory and antidiabetic activities. One-way analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by the Fisher's least significant difference (LSD) test. $p<0.05$ were reported as "statistically significant".

RESULTS

In this study, antiinflammatory and antidiabetic activities of ethanol and aqueous extracts from aerial parts of 14 *Ballota* taxa growing in Turkey were evaluated (Table 1).

Antiinflammatory Activity

Antiinflammatory activity was performed by evaluating the stabilization effects of extracts on hemolyzed erythrocyte membrane. HRBCs membrane stabilization activity of ethanolic and water extracts of *Ballota* sp. are given in Figure 1 and Figure 2.

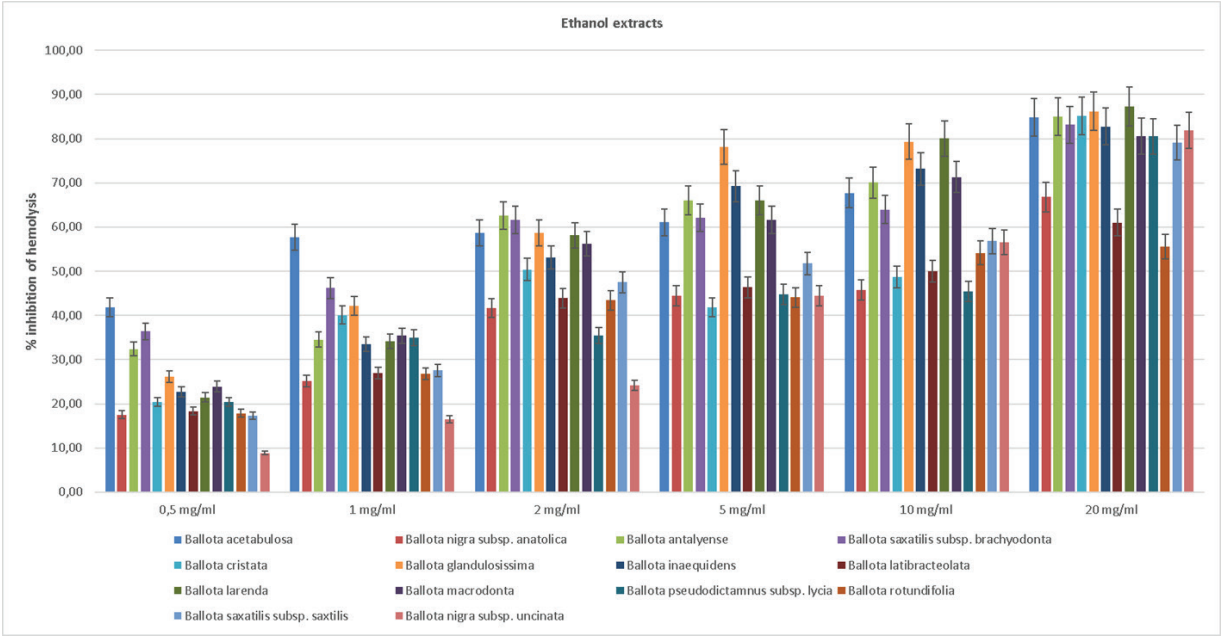

Figure 1. Percent inhibition of hemolysis of HRBC membrane in the presence of different concentrations of *Ballota* species.

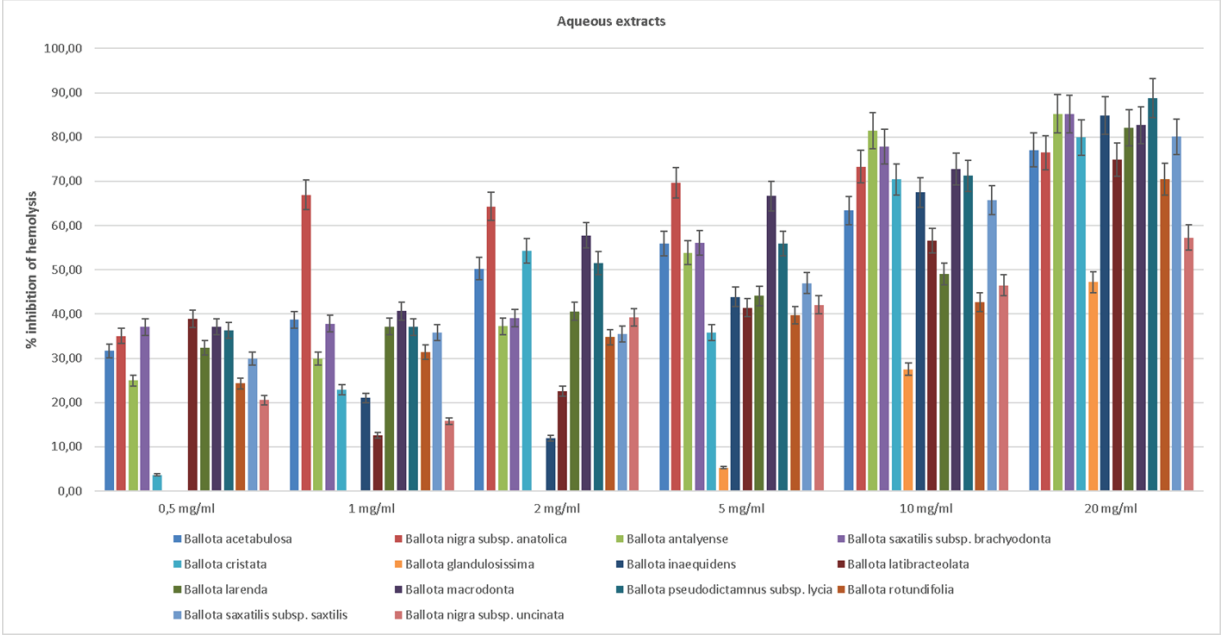

Figure 2. Percent inhibition of hemolysis of HRBC membrane in the presence of different concentrations of aqueous extracts of *Ballota* sp.

Table 2. *In-vitro* antiinflammatory activities by using HRBC membrane stabilizing method.

Plant name	IC_{50} (mg/ml) Mean ± SD	
	Ethanol extract	Aqueous extract
Control	-	-
Ballota acetabulosa	4.30±0.18*	7.24±0.01*
Ballota nigra subsp.*anatolica*	11.59±0.20*	3.18±0.10*
Ballota antalyense	5.46±0.02*	6.93±0.08*
Ballota saxatilis subsp. *brachyodonta*	5.31±0.02*	6.23±0.03*
Ballota cristata	8.45±0.06*	8.79±0.08*
Ballota glandulosissima	4.59±0.14*	20.25±0.04*
Ballota inaequidens	6.17±0.17*	9.69±0.17*
Ballota latibracteolata	12.06±0.44*	10.23±0.36*
Ballota larendana	5.61±0.06*	8.69±0.41*
Ballota macrodonta	6.47±0.15*	5.12±0.11*
Ballota pseudodictamnus subsp. *lycia*	9.65±0.05*	5.90±0.04*
Ballota rotundifolia	12.88±0.07*	11.49*[a]
Ballota saxatilis subsp *saxatilis*	8.67±0.07*	8.04±0.07*
Ballota nigra subsp. *uncinata*	9.89±0.03*	13.87±1.02*
Acetylsalicylic acid	0.28±0.01*	

*A p-value less than 0,05 was considered statistically significant compared to control. [a]SD<0.01 mg/ml

14 *Ballota* ethanolic extracts at all the doses (0.5–20 mg/ml) were found to protect the human erythrocyte membrane against lysis induced by heat. In the present study, the ethanolic extracts showed higher membrane stabilization profile than aqueous extracts except *B. nigra* subsp. *anatolica*, *B. latibracteolata*, *B. rotundifolia* and *B. saxatilis* subsp. *saxatilis*. As shown in Table 2, IC_{50} values of antiinflammatory effect of ethanolic and aqueous extracts of 14 *Ballota* samples ranged from 4.30-12.88 and 3.18-20.25 mg/ml, respectively. Under the same experimental condition, inhibition of hemolysis of acetylsalicylic acid was 0.28 mg/ml.

On the basis of this study, it was concluded that aqueous extracts of *Ballota nigra* subsp. *anatolica* (IC_{50}=3.18 mg/ml) exhibited the maximum antiinflammatory effect following by ethanolic extracts of *Ballota acetabulosa* and *Ballota glandulosissima* with a value of 4.30 and 4.59 mg/ml respectively.

Antidiabetic activity

In-vitro antidiabetic activity of the plant extracts was evaluated by measuring their inhibitory effect on α-glucosidase level and results were given in Table 3. Both aqueous and ethanol extracts of *Ballota glandulosissima* exhibited the maximum α-glucosidase inhibitory activity with IC_{50} values of 2.18 and 2.30 µg/ml, respectively. Generally, the aqueous extracts showed higher α-glucosidase inhibitory activity than the ethanol extracts. On the other hand, the ethanolic extract of *B. saxatilis* subsp. *brachyodonta* was found much stronger inhibition value (IC_{50}= 2.63 µg/ml) on α-glucosidase, compared with water extract (19.84 µg/ml). Similar results were also found for ethanol and water extracts of *B. cristata* with IC_{50} values of 2.60 and 17.82 µg/ml. The IC_{50} value of the reference compound, acarbose was 0.898 µg/ml.

DISCUSSION

Ballota species have been reported as being rich in phenolic compounds. They contain diterpenoids, flavonoids, phenylpropanoids, essential oils, tannins and saponins (Sever Yılmaz & Saltan Çitoğlu, 2003). In the present study, we applied membrane stabilization method and *in-vitro* α-glucosidase inhibitory activity to evaluate antiinflammatory and antidiabetic activities of 14 *Ballota* taxa growing in Turkey, respectively. According to our results; the ethanolic extracts showed higher membrane stabilization profile than aqueous extracts generally. Aqueous extracts of *Ballota nigra* subsp. *anatolica* exhibited the maximum antiinflammatory effect following by ethanolic extracts of *Ballota acetabulosa* and *Ballota glandulosissima*. And for antidiabetic activity, it was concluded that aqueous and ethanol extracts of *Ballota glandulosissima* (IC_{50}=2.18 and 2.30 µg/ml respectively) exhibited the maximum α-glucosidase inhibitory activity. In present study, among the *Ballota* species, the ethanolic extracts of B. *glandulosissima* showed both significant antidiabetic and antiinflammatory activity with 2.298 µg/ml and 4.59 mg/ml IC_{50} value, respectively.

According to our previous studies, *B. glandulosissima* and *B nigra* subsp. *anatolica* were found to have high antioxidant potential and *B. glandulosissima* was found as the richest species

Table 3. *In vitro* antidiabetic activities of ethanol and aqueous extracts of *Ballota* sp. by α-glucosidase inhibitory method.

Plant name	IC_{50} (µg/ml) Mean ± SD	
	Ethanol extract	Aqueous extract
Control	-	-
Ballota acetabulosa	26.93±0.03*	11.04±0.01*
Ballota nigra subsp.*anatolica*	19.21±0.01*	7.56±0.01*
Ballota antalyense	3.22±0.01*	3.68±0.02*
Ballota saxatilis subsp. *brachyodonta*	2.63± 0.01*	19.84±0.01*
Ballota cristata	2.60±0.01*	17.81±0.01*
Ballota glandulosissima	2.30± 0.01*	2.18±0.01*
Ballota inaequidens	15.67±0.03*	4.49±0.01*
Ballota latibracteolata	5.52± 0.01*	3.22±0.01*
Ballota larendana	14.83±0.01*	3.99±0.01*
Ballota macrodonta	4.56± 0.01*	4.52±0.01*
Ballota pseudodictamnus subsp. *lycia*	6.53± 0.01*	3.71±0.02*
Ballota rotundifolia	7.82± 0.01*	3.87±0.01*
Ballota saxatilis subsp *saxatilis*	9.16± 0.01*	4.50±0.02*
Ballota nigra subsp. *uncinata*	18.23±0.01*	6.07±0.01*
Acarbose	0.90± 0.01*	

with respect to flavonoid content We also studied the antiinflammatory activities of *B. inaequidens* and *B. glandulosissima in-vivo* and aqueous extract of *B. inaequidens* was found to have better antiinflammatory activity on carrageen induced hind paw edema in rats and the water extract *B. glandulosissima* showed antiinflammatory activity, but in the present study, ethanolic extract of *B. glandulosissima* is found to have higher membrane stabilizing activity (Özbek, et al., 2004; Sever Yılmaz et al., 2006; Erdoğan Orhan, Sever Yılmaz & Altun, 2010; Sever Yılmaz, Ergene & Saltan Çitoğlu, 2015). Kumatakenin, pachypodol, 5-hydroxy-7,3',4'-trimetoxyflavone, velutin, salvigenin, retusin, corymbosin were found in *B. glandulosissima* (Çitoğlu, Sever & Antus, 2003). On the other hand, the opposite results were observed for *B.nigra subsp. anatolica* which aqueous extract showed the strongest membrane stabilizing activity. Considering our earlier studies on chemical profile of *Ballota* species, it could be assumed that the antiinflammatory and antidiabetic potential of *B. glandulosissima* and *B. nigra* subsp. *anatolica* were related to flavonoids and phenolic compounds and besides, the results of present study are in good agreement with our earlier reports. Earlier reports pointed out the strong correlation between phenolic compounds and antioxidant activity. Uysal et al. mentioned that the antioxidant activity of water extract of *B. macrodonta* was higher than methanolic extract connected with phenolic contents (Uysal et al., 2018). It is also well known that strong antioxidant activity could be linked to both antiinflammatory and antidiabetic effects. So, in conclusion, *B. acetabulosa, Ballota glandulosissima Ballota nigra* subsp. *anatolica* could be a good candidate for the treatment of inflammation and on the other hand, *Ballota saxatilis* subsp. *brachyodonta*, *Ballota cristata*, *Ballota glandulosissima* were found to significant antidiabetic activities, directly related to the total amount of polyphenols and flavonoid content. The further step of this study was to isolate and identify the antiinflammatory and antidiabetic components of potential *Ballota* species.

CONCLUSION

Recently, the beneficial effects of natural products have become popular on the basis of low toxicity but it is clear that an evidence base approach is necessary for this issue. *Ballota* species have traditional use and are good candidates for treatment of inflammation and diabetes. Our results also support the medical profile of *Ballota* species but, of course, further studies are needed to be sure about the efficacy and safety.

Fundings

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Peer-review: Externally peer-reviewed.

Ethics Committee Approval: The study was approved by the ethics committees of the Faculty of Medicine of Ankara University, Ankara-Turkey (26.10.2015/16-695-15).

Author Contributions: Conception/Design of Study- A.C.A., B.S.Y., G.S.İ., T.Ç.; Data Acquisition- A.N.Y.E., B.S.Y., S.Y.S.; Data Analysis/Interpretation- A.C.A., S.Y.S., B.S.Y.; Drafting Manuscript- A.C.A., S.Y.S., B.S.Y.; Criti-

cal Revision of Manuscript- A.C.A., S.Y.S., A.N.Y.E., T.Ç., G.S.İ., B.S.Y.; Final Approval and Accountability- A.C.A., S.Y.S., A.N.Y.E., T.Ç., G.S.İ., B.S.Y.

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

Financial Disclosure: Authors declared no financial support.

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Istanbul J Pharm 51 (3): 333-340
DOI: 10.26650/IstanbulJPharm.2021.877929
Original Article

Study of *in vitro* antiinflammatory and immunomodulatory effect of Ayurvedic plants – Murva

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Cite this article as: Mathew, A. A., Asirvatham, R., Goowtham, A., & P. A., Daisy (2021). Study of *in vitro* antiinflammatory and immunomodulatory effect of Ayurvedic plants – Murva. *İstanbul Journal of Pharmacy, 51*(3), 333-340.

ABSTRACT

Background and Aims: Toxic chemicals, pathogens and damaged cells trigger the inflammatory process and cause disease in the kidney, heart, brain, liver, lung and reproductive system. The present study evaluates the anti-inflammatory and immunomodulatory effect of Ayurvedic plants under the Murva category.

Methods: *Marsdenia tenacissima* is an acceptable source of Murva. *Sansevieria roxburghiana* is considered as Murva in west Bengal. Methanol extracts of both plants were used to evaluate anti-inflammatory and immunomodulatory effects by certain *in vitro* methods. The *in vitro* anti-inflammatory effects of methanol extract *Marsdenia tenacissima* (MEMT) and methanol extract of *Sansevieria roxburghiana* (MESR) were assessed by heat and hypotonic induced haemolysis on the red blood cells of rats. Nitro blue tetrazolium (NBT) assay and inhibition of TNF-α release in DAL cell lines were conducted to assess the immunomodulatory potential of extracts.

Results: MEMT, MESR and a combination of MEMT&MESR showed significant ($p<0.001$) inhibition of haemolysis on heat and hypotonic induced methods compared with the standard Diclofenac Sodium. In NBT reduction test, MEMT showed a more significant ($p<0.001$) result than MESR. Similarly, the inhibition of TNF-α release was also significantly enhanced by 400 µg/mL of MEMT.

Conclusion: The present research results revealed that Murva is a safe Ayurveda drug for the treatment of cancer and cardiac disease with the protection of systemic immunity and set free from an inflammatory condition.

Keywords: Murva; *Marsdenia tenacissima, Sansevieria roxburghiana, in vitro* antiinflammatory, immunomodulatory, NBT assay

INTRODUCTION

Inflammation is the response of the immune system when exposed to harmful stimuli like pathogens, toxic compounds, damaged cells or radiation. Inflammation is a defence mechanism and acts by removing harmful stimuli and by initiating a healing process. The inflammatory process is mediated by the migration of leukocytes to the affected site and the release of inflammatory mediators. Chronic inflammation occurs when acute inflammation fails to correct or treat the affected site which leads to the development of cardiovascular diseases, cancer, arthritis, and type 2 diabetes mellitus (Chen L et al., 2018). Inflammation is the major component in tumour progression where most of the cancer arises from infection or chronic irritation and inflammation. The tumour cells are surrounded by inflammatory cells which release inflammatory mediators such as selectin, chemokines and their respective receptors. These mediators initiate cancer invasion, migration and metastasis process. Thus, an anti-inflammatory drug plays an effective role in the early tumour stage and malignant conversion (Huang, Zhang, & Ding, 2018). Inflammation is also

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Submitted: 18.02.2021
Revision Requested: 30.04.2021
Last Revision Received: 04.06.2021
Accepted: 24.06.2021
Published Online: 22.11.2021

caused by drug resistance or by inflammatory mediators and causes the therapeutic failure of various first-line anticancer drugs such as doxorubicin, cisplatin, 5-fluorouracil, and paclitaxel (Bag, Devi & Bhaigyabati, 2015). Similarly, inflammation is also linked to cardio stress. The affected myocyte has elevated levels of endothelial adhesion molecules which affect the release and production of inflammatory mediators such as chemokine, cytokine production and release (Chen et al., 2018). However, a single drug which compromises all the above incidents is quite difficult to find. Hence a new strategy was developed in pharmaceutical industries to establish a formulation with extended action of cardio protection from doxorubicin and anticancer activity free from myelosuppression and anti-anaemic effect. Under this comprehensive concept, medicinal herbs and their formulations have recently received greater attention in regard to the treatments of various life threatening diseases because of their efficacy and rapid curative properties. Among the herbal preparations, Ayurvedic formulations have been placed in the first position for thousands of years due to their lower toxicity and wider acceptability. Ayurveda is a Sanskrit word which means longevity and which is a traditional system of medicine in India and other south Asian countries. Ayurvedic treatment is based on the establishment of equilibrium in different elements of the human body, intellect, mind and soul (Lokhande, Jagdale, & Chabukswar, 2006). These drugs mainly act by stimulating the specific function of the organ, by altering hormones, affecting immunity and neurotransmitter as well as through antioxidant mechanism. It is composed of herbal preparations that are combined with supplements at different levels and are safe, effective and indigenous remedies for the people in India and China (Ven Murthy, Ranjekar, Ramassamy, & Deshpande, 2010; Arya& Guota, 2011). In Ayurveda, various therapeutic approaches are used which are based on years of experience, empiricism, and observation (Gogtay, Bhatt,& Dalvi, 2002) The major problem in Ayurveda is the standardisation and identification of medicinal plants and formulation because some drugs have more than one botanical source which creates difficulty in identification of the sources. One of such Ayurvedic plants is Murva. It is a controversial drug, a combination of 11 medicinal plant roots found in different parts of India. Due to its availability in south India, *Marsdenia tenacissima (Roxb.)* Wight et Arn., (Asclepiadaceae) (MT) is an acceptable source of Murva. However, *Sansevieria roxburghiana* (Schult. & Schult.f. (SR) (*S. zeylanica* Roxb.) (Asparagaceae) is also considered Murva in west Bengal, hence both plants were selected for this study (Mathew, Asirvatham, & Tomy, 2021) The remaining plants in Murva, their scientific names, and their source locations are *Helicteres isora* L (Sterculiaceae) from Punjab, *Maerua arenaria* Hook. f. & Thomson (Capparaceae) from Bihar, *Chonemorpha fragrans* (Moon) Alston (Apocynaceae) from Kerala, *Clematis triloba* Thunb. (Ranunculaceae), *Wattakaka volubilis* (Linn. f.) Stapf (Asclepidaceae) and *Salvadora persica* L. (Salvadoracae) from South India, *Argyreia nervosa* (Burm.f.) Bojer, (Convolvulaceae), *Maerua oblongifolia* A.Rich. (Capparaceae), and *Dregea volubilis* Benth. ex Hook.f. (Apocynaceae) from other regions of India (Mathew et al., 2021). Traditionally, Murva is used for the treatment of anaemia, diabetes, stomach disorder, typhoid, cough, fever and urinary tract infections. MT is traditionally used as a drug in clinical conditions like abdominal pain caused by worm infections, Vata conditions like constipation, light and interrupted sleep, nervousness, anxiety, tremors, fever and also disorders of heart, skin and neuroprotection (Tiwari, Singh& Tiwari 2018). In Chinese folk medicine, stems are used for the treatment of cancer and asthma. Traditionally the root is used in the treatment of fever by local practitioners (Hatapakki & Hukkeri, 2011) whereas the whole plant extract of SR is traditionally indicated in used as cardiotonic, febrifuge, expectorant, purgative, and tonic, and indicated in the treatment of glandular enlargement, purgative, and rheumatism. Mucilaginous rhizome is used for sustained chronic persistent cough, consumptive complaint, for mitigating common cough, cold and ear pain. The tender shoot juice administration was found to be effective in children for removing viscid phlegm from throats. The root is used for snakebite and haemorrhoids. In Bangladesh, young leaf juice is used for ear infection (Obydulla, 2016). The roasted leaves are traditionally used as emollient but, none of them have been experimentally proven its anti-inflammatory and immunomodulatory effect. The current study aimed to evaluate the anti-inflammatory and immunomodulatory potential by stabilization of cell membrane HRBC assay.

MATERIALS AND METHODS

Plant material collection

The roots of *Marsdenia tenacissima* (MT) and rhizome and roots of *Sansevieria roxburghiana* (SR) were collected in October 2018 from Cherthala, Kerala, India. Dr. K. Madhava Chetty identified and authenticated the plant MT, and the herbarium specimen (voucher number 1132) was deposited to the Department of Botany in Sri Venkateswara University Tirupathi, A.P. Similarly, SR also authenticated the herbarium specimen (No: AAM001) which was deposited to Dr. Jose Mathew, Department of Botany, Santana Dharma College, Alappuzha, Kerala. India.

Extraction procedure

The Roots of MT, Rhizome and roots of SR were washed and cleaned, and dried at room temperature (shade dry). About 300g of the coarsely powdered drug was successively extracted using Soxhlet apparatus with increasing polarity of solvents using petroleum ether (50-60^{o} C for 72 hours), chloroform (60-70^{o} C for 48 hours), methanol (70-80^{o} C for 48 hours), and marc from the methanol extract was macerated with chloroform-water (10:90) for 24 hours to obtain the aqueous extract (Hepsibah & Jothi, 2017). Petroleum ether extract of MT (PEMT), SR (PESR), chloroform extract of MT (CEMT), SR (CESR), methanol extract of MT (MEMT), SR (MESR) and aqueous extract of MT (AEMT), SR (AESR) were collected by rotary evaporator and then dried and stored in an airtight container for experimental purposes.

Cancer cell line

The transplantable Dalton ascetic lymphoma cells (DAL) were obtained from Amala cancer research institute, Thrissur, Kerala. The DAL cells were kept in the ascetic form *in vivo* through sequential passages in Swiss albino mice, by intraperitoneal transplantation of $2x10^{6}$ cells /mouse after every 14 days. Eight days after transplantation, ascetic fluid was taken from the DAL bearing mouse.

Preliminary phytochemical analysis

Phytochemicals are naturally occurring chemical compounds obtained from plants. The term commonly refers to those chemicals that might have a role in health, but are not confirmed as essential nutrients (da Silva et al., 2016; Gul, Jan, Faridullah, Sherani, & Jahan, 2017). Test for alkaloids, carbohydrates, glycosides, phytosterols, coumarins, flavonoids, phenolic compounds, tannins, saponins, fixed oil, protein and amino acids were conducted as described by Trease & Evans, 1989.

Tests for alkaloids

- Drageendorff's Test (Potassium bismuth iodide solution)

Two millilitres (2 mL) of acidic solution of plant extract were neutralized with 10% ammonia solution. Dragendorff's reagent was added and turbidity or precipitate was observed as indicative of presence of alkaloids.

- Wagner's Test (Potassium iodide solution)

2 mL of plant extract were boiled with 5 mL of 2% HCl on a steam bath. The mixture was filtered and 1 mL portion of the filtrate was treated with 2 drops Wagner's reagent. A reddish-brown precipitate indicates the presence of alkaloids.

- Mayer's Test (Potassium mercuric iodide solution)

Drops of Mayer's reagent were added to a portion of the acidic solution in a test tube and observed for an opalescence or yellowish precipitate indicative of the presence of alkaloids.

- Hager's Test (Iodine-picric acid)

For this test procedure, few drops of Hager's reagent (saturated picric acid solution) were added to 2 mL of the respective plant extract. Bright yellow precipitate formation indicated the existence of alkaloids.

Test for carbohydrate

- Molisch Test

A few drops of Molisch's solution were added to 2 mL of aqueous solution of the extract, thereafter a small volume of concentrated sulphuric acid was allowed to run down the side of the test tube to form a layer without shaking. The interface was observed for a purple colour as indicative of positive for carbohydrates.

- Seliwanoff's Test

5 mL of Seliwanoff's reagent were added in a test tube containing 1 mL of plant extract, heated using hot water. The colour of the test tube changed to red, indicating keto sugar (Fructose and Sucrose) was present in the solution.

- Benedict's Test

A mixture which contains 2 mL of plant extract and Benedict's solution (approximately 5 mL) was heated in a test tube for around two minutes and was then allowed to cool. Red coloured precipitate indicated the presence of carbohydrate.

Test for glycosides

- Legal Test

The plant extract was dissolved in 1 mL of water, with a few drops of 10% sodium hydroxide and 1 mL of 0.3% nitroprusside sodium reagent. The mixture turned a dark red colour almost instantly.

- Baljet Test

Dissolved the plant extract in the in 3 mL of methanolic sodium picrate solution. Added 1 mL of N-sodium hydroxide solution to the liquid. The mixture acquired at once a light wine-red colour.

- Borntrager's Test

5 mL of plant extract were added in 5 mL of 5% ferric chloride solution and 5 mL dil. hydrochloric acid, heated for 5 minutes in water bath. Cooled and added 3 mL of benzene or organic solvent. Shook well. Separated organic layer, added equal volume of 10% ammonia solution. The formed rose pink/red at ammonia layer showed the presence of glycosides.

- Keller-Killiani Test

5 mL of the plant extract were added to3 mL of concentrated acetic acid. Added 1 drop of iron (III) chloride test solution to the liquid and carefully transferred it on concentrated sulphuric acid. A reddish-brown ring formed at the interface, the upper acetic acid layer soon turned bluish green.

Test for phytosterol

- Liebermann-Burchard Test

The amount of 0.5 g of the extract was dissolved in 10 mL anhydrous chloroform and filtered. The solution was divided into two equal portions for the following tests. The first portion of the solution above was mixed with 1 mL of acetic anhydride followed by the addition of 1 mL of concentrated sulphuric acid down the side of the test tube to form a layer underneath. The test tube was observed for green colouration as indicative of steroids.

- Salkowski Test

5 mL of plant extract were mixed in 2 mL of chloroform followed by the careful addition of 3 mL concentrated sulphuric acid to form a layer. A layer of the reddish-brown coloration was formed at the interface thus indicating a positive result for the presence of terpenoids.

Test for coumarins

A volume of 1 mL of 10% NaOH solution was added to 1 mL of the plant extract. Yellow colour was formed when it was placed in a water bath. It confirmed the existence of coumarins in the tested samples.

Test for flavonoids

- Shinoda Test

Pieces of magnesium ribbon and concentrated HCl were mixed with aqueous crude plant extract after few minutes and pink colour showed the presence of flavonoid.

Test for phenolic compounds

- $FeCl_3$ test

A few drops of $FeCl_3$ solution were added to 1 mL of plant extract samples. Blackish red precipitate revealed the existence of flavonoids in the test samples

Test for tannin

- Gelatin Test

To a 1% gelatine solution, added a drop of 10% sodium chloride. If a 1% solution of tannin is added to the gelatine solution, tannins cause precipitation of gelatine from solution.

Test for protein and amino acid

- Biuret Test

A quantity (2 mL) of the extract was put in a test-tube and 5 drops of 1% hydrated copper sulphate were added. A quantity, 2 mL of 40% sodium hydroxide, was also added and the test tube shaken vigorously to mix the contents. A purple coloration shows the presence of proteins.

- Xanthoprotein Test

1 mL of extracts was treated with 1 mL of concentrated HNO_3. A white precipitate was formed then boiled and cooled. Then 20% of NaOH or NH_3 was subsequently added, which leads to the formation of an orange colour, which revealed the presence of aromatic amino acids.

- Lead Acetate Test

A fraction of the extracts was treated with 1 mL of lead acetate. A white precipitate formed, which indicated the presence of proteins.

Test for saponins

1 mL of methanol extract was diluted with distilled water to 20 mL and shaken for 15 minutes in a graduated cylinder. The presence of saponin was confirmed by the formation of a layer of foam.

Test for fixed oils

- Spot Test

A quantity of 0.1 g of the extract was pressed between filter paper and the paper observed. Translucency of the filter paper indicated the presence of oils.

- Saponification Test

A few drops of 0.5 N alcoholic potassium hydroxide were added to 1 mL of plant extract along with drop of phenolphthalein, the mixture was heated 2 hours. Formation of soap or partial neutralization of alkali indicated the presence of fixed oils and fats.

Cytoprotective assays

Preparation of RBC suspension

The study protocol (SJCP/IAEC/2018-4/35) was approved by the Institutional Animal Ethics Committee (IAEC), St. Joseph's College of Pharmacy Cherthala, Kerala, India. About 3ml of fresh blood were collected from the male rat and transferred to heparinised tube. The tube was centrifuged for 10 min at 3000 rpm. 40%w/v suspension was prepared by dissolving red blood pellets with normal saline. Then the solution was reconstituted with isotonic buffer (10mM sodium phosphate buffer, pH 7.4) and it was used for the experiment's purposes (Raju, Seeja, Deshami, Sinchu, & Malu, 2015). .

Human Red Blood Cell (HRBC) membrane stabilization test

By using normal saline, a 10 % blood suspension was prepared. The reaction mixture contained 1 mL of 10% RBC suspension and 1mL of different concentrations of MEMT, MESR and a combination of MEMT&MESR. Instead of 1 mL of test drug, normal saline was added in control test tube. Standard drug Diclofenac sodium (200 µg/mL) concentration was used. All the reaction mixtures were incubated for 30 minutes at 56°C in the water bath. After incubation, the test tube was cooled and centrifuged for 5 min at 2500 rpm. The haemoglobin content in the supernatant solution was estimated using a colorimeter at 560 nm. Each test was carried out in a triplicate manner with all samples (Raju et al., 2015).

Percentage membrane stabilization activity was determined by the formula,

Percentage inhibition $=A_{control}-A_{test}/A_{control}$

$A_{control}$=absorbance of control; A_{test}=absorbance of test

Heat-induced haemolysis

1mL of isotonic phosphate buffer was added to the 1 mL of different concentration (100, 200,400, 600, 800 and 1000µg/mL) samples of MEMT, MESR and combination of MEMT & MESR in a set of 6 centrifuge tubes. All the tests were done in a triplicate manner. 1mL of diclofenac (200 µg/mL) served as standard and 1mL of the vehicle taken as control. HRBC suspension was added to all tubes and mixed gently tubes were incubated in a regulated water bath at 54°C for 20 min. Another set was maintained in a freezer at -10°C for 20 min and followed by centrifuge at 3000 rpm for 3min. Supernatant haemoglobin content was determined by spectrophotometry under 540 nm (Raju et al., 2015).

The inhibition of haemolysis (%)=1-((OD2-OD1)/ (OD3-OD1)X 100

Where OD1 =absorbance of the test sample (unheated)

OD2= absorbance of the test sample (heated)

OD3= absorbance of the control sample (heated)

Hypotonicity induced haemolysis

Hypotonic solution (1 mL) of various graded dose of extracts (100,200,400,600,800 and 1000 µg/mL) were prepared and taken as in triplicate. In another set of the centrifuge tube, 1 mL of the isotonic solution containing a graded dose of extracts was also taken in triplicate manner.1 mL of vehicle (distilled water) was taken as control and 1 mL of 200 µg /mL of diclofenac for standard. HRBC suspension (1mL) was added to all test tubes and mixed gently. The mixture was incubated at room temperature for 1 hour followed by centrifuge at 3000 rpm for 3 min. Absorbance was determined spectrophotometrically at 540 nm using a supernatant containing haemoglobin (Raju et al., 2015).

The inhibition of haemolysis (%)=1-((OD2-OD1)/ (OD3-OD1) X 100

Where OD1 =absorbance of the isotonic test sample

OD2=absorbance of the hypotonic test sample

OD3= absorbance of control hypotonic sample

NBT assay on haemocytes

The reduction of NBT to insoluble blue formazan was used as an indication for superoxide generation, although it is not entirely specific for O_2^-. A measured volume of haemocytes was taken in triplicate, using 96 well microtiter plate (Sigma M-0156) and incubated with different concentrations of MEMT, MESR in humid conditions, for 30 min at room temperature for adherence of the haemocytes. The supernatants were added with 0.3% NBT and absolute methanol. The formazan deposits were dissolved in 120 mL 2 M KOH and 140 mL DMSO. After homogenization of the contents in the wells, the absorbance was read at 620 nm in a spectrophotometer (Heroor, Beknal, & Mahurkar, 2013).

TNF-α Inhibition assay

Inhibition of TNF-α release in lipopolysaccharide (LPS) stimulated DAL cells were evaluated by a slight modification of Prabhakar, Brooks, Lipshlitz, & Esser, 1995 This reference is not present in the reference list procedure. Different concentrations of MEMT, MESR and LPS were added to initiate inflammation in a measured volume of cell line and kept aside for 3 hours. Then the cells ($1X10^6$ cells/mL) were moved to a 96 well microplate and incubated for 18 hours (overnight) at 37°C followed by centrifuge (1800 g, 5 min, 16°C) after which the supernatant was collected. TNF-α was measured from the supernatant by the cytokine specific sandwich quantitative enzyme-linked immunosorbent assay (ELISA). The inhibition of TNF-α release by LPS-stimulated DAL cells was estimated by the ratio between the TNF-α amount secreted by treated cells (pg/mL) and the level of this cytokine (pg/mL) was observed for untreated cells stimulated with LPS (Sakthivel & Guruvayoorappan, 2013). Percentage of THF-α inhibition was reported as well as calculated from the formula of [1 – (cytokine secretion of treated cells/ cytokine secretion of cells cultivated with solvent control) 100].

RESULTS

All the extracts were subjected to a qualitative analysis of various phytoconstituents such as alkaloids, carbohydrates, proteins and amino acids, anthraquinone, glycosides, steroids, flavonoids, tannins, phenolic compounds, saponins, fats and fixed oil. Both plant extracts showed the presence of carbohydrates, alkaloids, glycosides, saponins, flavonoids, tannins, phytosterols, amino acids and proteins where the various literature showed the presence of glycosides of z-deoxy sugars, which on hydrolysis gave genins like cissogenin, tenasogenin, tenacissigenin, tenacigenoside I, tenacissoside C, tenacigenoside K, tenacigenosides G, tenacissoside H, marsdenoside B -H and11α-O-2-

Table 1. Phytochemical analysis of the Pet. ether, $CHCl_3$, methanol and aqueous extracts of MT & SR.

Test	Pet.ether		$CHCl_3$		Methanol		Water	
	MT	SR	MT	SR	MT	SR	MT	SR
Carbohydrate	-	-	-	-	-	-	+	+
Alkaloid	-	-	-	-	+	+	-	-
Anthraquinone glycoside	-	-	-	-	-	-	-	-
Glycoside	-	-	-	-	+	+	-	-
Tannin	-	-	-	-	+	+	-	-
Flavonoid	-	-	+	+	+	+	-	-
Saponin	-	-	+	+	+	+	-	+
Amino acid and Protein	-	-	-	-	+	+	-	+
Phytosterol	+	+	-	-	+	+	-	-
Phenolic compound	-	-	-	-	+	+	-	-
Coumarins	-	-	-	-	-	-	-	-
Fixed oil	-	-	-	-	-	-	-	-
Mucilage	-	-	-	-	-	-	-	+

Definition of abbreviations: Pet.ether- Petroleum ether, $CHCl_3$- Chloroform, MT- *Marsdenia tenacissima*, SR- *Sansevieria roxburghiana*, + Present, - Absent

Methyl butyryl-12β-O-acetyltenacigenin B, flavonoid, alkaloid, tannins, carotenoids, saponins and anthocyanin's proteins (Obydulla, 2016). The standard procedure was followed to test the phytochemical constituent of these plant extracts. The phytochemical screening results are shown in Table 1.

Table 2 shows the effect of MEMT, MESR & Combination of MEMT & MESR on membrane protection on HRBC. Concentration dependent percentage inhibition of cell membrane protection was found in this assay, where a combination of MEMT and MESR (1000µg/mL) showed the maximum inhibition of 65.12±0.73%, MEMT (58.7±0.61%) and MESR (42.69±0.48%). Standard Diclofenac sodium at 200 µg/mL concentration showed 76.5±0.78 % inhibition. In heated solution, 1000µg/mL of combination of MEMT&MESR showed an increase in inhibition of 77.01±0.67% whereas only MEMT and MESR showed 65.97±1.71% and 44.89±0.98% respectively. In the case of hypotonic induced haemolysis, the combination of MEMT &MESR (1000µg/mL) showed the maximum protection 84.25±0.31%. Higher concentration of MEMT 1000 µg/mL showed percentage inhibition of 77.65±0.29% and 54.47±0.77% for 1000µg/mL MESR.

The effects of MEMT and MESR on NBT reduction are shown in Table 3. MEMT showed significant ($p<0.001$) inhibition of haemolysis compared with MESR which showed a less significant ($p<0.01$) effect.

Table 3. Effect of Murva extracts on NBT reduction test.

Extract name	Sample concentration (µg/mL)	Mean in %±SD
MEMT	50	11.6±1.4
	100	23.5±3.4
	200	42.6±3.8
	400	68.4±2.9
	800	88.4±1.3
	1000	91.5±2.2
MESR	50	1.1±2.2
	100	08.5±3.1
	200	16.4±1.3
	400	23.5±1.1
	800	46.2±4.3
	1000	52.6±2.3

Definition of abbreviations: MEMT- Methanol extract of *Marsdenia tenacissima*, MESR- Methanol extract of *Sansevieria roxburghiana.* All the assays are performed in triplicate and results are expressed in mean %±standard deviation (SD).

Table 2. *In vitro* anti-inflammatory activity effect of MEMT, MESR & Combination on membrane protection on HRBC.

Concentration and name of extracts (µg/mL)		Percentage inhibition		
		Membrane stabilization	Heated solution	Hypotonic
MEMT	100	23.49±0.37	47.18±0.67	29.39±0.72
	200	37.32±0.49	49.46±0.48	33.08±0.48
	400	41.46±0.49	52.57±0.83	40±0.38
	600	49.35±0.61	54.92±0.48	46.99±0.58
	800	56.83±0.74	58.86±0.50	67.81±0.38
	1000	58.7±0.61	65.97±1.71	77.65±0.29
MESR	100	13.98±0.37	13.78±0.59	9.90±0.39
	200	17.88±0.37	20.82±0.58	14.48±0.39
	400	23.98±0.51	29.65±1.55	20.57±0.38
	600	26.02±0.51	33.39±0.48	38.98±0.58
	800	39.51±0.49	37.52±1.44	47.11±0.58
	1000	42.69±0.48	44.89±0.98	54.47±0.77
Combination of extracts	100	39.59±0.51	60.76±0.50	58.86±0.50
	200	40.81±0.37	64.32±1.05	70.47±0.52
	400	44.47±0.74	67.36±0.58	74.29±0.50
	600	48.29±0.88	71.24±0.57	81.08±0.61
	800	53.66±1.71	72.89±1.71	77.45±0.29
	1000	65.12±0.73	77.01±0.67	84.25±0.48
Diclofenac	200	76.50±0.78	84.12±0.31	86.29±0.58

Definition of abbreviations: MEMT- Methanol extract of *Marsdenia tenacissima*, MESR- Methanol extract of *Sansevieria roxburghiana.* HRBC- Human Red Blood Cell. All the assays are performed in triplicate and results are expressed in mean±standard deviation (SD).

Table 4. Inhibition of TNF-α release by LPS-stimulated assay on DAL cells.

Extract name	Concentration of extracts (µg/mL)	Inhibition of TNF-α (%±SD)
MEMT	100	12.4±2.1
	200	59.6±1.4
	400	92.6±2.1
MESR	100	10.3±1.4
	200	48.8±1.8
	400	90.7±2.7
Control	-	12.6±2.1
Dexamethasone	10	87.5±1.3

Definition of abbreviations: TNFα- Tumour necrosis factor alpha, LPS- Lipopolysaccharide, MEMT- Methanol extract of *Marsdenia tenacissima*, MESR- Methanol extract of *Sansevieria roxburghiana*. All the assays are performed in triplicate and results are expressed in mean %±standard deviation (SD).

Effect of MEMT and MESR on inhibition of TNF-α release from DAL cells by LPS-stimulated assay results are shown in Table 4. MEMT showed significant ($p< 0.001$) inhibition of TNF-α release compared with MESR which showed less significant ($p<0.01$) effect.

DISCUSSION

There is a link between cancer and inflammation as many researchers have reported that anti-inflammatory or immunomodulatory phytoconstituents from natural sources have an anti-cancer effect. This anti-cancer effect is due to the stimulation or inhibition of particular cellular inflammatory actions and the related molecular signalling pathways (Huang, Lin, Liao, Young & Yang, 2008) Both the plants have an anti-cancer effect and are traditionally used for the treatment of cancer. MT showed an anti-cancer effect on oesophageal carcinoma (Fan et al., 2015) whereas SR showed the cytotoxic effect on EAC cell line (Haldar, Kar, Bala, Bhattacharya, & Mazumder, 2010). Inflammation is a complex biological reaction of vascular tissues to noxious stimuli.

Inflammation is connected with pain and also includes various processes like the surge of protein denaturation and vascular permeability, and membrane alteration (Raju et al., 2015). Inflammations occur due to leukocyte infiltration. The cells release lysosomal contents such as bactericidal enzymes and protease, which induce damage and inflammation. Lysosomal membrane damage triggers the release of the phospholipids into lysophospholipid and free fatty acid such as arachidonic acid which acts as the precursor for the inflammatory mediators such as prostaglandins, leukotrienes and platelet-activating factor stabilisation of lysosome membrane preventing the lysis and subsequent release of mediators (Galhena et al., 2012). The inhibition of haemolysis is considered as a marker of the anti-inflammatory activity of plant extracts. Since there is a close similarity for RBC with the lysosomal membrane, protection from RBC lysis via hypotonicity or heat might indicate the stabilization of lysosomal membranes and is used as a biochemical indication of anti-inflammatory activity. Researchers have already reported that various phytoconstituents (triterpenoids, flavonoids, and various secondary plant metabolites) can mediate analgesic and anti-inflammatory effects through its membrane-stabilizing actions (Yamada, Webber, Kirillova, Peschon, & Fausto, 1998) RBC exposed to noxious stimuli like heat, hypotonicity, or chemicals such as methyl salicylate and phenyl hydrazine that mediate the membrane damage. Membrane stabilization prevents the leakage of the inflammatory mediators (Raju et al., 2015). The results MEMT, MESR and combination of MEMT& MESR also show membrane stabilization and percentage inhibition of haemolysis in a dose-dependent manner by inhibiting both hypotonicity and heat-induced lysis of erythrocytes compared with the standard drug diclofenac sodium.

The quantification of NBT reduction proved that it was a valuable tool to measure the health condition and immunological status of a cell or organ where the haemocytes generate reactive oxygen intermediates which react with numerous biomolecules like carbohydrates, proteins nucleic acids and lipids. This interaction causes the damage of the cells and functional units which leads to a reduction of the immune response at a cellular and organismal level. NBT reduction assay is an accurate qualitative method for detecting the production of superoxide by haemocytes (Muñoz et al., 2000) The generated superoxide radicals from NBT reduction assay were scavenged by MEMT, MESR and the combination of MEMT& MESR. This significant effect is due to neutrophils present in peripheral blood, which change the yellow compound NBT into a compound with a deep blue colour (Formazone). This blue compound can be clearly detected in microscope. This reduction assay is also concentration-dependent. As a result of the host defence mechanism, upon exposure to various inflammatory stimuli such as lipopolysaccharide (LPS), macrophages initiate the release of various mediators like TNF-α and NO. In tumour progression, process inhibition of TNF expression occurs and is used to find out the possible mechanism of any anti-cancer drug. Researchers reported that a drug with the anti-inflammatory or immunomodulatory property also inhibits TNF expression in the host cell for defence purpose (Yamada et al.,1998). The present study results reveal that MEMT, MESR and a combination of MEMT& MESR possess concentration-dependent inhibition on TNF-α release.

Our results indicate that MEMT, MESR and a combination of MEMT& MESR contain various biologically active constituents which are responsible for the anti-inflammatory, immune regulatory potency and various pharmacological actions.

CONCLUSIONS

In conclusion, besides reports on various pharmacological actions of Murva, no other literature is available for the evaluation of its anti-inflammatory as well as immunomodulatory activity even though it has been used for the treatment of various diseases in Ayurveda and traditional medicine for many years. In the present research the results proved that both the

plant under the Murva category (SR and MT) and the local, safe Ayurveda drug can be used to treat cancer and cardiac disease without affecting the immune system and set the patients free from other inflammatory conditions.

Peer-review: Externally peer-reviewed.

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

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

Financial Disclosure: Authors declared no financial support.

Acknowledgement: The authors gratefully thanks to Director Dr. Sr. Betty Carla from St. Joseph's College of Pharmacy, Cherthala, Kerala for providing support and facilities for this research work.

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Istanbul J Pharm 51 (3): 341-347
DOI: 10.26650/IstanbulJPharm.2021.974035
Original Article

Antioxidant and antimicrobial activities of various extracts from *Stachys cretica* subsp. *bulgarica* Rech.f., *Stachys byzantina* K. Koch and *Stachys thirkei* K. Koch

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Cite this article as: Gulsoy Toplan, G., Taskin, T., Mataraci Kara, E., & Ecevit Genc, G. (2021). Antioxidant and antimicrobial activities of various extracts from *Stachys cretica* subsp. *bulgarica* Rech.f., *Stachys byzantina* K. Koch and *Stachys thirkei* K. Koch. *İstanbul Journal of Pharmacy, 51*(3), 341-347.

ABSTRACT

Background and Aims: *Stachys* L. species, which are members of the Lamiaceae family, have long been applied for their therapeutic benefits especially to cure cough, infection, genital tumors, ulcers, inflammatory illnesses, and wounds in Anatolian traditional medicine. In the current study, various extracts prepared from the aerial parts of *Stachys cretica* subsp. *bulgarica* Rech.f. (SC), *Stachys byzantina* K. Koch (SB), *Stachys thirkei* K. Koch and were tested for their in vitro antioxidant, antibacterial, and anticandidal properties.

Methods: The aerial parts of three *Stachys* species were sequentially extracted using n-hexane, chloroform, and methanol. Aqueous extracts of each sample was also prepared by infusion process. The total phenolic content of each extract was determined and the contribution of the biological activities in the samples was evaluated. To assess the antioxidant capacity, samples were studied using CUPRAC activity, DPPH• free radical scavenging, and FRAP methods. The antimicrobial activity of the extracts was tested against 7 bacteria and 3 yeast.

Results: The infusion and methanol extract exhibited the strongest antioxidant potential and also had the highest percentage of phenolics among the studied extracts. The *n*-hexane extracts of all studied species showed considerable antifungal activity with MIC values ranging from 312.5-78.12 mg/L.

Conclusion: According to our results, three *Stachys* species were found to be beneficial for their antioxidant and antimicrobial properties.

Keywords: *Stachys*, antioxidant activity, total phenolic, antimicrobial activity

INTRODUCTION

Plants have been evaluated for different purposes throughout humankind's history such as food, hunting, prevention and treatment of diseases, and preparation for spiritual ceremonies. Several studies have confirmed the beneficial properties of plants in

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Submitted: 24.07.2021
Revision Requested: 22.09.2021
Last Revision Received: 31.10.2021
Accepted: 01.11.2021
Published Online: 00.00.0000

the pharmaceutical, food, and cosmetic industries and that has led to an increase in their economic value (Silva, & Fernandes Júnior, 2010). In particular, Lamiaceae species offer a broad range of activities due to their rich chemical composition (Frezza, Venditti, Serafini, & Bianco, 2019).

Stachys L. is classified as one of the most extensive genera of the Lamiaceae family, containing over 300 species with almost worldwide distribution (BilušićVundać, 2019; Tundis, Peruzzi, & Menichini, 2014). The flora inTurkey is represented by approximately 90 species including 115 taxa, 54 of which are endemic (Akcicek, Dirmenci, & Dündar, 2012). The species of *Stachys* are annual, perennial herbs or tiny shrubs with simple leaf segments that attach directly to the stem or are sessile. They are commonly known as 'Dağ Çayı' in Anatolian folk medicine and utilized as an appetizer, healer for digestive complaints, stimulant, antispasmodic, and also as carminative (Baytop, 1999; Goren, 2014). Ethnopharmacological usage of *Stachys* species is supported by several studies in the worldwide literature, mainly demonstrated by antibacterial, anti-*Helicobacter pylori*, anti-inflammatory, anticancer, and antioxidant properties (Salehi, Sonboli, &Asghari, 2007; Khanavi et al., 2009; Goren et al., 2011; Tomou, Barda, & Skaltsa, 2020; Tundis et al., 2014). The promising findings shown in research conducted on several antioxidant test systems also indicate the great potential for preventing diseases that correlate with the deficiency of antioxidant mechanisms (Erdemoglu, Turan, Cakıcı, Sener, & Aydın, 2006; Kukić, Petrović, & Niketić, 2006; Hajdari, Novak, Mustafa, & Franz, 2012; Tundis, et al., 2014). The diversity in its pharmacological properties can be explained via its containing multiple classes of secondary metabolites in combination (Pieters & Vlitenick, 2005). An extensive range of investigations have been conducted on the phytochemistry of *Stachys* species that have revealed the presence of iridoids, di- and triterpenes, alkaloids, phenylethanoid glycosides, flavonoids, phenolic acids, and essential oil (Kaya, Demirci, & Baser, 2001; Asnaashari et al., 2010; Demirtas, Gecibesler, & Yaglioglu, 2013; Tomou et al., 2020).

Free radicals have become of concern since they appear to play a role in a wide range of diseases and food deterioration (Fang, Yang, & Wu, 2002). Multiple studies have indicated that the production of reactive oxygen species (ROS) and the resulting oxidative stress are important in the initiation and progression of many major disorders, including cancer and degenerative diseases (Valko et al., 2007). According to the literature, phenolic compounds have significant effects on the protection of degenerative disorders due to slowing down free radical reactions and reducing lipid oxidation (Toplan et al., 2017). Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are commonly employed in the foodservice industry as synthetic antioxidants to prevent oxidative degradation in prepared foods (Branen, 1975). Because of the potentially harmful consequences of these agents, there has been a tremendous rise in international importance and utilization of plants for their antioxidant, anti-aging, and antimicrobial benefits, and besides, they have fewer adverse effects (Li et al., 2012). Therefore, this encourages researchers to investigate plants, especially those rich in phenolics, for their natural antioxidants which keep the human body healthy and preserve food from rotting.

Today, antibiotic resistance is a significant public health problem. There is substantial evidence of resistance to nearly all of the antimicrobial drugs now in use, and this evidence is growing (Kwapong, Soares, Teo, Stapleton, & Gibbons, 2020). In recent decades, the amount of new antibacterial medications has decreased and the design and discovery of new antibacterial chemicals have become one of the key fields of antibacterial research (Coqueiro, Regasini, Stapleton, da Silva Bolzani, & Gibbons, 2014). Another key issue in the food, beverage, cosmetic, and pharmaceutical industries is microbial contamination (Tiwari et al., 2009). Researchers worldwide are keen to discover new natural antimicrobial compounds in response to growing cases of microbe resistance to currently used preservatives. Numerous investigations have approved the promising antibacterial and antifungal characteristics of plant extracts as well as secondary metabolites (Pieters & Vlietinck, 2005). Nowadays, a large percentage of prescribed medicines consist of plant-based products (Atanasov, Zotchev, Dirsch, & Supuran, 2021).

Three *Stachys species,* namely *Stachys cretica* subsp. *bulgarica, S. byzantina* and *S. thirkei* whose traditional names are 'Kızıl Deliçay', 'Bozkarabaş', and 'Kestere' respectively, are three of the most used species in Turkish traditional medicine; their infusions or decoctions are consumed as a tea by local people for mainly gastrointestinal problems (Satıl & Acar, 2020). In the current study, the antioxidant and antimicrobial activities of different extracts obtained from the aerial parts of these three *Stachys* species were screened for their total phenolic content.

MATERIALS AND METHODS

Plant material

The aerial parts of *Stachys cretica* subsp. *bulgarica* (SC)*, S. byzantina* and *S. thirkei* were collected from Saray-Güngörmez, Tekirdağ, and Kastamonu during the flowering stage. After that, the collected plants were dried at room temperature in a dark storeroom. Dried plant material was ground usinga laboratory mill before the experiments. Specimens were identified by one of us (Gulay, Ecevit-Genç) and vouchers were deposited in in Herbarium of İstanbul University Faculty of Pharmacy (ISTE). The herbarium number of each plant are given in Table 1.

Table 1. The location of the collected plants with herbarium number.

Species	Location	Date	Herbarium Number	Sample Code
Stachys cretica subsp. *bulgarica* (SC)	Tekirdağ	15.07.2015	ISTE 117262	SC
Stachys byzantina (SB)	Ilgaz Yolu- Kastamonu	02.08.2015	ISTE 117263	SB
Stachys thirkei (ST)	Saray-Güngörmez	15.07.2015	ISTE 117264	ST

Extraction of samples

The aerial parts of each plant were crushed and extracted sequentially with *n*-hexane, chloroform, and methanol using a Soxhlet apparatus. A rotary evaporator was used to evaporate the solvents under reduced pressure, with a maximum temperature of 50°C. Following solvent evaporation, the crude extracts were kept at +4 °C until analysis and used in all studies.

The aqueous extracts of aerial parts of each plant were prepared by infusing 10.0 g of dried material in 200 mL of distilled water for 15 min at 80 °C. The infusion was filtered, and the filtrates were frozen and stored at -80°C in an ultra-low degree freezer. After that, the solution was lyophilized, and the freeze-dried product was stored at -20°C until the screening.

Total phenolic content of the samples

4.5 mL of distilled water was added to 0.1 mL of the extracts produced at 0.5-5 mg/mL concentrations. The absorbance of a blue color after 2 hours at room temperature was measured at 760 nm against reference standards using 0.1 mL of Folin-Ciocalteu reagent (diluted 1/3 with distilled water) and 0.3 mL of 2% carbonate solution. The total phenolic content was measured as milligram gallic acid equivalents per milligram of extract (Taskın, Taskın, & Rayaman, 2018).

Antioxidant capacity of the samples

The cupric reducing antioxidant capacity (CUPRAC)

CUPRAC technique was used to assess the antioxidant capacity of the samples. 60 µL Cu (II) (1.10-2 M), neocuproine ethanolic solution (7.3.10-3 M), and 1 M ammonium acetate buffer solution were added to a microplate well. The samples were diluted with solvent mixture of methanol:DMSO (2:1 v/v). 60 µL of the diluted extracts and 10 µL pure ethanol were added to the original mixture. The absorbance of the solution was measured at 450 nm against a reagent blank after ten seconds of vortexing. The Trolox equivalents (mM Trolox/mg extract) were used to calculate the CUPRAC values of the samples. (Apak, Güçlü, Özyürek, & Karademir, 2004).

DPPH radical scavenging activity

The ability of free radical scavenging of the four different extracts was tested using the DPPH technique. To summarize, 240 mL of DPPH solution (0.1 mM) was combined with 10 mL of extracts (5 mg/mL-0.5 mg/mL) at varied concentrations. The combination was then held at room temperature for another 30 minutes before being used. Using a microplate reader set at 517 nm, the absorbance of the mixture was measured in comparison to a standard (Wei et al., 2010).

Reducing power capacity (FRAP technique)

The FRAP technique was used to test the capacity of the samples (5 mg/mL-0.5 mg/mL concentrations) to reduce ferric ion. In brief, FRAP reagent (3.8 mL) was combined with samples (0.2 mL) and the absorbance of the combination was measured at 593 nm 4 minutes later. The standard curve was made using $FeSO_4.7 \times H_2O$, and the FRAP values of the samples were expressed in mM Fe^{2+}/mg extract (Benzie & Strain, 1996).

Antimicrobial activities of the samples

Determination of minimum inhibitory concentrations (MICs)

Staphylococcus aureus ATCC 29213, *S. epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Proteus mirabilis* ATCC 14153, *Candida albicans* ATCC 10231, *C. parapsilosis* ATCC 22019, and *C. tropicalis* ATCC 750 were used in this study for *in vitro* antimicrobial activities of various extracts obtained from three different *Stachys* species. The antimicrobial activity assay of the samples against these strains was determined using the broth microdilution technique as described by the Clinical and Laboratory Standards (CLSI, 1997; CLSI, 2020). The MIC was defined as the lowest concentration of antibiotics giving complete inhibition of visible growth. The following standard antibacterial and antifungal agents were used as standard compounds: Cefuroxime-sodium, cefuroxime, ceftazidime, amikacin, amphotericin B, and clotrimazole. RPMI-1640 medium for the yeast strain and Mueller-Hinton broth for bacteria were used as negative controls.

Statistical analysis

Results were expressed as the mean ± standard deviation (SD) of three independent and parallel measurements. One-way analysis of variance (ANOVA) was performed, and significant differences between means were determined using Tukey's multiple comparisons test. Statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION

The yield and total phenolic contents of the samples

Aerial parts of *Stachys cretica* subsp. *bulgarica, S. byzantina* and *S. thirkei* were extracted successively using several solvents to obtain crude extracts and the results of fractions yielded and also their total phenolic content are demonstrated in Table 2.

Total phenolic compounds contained in plants were determined by the FCR method. Compared with other extracts, infusion and methanol extracts prepared from plants were found to have higher quantities of phenolic compounds. The total amount of phenolics contained in the methanol extracts prepared from three plants is as follows: SC methanol (0.101 mg GAE/mg extract)> SB methanol (0.090 mg GAE/mg extract)>ST methanol (0.058 mg GAE/g extract)

In this investigation, it was discovered that methanol and infusion extracts prepared from aerial parts of the plants had significant amounts of phenolic compounds and, as a result, showed substantial biological activity, which is in line with the literature.

Antioxidant capacity of the samples

The antioxidant capacity of the samples was measured by CUPRAC activity, DPPH• free radical scavenging, and FRAP methods. The results of the three different methods aredemonstrated in Table 3.

Table 2. The yield and total phenolic content of the extracts (beginning with 10 g of material).

Samples	Extracts	Yield	Total phenolics
Stachys cretica subsp. *bulgarica* (SC)	*n*-hexane	0.1177	0.00237±0.00180
	chloroform	0.194	0.0496±0.0014
	methanol	1.6308	**0.1010±0.0117**
	infusion	0.224	**0.0684±0.0058**
Stachys byzantina (SB)	*n*-hexane	0.1157	0.0173±0.0109
	chloroform	0.1903	0.0386±0.0062
	methanol	2.3717	**0.0901±0.098**
	infusion	0.3784	**0.0630±0.0057**
Stachys thirkei (ST)	*n*-hexane	0.1462	0.0032±0.0056
	chloroform	0.1278	0.0134±0.0030
	methanol	1.236	**0.0578±0.0104**
	infusion	0.197	**0.0523±0.0027**

Table 3. Antioxidant activity of the various extracts from three *Stachys* species.

Samples	DPPH (mg AaE/mg extract)	FRAP assay (mM Fe^{2+} /mg extract	CUPRAC (mM trolox/mg extract)
SC-H	0.00496±0.004288	0.122 ± 0.01^{9}*	0.055 ± 0.00^{3}*
SC-C	0.013456±0.000626	0.337 ± 0.02^{7}*	0.097 ± 0.00^{2}*
SC-M	0.062819±0.000371	0.454 ± 0.00^{7}*	0.102 ± 0.00^{3}*
SC-I	0.049691±0.01142	0.459 ± 0.00^{2}*	0.097 ± 0.00^{1}*
SB-H	0.00231± 0.001782	0.197 ± 0.03^{0}*	0.052 ± 0.00^{4}*
SB-C	0.004962±0.001384	0.339 ± 0.01^{2}*	0.072 ± 0.00^{5}*
SB-M	0.0627±0.000412	0.476 ± 0.00^{2}*	0.098 ± 0.00^{1}*
SB-I	0.055988±0.000713	0.434 ± 0.00^{5}*	0.095 ± 0.00^{4}*
ST-H	0.00258±0.000879	0.038 ± 0.00^{6}*	0.051 ± 0.00^{2}*
ST-C	0.00609±0.000713	0.331 ± 0.03^{5}*	0.079 ± 0.00^{2}*
ST-M	0.039118±0.005585	0.363 ± 0.03^{3}*	0.089 ± 0.00^{6}*
ST-I	0.05076±0.001029	0.451 ± 0.00^{5}*	0.092 ± 0.00^{4}*
BHT		1.1±0.12	
BHA			1.622±0.12

Values are mean of triplicate determination (n=3) ±standard deviation; *p<0.05 compared with the positive control
H: *n*-hexane extracts, C: Chloroform extracts; M: Methanol extracts; I: Infusion; BHA: butylated hydroxyanisole; BHT: butylated hydroxytoluene; AaE: ascorbic acid equivalent

The cupric reducing antioxidant capacity of infusion and methanol extracts of three plants were found to be quite similar in this investigation. The methanol extracts of the SB (0.098 mM trolox/mg extract) and SC (0.102 mM trolox/mg extract) plants, as well as the infusion of ST (0.092 mM trolox/mg extract) plant, were found to have the greatest CUPRAC value.

The methanol extract produced from SC (0.102 mM trolox/mg extract) had a higher cupric reducing antioxidant capacity than the other extracts, according to the cupric reducing antioxidant capacity of the plants. When the cuprac values of all extracts were compared to the standard substance (1.622 mM trolox/mg extract), it was discovered that they had a reduced activity potential.

As a result of DPPH radical scavenging activities of the methanol extracts from SB (**0.063** mg AaE/mg extract) and SC (**0.062** mg AaE/mg extract) plants, as well as the infusion extract from the ST (0.051 mg AaE/mg extract) plant, were shown to have a greater radical scavenging capability than the other extracts in the present investigation. When the plants utilized in this study were examined, it was discovered that the radical scavenging

activity of the SB and SC was quite similar. In comparison to the two other species, ST exhibited to have low radical scavenging activity.

A general association between reductive capacity and the presence of antioxidant agents is that they break free radical chains by donating hydrogen atoms. Reducing power assays are commonly used to determine whether an antioxidant is capable of transforming a Fe^{+3} to Fe^{+2} (El Atki, et al., 2020). Hence, the FRAP assays were employed to determine the ferric reducing power of the samples. The FRAP values of infusion and methanol extracts produced from SB and SC were found to be extremely near to each other in the FRAP experiment. Infusion of SC (0.459 mM Fe^{2+} /mg extract) and ST (0.451 mM Fe^{2+} /mg extract), as well as methanol extract of the SB (0.476 mM Fe^{2+} /mg extract), showed to have greater iron (III) ion reducing power activity than other extracts. When the plants' iron-reducing activities were evaluated, the methanol extract of SB was found to have the greatest FRAP value. All of the extracts obtained from the plants showed to have a lower iron reduction capacity than the positive control, BHT compound (1.1 mM Fe^{2+} /mg extract).

Numerous investigations confirmed not only the antioxidant potential of many *Stachys* species but also the rich diversity of their chemical composition many of which reduce free radical damage (Jassbi, Miri, Asadollahi, Javanmardi, & Firuzi, 2014; Sarikurkcu, Kocak, Uren, Calapoglu, & Tepe, 2016; Bahadori, Zengin, Dinparast, & Eskandani, 2020). This is the first study to identify and compare the antioxidant capacity of different solvent extracts of these three species. It is well-known that the antioxidant capacities of extracts are influenced by many factors including extraction procedure, polarities of used solvents, and also polymorphic properties and diversity of the species (BilušićVundać, 2019; Tomou, et al., 2020). Thus, different types of complementary methods are recommended to evaluate the antioxidant potential of the extracts. Generally, the highest antioxidant activity is observed in polar extracts of plants such as methanol, infusion, and decoction due to containing high amounts of phenolic compounds (Ertas, &Yener, 2020). As a result of the evaluation of the various solvents' extract from three *Stachys* species in several assays, infusion and methanol extracts showed the highest antioxidant capacity with the highest total phenolic content compared with the other extracts.

Antimicrobial activity of the samples

To provide comparable data for samples, the samples obtained from three *Stachys* species and prepared with various types of solvents were tested against a panel of three Gram-positive bacteria and four Gram-negative bacteria by using the broth micro dilutions technique according to the Clinical Laboratory Standards Institute (CLSI) recommendations (CLSI, 1997; CLSI, 2020). Well-known commercial antibiotics were used as the standard drugs and the minimal inhibitory concentrations (MIC) values compared with the standard drugs are presented in Table 4. The extracts exhibited moderate to mild antimicrobial activities compared with the standards. Nevertheless, infusion of all species was not shown to have any antimicrobial activity. Depending on the antibacterial results of all compounds, it was observed that all of the tested *Stachys'* samples displayed no inhibitory activity against any Gram-negative bacteria except methanol extract of SC for *E. coli*. *In addition to this, n-hexane extracts of SCB also showed antimicrobial activity against tested Gram-positive bacteria with the MIC values ranging from 312.5 to 625 mg/L.*

Antifungal activity of each extract was tested against three pathogenic yeasts, namely *C. albicans*, C. *parapsilosis*, and *C. tropicalis*. Among all the extracts studied for antifungal potency, *n*-hexane extracts of each species showed strong activity against *C. parapsilosis* and *C. tropicalis*. The most resistant yeast against the samples was determined as *C. albicans*. The *n*-hexane extracts of SC and ST exhibited strong anticandidal activity

Table 4. The antimicrobial and antifungal activity of the various extracts from three *Stachys* species (MIC, µg/mL).

Strains	SC-H	SC-C	SC-M	SB-H	SB-C	SB-M	ST-H	ST-C	ST-M
P. aeruginosa ATCC 27853	>2500	>2500	>2500	>2500	>2500	>2500	>2500	>2500	>2500
E. coli ATCC 25922	>2500	>2500	**312.5**	>2500	>2500	>2500	>2500	>2500	>2500
K. pneumoniae ATCC 4352	>2500	>2500	>2500	>2500	>2500	>2500	>2500	>2500	>2500
P. mirabilis ATCC 14153	>2500	>2500	>2500	>2500	>2500	>2500	>2500	>2500	>2500
E. faecalis ATCC 29212	**312.5**	>2500	>2500	>2500	**156.2**	>2500	>2500	>2500	>2500
S. epidermidis ATCC 12228	625	>2500	>2500	>2500	>2500	1250	>2500	625	625
S. aureus ATCC 29213	>2500	>2500	>2500	>2500	>2500	>2500	>2500	>2500	>2500
C. albicans ATCC 10231	>2500	>2500	>2500	>2500	>2500	>2500	>2500	>2500	>2500
C. parapsilosis ATCC 22019	**78.12**	>2500	>2500	**156.2**	>2500	>2500	**78.12**	>2500	>2500
C. tropicalis ATCC 750	**156.2**	>2500	>2500	**312.5**	>2500	>2500	>2500	>2500	>2500

H: *n*-hexane extracts, C: Chloroform extracts; M: Methanol extracts; I: Infusion
Reference compounds: Ceftazidime: 2.4 mg/L For *P. aeruginosa,* Cefuroxime-Na: 4.9 mg/L for *E. coli* and *K. pneumoniae,* Cefuroxime-Na: 2.4 mg/L for *P. Mirabilis,* Cefuroxime-Na: 1.2 mg/L for *S. aureus*, Cefuroxime: 9.8 mg/L for *S. epidermidis*, Amikacin: 128 mg/L for *E. faecalis*, Clotrimazole: 4.9 mg/L *C. albicans*, Amphotericin B: 0.5 mg/L for *C. parapsilosis*, Amphotericin B: 1 mg/L for *C. tropicalis.*

against *C. parapsilosis* with 78.12 mg/L MIC values whereas the *n*-hexane extracts of SB and SC exhibited moderate anticandidal activity against *C. tropicalis* with MIC values ranging from 312.5-156.2 mg/L. As to the antibacterial activity of chloroform extracts, *E. faecalis* and *S. epidermis* were found to be susceptible to the SB and ST samples.

According to the literature, the essential oil and extracts of *Stachys* species possess remarkable antimicrobial activity related to the polarities of extracts, composition of samples, and species' growing conditions (İşcan et al., 2012; Leblebici, Kaygusuz, Korkmaz, & Darcan, 2016). Previously, the antimicrobial activity of essential oil from several *Stachys* species were investigated using the disc diffusion method and among them, considerable antifungal activity from the essential oil of *Stachys cretica* subsp. *Bulgarica* was observed against *C. albicans* (Goren et al., 2011). In another study, the *n*-butanolic and light petroleum extracts of *Stachys cretica* subsp. *lesbiaca* and *S. cretica* subsp. *trapezuntica* showed antifungal activity whereas no antibacterial activity was observed in either ethanol, dichloromethane, or ethyl acetate extracts (Şerbetçi et al., 2010). The methanol extracts of *S. byzantina*, *S. inflata*, *S. lavandulifolia,* and *S. taxa* were tested against Gram-positive bacteria and yeast, in contrast, in the other studies no antifungal activities were determined in the extracts whereas remarkable concentration-dependent antibacterial activity was determined in both disc diffusion and MIC methods (Saeedi, Morteza-Semnani, Mahdavi, & Rahimi, 2008).

CONCLUSION

Interest in ethnopharmacology has recently increased due to the recent development of phytochemistry methodologies (Bremner & Heinrich, 2002). Aromatic plants are of great potential for both antioxidant and antimicrobial activities owing to the rich synergy of different types of secondary metabolites (Tomou et al., 2020). In the present study, the antimicrobial and antioxidant activities of four type of extracts obtained from *Stachys cretica* subsp. *bulgarica*, *S. byzantina* and *S. thirkei* were carried out and their total phenolic contents were determined.

In several investigations was noticed that many progressive illnesses are related to the imbalance of oxidative stress in the body. Thus, finding alternative extracts or compounds in the plant kingdom becomes one of the most important targets in the scientific area. The present study results indicated that the polar extracts of all investigated *Stachys* species showed considerable antioxidant effects and also had high amounts of phenolic contents. Furthermore, the increased bacterial resistance is one of the significant global health concerns all around the world. As to the MIC values, the extracts of *Stachys cretica* subsp. *bulgarica*, and *S. byzantina* exhibited strong to moderate antimicrobial activity while the extracts of *Stachys thirkei* exhibited moderate to poor antimicrobial activity. Consequently, these findings highlight the value of these three *Stachys* species. But still more research into the chemical compositions, molecular mechanisms, and adverse impacts of extracts is needed to determine their potential.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- G.G.T., G.E.G.; Data Acquisition- G.G.T., T.T., E.M.K.; Data Analysis/Interpretation- G.G.T., T.T., E.M.K.; Drafting Manuscript- G.G.T.; Critical Revision of Manuscript- G.G.T., T.T., E.M.K., G.E.G.; Final Approval and Accountability- G.G.T., T.T., E.M.K., G.E.G.

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

Financial Disclosure: Authors declared no financial support.

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Istanbul J Pharm 51 (3): 348-356
DOI: 10.26650/IstanbulJPharm.2021.821070
Original Article

Investigation of the antimicrobial activities of solvent extracts of two endemic species from Turkey: *Campanula tomentosa* Lam. and *Verbascum mykales* Bornm.

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Cite this article as: Poyrazoglu Coban, E., & Barisik, E. (2021). Investigation of the antimicrobial activities of solvent extracts of two endemic species from Turkey: *Campanula tomentosa* Lam. and *Verbascum mykales* Bornm.. *İstanbul Journal of Pharmacy, 51*(3), 348-356.

ABSTRACT

Background and Aims: *Campanula tomentosa* Lam. and *Verbascum mykales* Bornm. are endemic species in Turkey. Extracts of these plants contain important natural compounds such as flavonoids, saponins and tannins. This study investigates the antimicrobial effects of leaf extracts of *C. tomentosa* and *V. mykales* against some bacteria and yeasts.

Materials and Methods: Leaves of plant samples were air-dried and ground into powder. Five solvents (ethyl acetate, methanol, acetone, chloroform, boiled water) were used for extraction. Experiments were conducted using these crude extracts on seventeen bacteria, three yeasts and three microfungi. The agar well diffusion method was used for the antimicrobial activities of the extracts. In addition, minimum inhibitory concentrations, minimum bacteriocidal concentration, minimal fungicidal concentrations were carried out.

Results: The ethyl acetate and methanol extracts of *C. tomentosa* and *V. mykales* were found to be highly effective against the tested microorganisms. According to the MIC values, the ethyl acetate extracts of *C. tomentosa* and *V. mykales* had a strong effect (4-8 µg/mL) against *Escherichia coli* ATCC 35218, *Micrococcus luteus* ATCC 9341, *Streptococcus pneumonia* ATCC 27336, *Pseudomonas aeruginosa* ATCC 35032, *Mycobacterium smegmatis* ATCC 607, *Proteus vulgaris* ATCC 33420, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 11778, and *Bacillus subtilis* ATCC 6633. The ethyl acetate extract of *C. tomentosa* had a moderate effect (64 µg/mL) against *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* ATCC 9763, and the ethyl acetate extract of *V. mykales* had a moderate effect (64 µg/mL) against *Aspergillus flavus* ATCC 9807 and *Aspergillus niger* ATCC 16404. However, the boiled water extract of *C. tomentosa* and *V. mykales* had no effect on the tested microorganisms.

Conclusion: *C. tomentosa* and *V. mykales* used in the study are endemic plants and their antimicrobial activities are being investigated for the first time. The ethyl acetate extract of both plants was found to be most effective against the Gram (+) and Gram (-) bacteria. However, all extracts of both plants were found to have fewer antimicrobial effects against used yeasts and microfungi. This study demonstrates that plant extracts are more effective against prokaryotic microorganisms than eukaryotes.

Keywords: *Campanula tomentosa* and *Verbascum mykales*, antimicrobial activity, agar well diffusion, MIC/MBC/MFC

INTRODUCTION

Humans have used plants as food, spices, textiles, perfumes, and medicines for centuries. The World Health Organization (WHO) has reported that approximately 20000 plants are used as medicine. The number of plants used as medicine is estimated to be

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Submitted: 04.11.2020
Revision Requested: 26.04.2021
Last Revision Received: 23.08.2021
Accepted: 16.09.2021
Published Online: 00.00.0000

around 500 in Turkey (Baytop, 1999; Faydaoğlu & Sürücüoğlu, 2011; Temel, Tınmaz, Öztürk & Gündüz, 2018; Yaldız & Çamlıca, 2018). Wild plants have been used for treatment since ancient times by people living in Anatolia as in other societies (Faydaoğlu & Sürücüoğlu, 2013). The method of treatment with herbs was applied for the first time in the civilizations of Sumer, Akkad and Assyria, which were established in Mesopotamia (Dar, Shahnawaz, & Qazi, 2016). Wild plants are used as alternative treatments all over the world today. Therefore, as an alternative to synthetic drugs, interest in the use of herbal medicines has increased in the developing world. Herbs are widely used as an alternative treatment in Europe, North America and some developed countries (Keskin, 2018).

The genus Campanula belongs to the Campanulaceae family and involves 300 species (Figure 1). Many Campanula species grow in Asia, the Black Sea and the Mediterranean region. There are many endemic Campanula species in Turkey. (Ozhatay, Kultur, & Aslan, 2009). Studies on the ethnobotanical use and biological activity of various *Campanula* species have been conducted by many researchers in Turkey (Buruk, Sokmen, Aydin, & Erturk, 2006; Benli, Bingöl, Geven, Güney, & Yiğit; 2008; Tosun, Kahrıman, Çoşkunçelebi, Genç, Alpay Karaoglu, & Yaylı, 2011; Sinek, Yılmaz İskender, Yaylı, Alpay Karaoglu, & Yaylı, 2012; Usta, Birinci Yildirim, & Ucar Turker, 2014). A large number of substances from the root, stem and leaf structures of plants that can inhibit the growth of microorganisms were analyzed. The flavonoids and anthocyanins such as cyanidinand delphinidin were isolated from the *Campanula* genus. *Campanula* species have been used in folk medicine for therapy of tonsillitis, laryngitis, and bronchitis. Furthermore, they have antioxidant, antiviral, and antiallergic properties (Alhage, Elbitar, Taha, & Benvegnu, 2018; Herkul & Köroğlu, 2019).

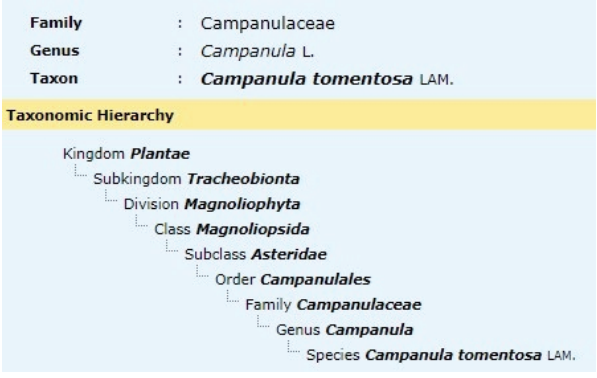

Figure 1. General taxon information of *Campanula tomentosa* Lam. (Turkish Plants Data Service) (www.tubives.com).

Verbascum belongs tothe Scrophulariaceae family and comprisesof 323 species in the world (Figure 2). The genus includes 245 species in Turkey and the endemism ratio of this genus is very high (79%) (Dulger & Dulger, 2018). The *Verbascum* species has been used as folk medicine since ancient times all over the world. Particularly, flowers and leaves of plants have been used to treat respiration disorders in phytotherapy. Ingredients of the plant, such as flavonoids, glycosides, phenylethanoids, iridoids, saponins, monoterpene and neolignans, have expectorant, diuretic and relaxing properties (Kahraman, Tatlı, Kart, Ekizoğlu, & Akdemir, 2018). Flowers containing plant phenyl porpanoids especially have anti-inflammatory effects (Karalija, Parić, Dahija, Bešta-Gajević, & Zeljković, 2018).

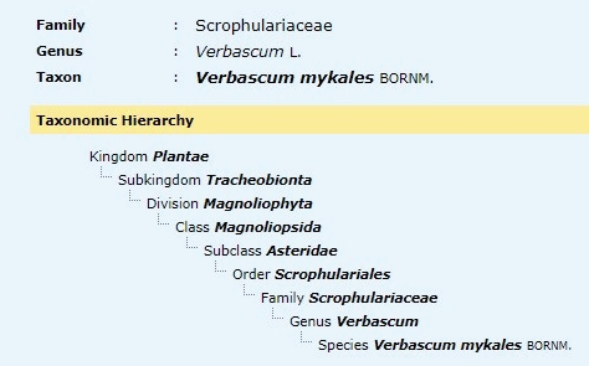

Figure 2. General taxon information of *Verbascum mykales* Bornm. (Turkish Plants Data Service) (www.tubives.com).

The antimicrobial effect of *Campanula lyrata* Lam. *subsp. lyrata* (leaf and flower) methanol extract was analyzed against *E. gallinarum* CDC-NJ-4, *E. faecalis* ATCC 29212, *B. subtilis* RSHI, *E. coli* RSHI, *Shigella* sp., *E. coli* ATCC 25922, *S. pyogenes* ATCC 19615, *S. aureus* ATCC 29213, *L. monocytogenes* ATCC 7644, *P. aeruginosa* ATCC27853, *S. cerevisiae* (Pakmaya), *C. albicans* 845981, *C. crusei* ATCC 6258 and *C. albicans* 900628. It was revealed that *C. lyrata subsp. lyrata* extract was effective against *B. subtilis* and *S. aureus*. The minimum inhibitory concentration of *C. lyrata subsp. lyrata* extract was determinated as 29 mg/mL for *B. subtilis* and 14.5 mg/mL for *S. aureus* (Benli, Bingol, Geven, Guney & Yigit, 2008). Antimicrobial activities of the dichloromethane, ethanol: water (70:30 v/v), water, and methanol extracts of *Verbascum macrurum* Ten. leaves were examined and it was demonstrated that the ethanol: water extract was the most effective (Guarino, 2002). The ethanolic extract of *V. qulebrium* Boiss. was tested against *S. aureus*, *S. typhi*, *S. pastorianus*, *E. coli*, *B. subtilis* and *P. aeruginosa* and the best inhibition effect was obtained against the Gram (+) bacteria *B. subtilis* and the yeast *S. pastorianus* (Khafagi, 2001). Antimicrobial effect of the extracts of *V. olympicum* Boiss., *V. prusianum* Boiss. and *V. bombyciferum* Boiss. were evaluated against Gram (+) and Gram (-) bacteria, and yeasts. It was shown that Verbascum species had antimicrobial activity against the Gram (+) bacteria and the yeast, but no activity was seen against the Gram (-) bacteria (Dulger, Kirmizi, Arslan & Guleryuz, 2002).

In this study, antimicrobial activities for the solvent extracts of *C. tomentosa* and *V. mykales*, two endemic plant species from Aydın-Turkey, were examined against some Gram (+) and Gram (-) bacteria, yeasts and microfungi.

MATERIALS AND METHODS

Plant materials

The sample of leaves of *C. tomentosa* was collected from Aydın, Doğanbey village (Turkey) in 2018 (Figure 3a, b) and the leaf sample of *V. mykales* was collected from Aydın, Söke/Samsun Mountain (Turkey) in 2017 (Figure 4a, b). The plants were authenticated by Dr. Özkan EREN. Leaf samples from these plants were collected in an amountsuitable to be used in the study

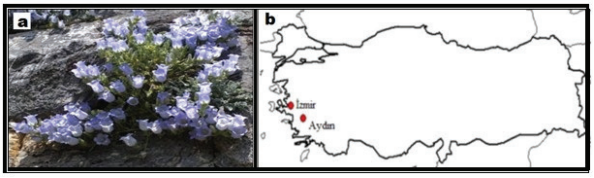

Figure 3. a. *Campanula tomentosa* Lam. (Eren ve Şentürk, 2018) **b.**The geographical distribution of *Campanula tomentosa* Lam. endemic species in Turkey (www.tubives.com).

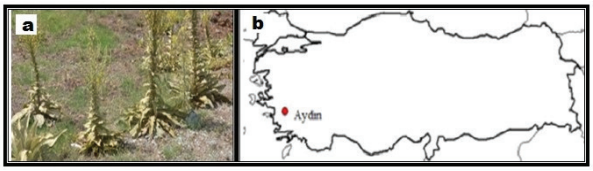

Figure 4. a. *Verbascum mykales* Bornm.(www.turkiyebitkileri.com) **b.**The geographical distribution of *Verbascum mykales* Bornm endemic species in Turkey (www.tubives.com).

without damaging the plant by Dr. Özkan EREN. Both plants are under protection in Turkey. The herbarium numbers of *V. mykales* and *C. tomentosa* are AYDN 2603 and AYDN 2604, respectively.

Preparation of plant extracts

Leaf of the plant samples were washed with distilled water and air-dried. Dried leaf was powdered and 15 grams of the materials were extracted separately in 150 mL of ethyl acetate, methanol, chloroform, acetone and boiled water in a Soxhlet apparatus for 6 h (Göse & Hacıoğlu Doğru, 2021). Then, the extract was filtered and concentrated by rotary evaporator. The dry powder extracts (0.5-1.0g) were kept at +4°C and the extracts were dissolved in 5% DMSO just before the activity studies were started (Törün, Çoban, Bıyık, & Barışık, 2017; Çoban, Bıyık, Törün, &Yaman, 2017).

Microorganisms and condition for cultivation

Seventeen bacteria, three yeasts and three microfungi were used to test the antimicrobial effect. The Gram (-) strains were *Escherichia coli* ATCC 35218, *Enterobacter aerogenes* ATCC 13048, *Salmonella typhimurium* ATCC 14028, *Klebsiella pneumoniae* ATCC 13882, *Pseudomonas aeruginosa* ATCC 35032, *Serratia marcescens* ATCC 13880, and *Proteus vulgaris* ATCC 33420. The Gram (+) strains were *Micrococcus luteus* ATCC 9341, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus pneumoniae* ATCC 27336, *Corynebacterium xerosis* ATCC 373, *Mycobacterium smegmatis* ATCC 607, *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 19112, *Bacillus cereus* ATCC 11778, and *Bacillus subtilis* ATCC 6633. The yeast strains were *Candida albicans* ATCC 10231, *Candida utilis* ATCC 9950, *Saccharomyces cerevisiae* ATCC 9763, *Aspergillus flavus* ATCC 9807, *Aspergillus niger* ATCC 16404 and *Aspergillus oryzae* ATCC 10124. The strains were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The bacterial strains were cultered in Tryptic Soy Agar (TSA) and Brain Heart Infusion Agar (BHIA) at 30-37°C for 24 h. The yeast strains were cultured in Malt Extract Agar (MEA) at 30°C for 24 h (Çoban, Erçin, Törün, & Bıyık, 2018; Bıyık, Onur, Törün, & Çoban, 2018). The microfungi strains were cultured in Potato Dextrose Agar (PDA) at 25-27°C for 5-7 days (Okoye, Uba, Dike, & Eziefule, 2020).

Antimicrobial assays

The antimicrobial activities of the two plants were determined by the agar well diffusion method (Collins, Lyne, Grange, & Falkinham, 2004; CLSI, 2004; CLSI, 2015; Balouiri, Sadiki, & Ibnsouda, 2016; EUCAST, 2019). The minimum inhibitory concentrations (MIC) were obtained by the broth dilution method (Jorgensen & Ferraro, 2009; CLSI, 2009; CLSI, 2013). The minimum bacteriocidal concentrations (MBC) and the minimum fungicidal concentrations (MFC) were tested (Zamri, Bakar, Noor, & Fuad, 2020; Stojkovi´c, Dias, Drakuli´c, Barros, Stevanovi´c, Ferreira, & Sokovi´c, 2020).

Disc diffusion method

Screening for antimicrobial activities were carried out by the agar well diffusion method against test microorganisms (Collins, Lyne, Grange, & Falkinham, 2004; CLSI, 2004; CLSI, 2015; EUCAST, 2019). The inoculum suspensions of the tested bacteria and yeasts were prepared from the broth cultures (18-24 h) and the turbidity adjusted using a 0.5 McFarland standard tube to give an equivalent concentration of 1×10^8 bacterial cells/mL, and 1×10^6 yeast cells/mL (Çoban, Erçin, Törün, & Bıyık, 2018; Oyeka, Asegbeloyin, Babahan, Eboma, Okpareke, Lane, Ibezim, Bıyık, Törün, & Izuogu, 2018). The microfungi suspensions were adjusted to 1×10^4 conidia cells/mL (Ismaiel & Tharwat, 2014). In order to test the antimicrobial activity of the plants, 20 mL of Mueller Hinton Agar (MHA) were poured in petri dishes and kept at room temperature to solidify. Then, they were inoculated with strains of bacteria, yeasts and fungi by taking 0.1 mL from the cell culture media. Then, a hole of 6 mm in diameter and depth was made on the top of the agar plates with a sterile stick and was filled with 50 µL of plant extract (1000µg/mL). Then, bacterial cultures were incubated at 30-37°C for 18-24 h, and yeast cultures were incubated at 27-30°C for 18-24 h. The fungi cultures were incubated at 25-27°C for 5-7 days. At the end ofthe incubation time, the diameters of the inhibition zones formed on the MHA were evaluated in mm. Discs containing Chloramphenicol (30 mg Oxoid), Gentamycin (10 mg Oxoid), Tetracycline (30 mg Oxoid), Erythromycin (15mg Oxoid), Penicillin (10 mg Oxoid), Ampicillin (10 mg Oxoid), Vancomycin (30 mg Oxoid), and Ofloxacin (5 mg Oxoid) for bacteria, Nystatin (100 mg Oxoid) for yeasts, and Clotrimazole (10mg Oxoid) for microfungi were used as positive controls. The measured inhibition zones of the extracts were compared with those of the reference discs (Çoban, Bıyık, Törün, &Yaman, 2017).

Dilution method

The antibacterial and antifungal activities of solvent extracts synthesized compounds were examined by preparing a microdilution broth (Jorgensen & Ferraro, 2009; CLSI, 2009; CLSI, 2013). The analysis was carried out in a sterile 96-well microtitre plate. The suspensions, adjusted as 1×10^8 bacterial cells/mL, 1×10^6 yeast cells/mL and 1×10^4 conidia cells/mL for the analysis, were used. Initially, 100 µL of Mueller Hinton Broth (MHB) was placed in each well. After, the extracts were added into the first well. Two-fold serial dilutions of the extracts were carried out to determine the MIC, within the concentration range 256 to 0.25 µg/mL. Next, 100 µL of microorganism suspension was added into each well. The bacterial cultures were incubated at

30-37°C, and yeast cultures were incubated at 27-30°C for 18-24 h. The fungi cultures were incubated at 25-27°C for 5-7 days. The lowest concentration of antimicrobial agent that resulted in complete inhibition of the microorganisms was represented as MIC (µg/mL). Streptomycin for bacteria, and fluconazole for yeasts and microfungi were used as positive controls in the dilution method. In each case, the test was performed in triplicate and the results were expressed as means.

Minimum Bacteriocidal Concentration (MBC) / Minimum Fungicidal Concentration (MFC)

As a result of MIC test was carried out MBC and MFC tests. From each clear well in the MIC assay, 10 µL was inoculated and spread onto MHA plates. Then the plates were incubated at 30-37°C for 18-24 hours for the bacteria, and at 25-27°C for 5-7 days for the fungi. The MBC and MFC were identified as the lowest concentration of extract that did not grow any bacteria and fungi on the MHA plates (Zamri, Bakar, Noor, & Fuad, 2020; Stojkovi´c, Dias, Drakuli´c, Barros, Stevanovi´c, Ferreira, & Sokovi´c, 2020).

Statistical analysis

Mean values and standard deviation calculations were made using SPSSv22 (Statistical Package for Social Sciences).

RESULTS AND DISCUSSION

Antimicrobial analysis

The antimicrobial activity of the ethyl acetate, methanol, chloroform, acetone and boiled water extracts of *C. tomentosa* and *V. mykales* were researched and the results are given Table 1 and 3. Also, the results of the reference antibiotics used are showed in Table 2.

Among the plant extracts tested, the ethyl acetate extracts of *C. tomentosa* and *V. mykales* indicated a high effect against

Table 1. Antimicrobial activities of the extracts of *C. tomentosa* and *V. mykales* against some microorganisms (Inhibition zone mm).

Test Microorganisms	Inhibition zones (mm)									
	Plant Extracts									
	Campanula tomentosa Lam.					*Verbascum mykales* Bornm.				
	EA	C	M	Ac	BW	EA	C	M	Ac	BW
Escherichia coli	17.33±2.51	_	_	_	_	25.33±2.51	_	_	_	_
Enterobacter aerogenes	17.66±2.51	_	_	_	_	19.33±2.08	_	_	_	_
Salmonella typhimurium	15.33±2.51	_	9.66±0.57	_	_	19.66±0.57	_	_	_	_
Micrococcus luteus	21.33±1.52	_	8.66±0.57	_	_	27.33±2.51	_	11.00±1.00	10.66±1.15	
Staphylococcus aureus	13.33±0.57	_	14.33±0.57	_	_	16.00±2.64	_	12.33±2.51	11.66±2.08	_
Staphylococcus epidermidis	17.00±2.64	_	9.33±0.57	_	_	19.33±1.15	_	10.00±0.00	11.66±2.08	_
Klebsiella pneumoniae	19.33±0.57	_	12.66±0.57	_	_	18.00±3.00	_	_	_	_
Streptococcus pneumoniae	15.66±0.57	_	9.33±0.00	_	_	25.00±2.00	_	11.66±2.88		_
Pseudomonas aeruginosa	12.00±1.00	_		_	_	23.00±2.64	9.66±0.57	23.33±2.88	18.66±2.08	_
Corynebacterium xerosis	19.00±2.64	_	12.33±0.57	_	_	19.66±0.57	_	_	_	_
Mycobacterium smegmatis	22.33±1.52	_	10.33±0.57	_	_	19.33±1.15	_	_	_	_
Listeria monocytogenes	19.33±0.57	_	11.00±0.00	10.33±0.57	_	19.00±1.73	_	_	_	_
Serratia marcescens	21.66±2.08	_	8.66±0.57	_	_	21.66±2.88	_	_	_	_
Proteus vulgaris	24.33±1.15	_	11.66±1.15	_	_	20.33±0.57	_	_	_	_
Enterococcus faecalis	23.00±1.00	_	10.66±1.15	_	_	15.00±1.73	_	_	_	_
Bacillus cereus	24.00±1.00	_	14.33±1.15	10.33±0.57	_	19.33±3.21	_	12.00±2.00	12.00±1.73	_
Bacillus subtilis	23.33±1.52	_	11.33±0.57	9.66± 0.57	_	23.00±1.73	_	11.33±1.52	11.66±1.52	_
Candida albicans	13.66±1.15		9.66±1.15	10.33±0.57	_	_	_	_	_	_
Candida utilis	_	_	_	_	_	11.00±1.00	_	_	11.33±2.30	_
Saccharomyces cerevisiae	12.33±1.15		10.00±0.00	_	_	_			_	_
Aspergillus flavus	_	_	_	_	_	12.33±2.51	_	_	_	_
Aspergillus niger	_	_	_	_	_	12.66±0.57	_	_	_	_
Aspergillus oryzae	_	_	_	_	_	_	_	_	_	_

(-): Zone did not occur
EA: Ethyl Acetate, M: Methanol, C: Chloroform, Ac: Acetone, BW: Boiled Water

Table 2. Inhibition zone diameter of the reference antibiotics to test microorganisms (mm).

Test Microorganisms	Inhibition zones (mm)									
	Reference antibiotics									
	C30	CN10	TE30	E15	P10	AMP10	VA30	OFX5	NS100	CTL10
Escherichia coli	24	21	15	11	16	-	23	28	NT	NT
Enterobacter aerogenes	19	20	14	-	-	-	-	19	NT	NT
Salmonella typhimurium	17	16	15	8	15	8	21	25	NT	NT
Micrococcus luteus	25	15	26	30	13	28	14	24	NT	NT
Staphylococcus aureus	23	20	22	23	12	20	13	23	NT	NT
Staphylococcus epidermidis	22	17	19	11	11	17	12	22	NT	NT
Klebsiella pneumoniae	21	19	20	14	18	-	23	27	NT	NT
Pseudomonas aeruginosa	22	20	20	21	14	-	18	29	NT	NT
Corynebacterium xerosis	20	17	25	26	14	27	21	22	NT	NT
Mycobacterium smegmatis	23	18	26	25	16	19	20	30	NT	NT
Listeria monocytogenes	19	14	12	-	10	12	25	29	NT	NT
Serratia marcescens	23	19	13	-	18	19	27	27	NT	NT
Proteus vulgaris	17	24	16	20	15	-	24	26	NT	NT
Enterococcusfaecalis	16	11	19	-	12	14	20	28	NT	NT
Streptococcus pneumoniae	24	20	25	15	19	14	29	28	NT	NT
Bacillus cereus	23	24	25	26	10	-	21	28	NT	NT
Bacillus subtilis	22	20	12	25	11	-	20	27	NT	NT
Candida albicans	NT	NT	NT	NT	NT	NT	NT	NT	22	NT
Candida utilis	NT	NT	NT	NT	NT	NT	NT	NT	21	NT
Saccharomyces cerevisiae	NT	NT	NT	NT	NT	NT	NT	NT	15	NT
Aspergillus flavus	NT	NT	NT	NT	NT	NT	NT	NT	NT	23
Aspergillus niger	NT	NT	NT	NT	NT	NT	NT	NT	NT	24
Aspergillus oryzae	NT	NT	NT	NT	NT	NT	NT	NT	NT	24

(-): Zone did not occur. NT: Not tested
C30: Chloramphenicol (30 mg Oxoid), CN10: Gentamycin (10 mg Oxoid), TE30: Tetracycline (30 mg Oxoid), E15: Erythromycin (15mg Oxoid), AMP10: Ampicillin (10 mg Oxoid), P10: Penicillin (10 mg Oxoid), VA: Vancomycin (30 mg Oxoid), OFX5: Ofloxacin (5 mg Oxoid), NS100: Nystatin (100 mg Oxoid), CTL10: Clotrimazole (10mg Oxoid).

some Gram (-) and Gram (+) bacteria (Table 1). The ethyl acetate extract of *C. tomentosa* showed strong activity (19-24 mm) against *M. luteus*, *K. pneumoniae*, *C. xerosis*, *M. smegmatis*, *L. monocytogenes*, *S. marcescens*, *P. vulgaris*, *E. faecalis*, *B. cereus*, and *B. subtilis*. On the other hand, the ethyl acetate extract of *V. mykales* Bornm. demostrated more powerful effects (18-27 mm) against *E. coli*, *E. aerogenes*, *S. typhimirium*, *M. luteus*, *S. epidermidis*, *K. pneumoniae*, *S. pneumonia*, *P. aeruginosa*, *C. xerosis*, *M. smegmatis*, *L. monocytogenes*, *S. marcescens*, *P. vulgaris*, *B. cereus*, and *B. subtilis* (Figure 5a, b). The ethyl acetate extract of *C. tomentosa* showed significant activity (12-17 mm) against *E. coli*, *E. aerogenes*, S. *typhimirium*, *S. aureus*, *S. epidermidis*, *S. pneumonia*, *P. aeruginosa*, *C. albicans*, and *S. cerevisiae*. The methanole extract of *C. tomentosa* showed a remarkable effect (12-14 mm) against *S. aureus*, *K. pneumoniae*, *C. xerosis*, and *B. cereus*. The same extract and the acetone extract of the plant indicated a slight effect (8-11 mm) against *S. typhimirium*, *M. luteus*, *S. epidermidis*, *S. pneumonia*, *M. smegmatis*, *L. monocytogenes*, *S. marcescens*, *P. vulgaris*, *E. faecalis*, *B. cereus*, *B. subtilis*, *C. albicans*, and *S. cerevisiae*. However, the chloroform and boiled water ex-

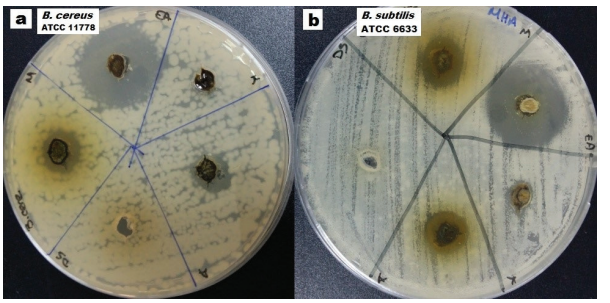

Figure 5. a. Effect of ethyl acetate, methanol, chloroform, acetone, boiled water extracts of *Campanula tomentosa* Lam. against *Bacillus cereus* ATCC 11778 **b.** Effect of ethyl acetate, methanol, chloroform, acetone, boiled water extracts of *Verbascum mykales* Bornm. against *Bacillus subtilis* ATCC 6633. EA: Ethyl Acetate, M:Methanol, K:Chloroform, A:Acetone, DS: Boiled Water.

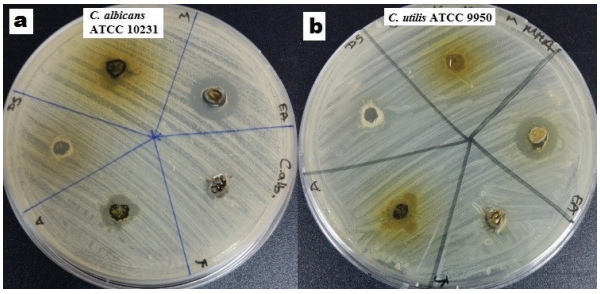

Figure 6. a. Effect of ethyl acetate, methanol, chloroform, acetone, boiled water extracts of *Campanula tomentosa* Lam. against *Candida albicans* ATCC 10231 **b.** Effect of ethyl acetate, methanol, chloroform, acetone, boiled water extracts of *Verbascum mykales* Bornm. against *Candida utilis* ATCC 9950. EA: Ethyl Acetate, M:Methanol, K:Chloroform, A:Acetone, DS: Boiled Water.

tracts of *C. tomentosa* had no effect against *C. albicans* (Figure 6a). The ethyl acetate extract of *V. mykales* Bornm. displayed a noteworthy effect (12-16 mm) against *S. aureus*, *E. faecalis*, *A. flavus*, and *A. niger*. The methanole and acetone extracts of the plant demostrated a high effect (18-23 mm) against *P. aeruginosa* while the same extracts had a moderate effect (12 mm) against *B. cereus*. However, the ethyl acetate and acetone extracts of *V. mykales* Bornm. had a low effect (9-11 mm) against *C. utilis* (Figure 6b). Otherwise, The boiling water extract of the plant has no effect against tested microorganisms.

According to the MIC/MBC/MFC values in Table 3, the ethyl acetate extract of *C. tomentosa* had a strong effect (8 µg/mL) against *M. smegmatis*, *P. vulgaris*, *E. faecalis*, *B. cereus*, and *B. subtilis*. On the other hand, the ethyl acetate extract of the plant showed a significant effect (16-64 µg/ mL) against other bacteria and yeasts. Also, the methanol and acetone extracts of the plant had a very low effect (128-256 µg/mL) against many bacteria and two yeasts. The ethyl acetate extract of *V. mykales* indicated a very strong effect (4-8 µg/mL) against *E. coli*, *M. luteus*, *S. pneumonia*, *P. aeruginosa*, and *B. subtilis*. Besides, the same extract of plant demostrated an appreciable effect (16-64 µg/mL) against other bacteria, one yeast and two microfungi. However, the methanole and acetone extracts of the plant showed a remarkable effect (8-16 µg mL^{-1}) against *P. aeruginosa*. Otherwise, the chloroform, methanole and acetone

Table 3. Antimicrobial activities of the extracts of *C. tomentosa* and *V. mykales* against some microorganisms (MIC/MBC/MFC), (µg/mL).

Test Microorganisms	MIC/MBC/MFC (µg/mL)									
	Campanula tomentosa Lam.				*Verbascum mykales* Bornm.				Reference antibiotics	
	EA	C	M	A	EA	C	M	A	STR	FLK
Escherichia coli	32/64	_	_	_	4/8	_	_	_	64	NT
Enterobacter aerogenes	32/64	_	_	_	16/32	_	_	_	64	NT
Salmonella typhimurium	32/64	_	256/> 256	_	16/32	_	_	_	64	NT
Micrococcus luteus,	16/32	_	256/> 256	_	4/8	_	_	_	32	NT
Staphylococcus aureus	32/64	_	64/128	_	32/64	_	>256/>256	128/ 256	32	NT
Staphylococcus epidermidis	32/64	_	256/> 256	_	16/32	_	>256/>256	128/ 256	32	NT
Klebsiella pneumoniae	16/32	_	64/128	_	16/32	_	_	_	64	NT
Streptococcus pneumoniae	32/64	_	256/> 256	_	4/8	_	>256/>256	_	128	NT
Pseudomonas aeruginosa	64/128	_	_	_	8/16	256/> 256	8/16	16/32	64	NT
Corynebacterium xerosis	16/32	_	64/128	_	16/32	_	_	_	64	NT
Mycobacterium smegmatis	8/16	_	128/ 256	_	16/32	_	_	_	128	NT
Listeria monocytogenes	16/32	_	128/256	256/> 256	16/32	_	_	_	64	NT
Serratia marcescens	16/32	_	256/> 256	_	16/32	_	_	_	64	NT
Proteus vulgaris	8/16	_	128/ 256	_	16/32	_	_	_	64	NT
Enterococcus faecalis	8/16	_	128/ 256	_	32/64	_	_	_	64	NT
Bacillus cereus	8/16	_	64/128	128/ 256	16/32	_	64/128	64/128	64	NT
Bacillus subtilis	8/16	_	128/ 256	256/> 256	8/16	_	128/ 256	128/ 256	64	NT
Candida albicans	64/128	_	256/ >256	128/ 256	_	_	_	_	NT	64
Candida utilis	_	_	_	_	128/ 256	_	_	128/ 256	NT	64
Saccharomyces cerevisiae	64/128	_	128/ 256	_	_	_	_	_	NT	64
Aspergillus flavus	_	_	_	_	64/128	_	_	_	NT	64
Aspergillus niger	_	_	_	_	64/128	_	_	_	NT	64

STR= Streptomycin, FLK= Fluconazole. (-) = No effect, NT: Not tested

extracts had a slight activity (128-256 µg/mL) against some bacteria and one yeast (Table 3).

The antimicrobial effect of methanol, acetone and ethyl acetate extracts obtained from *V. pinnatifidum* Vahl. and *V. antinori* Boiss. et Heldr were researched against Gram (+) and Gram (-) bacteria, and *C. albicans* ATCC 10231. It was found that the *V. antinori* extracts have a greater antimicrobial effect than *V. pinnatifidum* extracts against the test microorganisms (Göse & Hacıoğlu Doğru, 2021). The methanol, dichloromethane, and aqueous crude extracts of *C. retrorsa* flower, leaf and stem were tested against the microorganisms. It was found that the activities of the dichloromethane extracts of leaves and flowers of *C. retrorsa* have a moderate effect against *A. baumanii* and *C. albicans* and the methanol and aqueous crude extracts of *C. retrorsa* have no activity against the other bacteria tested and *C. albicans* (Alhage, Elbitar, Taha, & Benvegnu, 2020). Himalayan medicinal plants were used traditionally to treat pneumonia and tuberculosis. It has been revealed that the methanol extract of *V. thapsus* leaves has antibacterial activity against *S. aureus*, *S. pneumonia* and *M. tuberculosis* (Muhammad, Shandana, Khushboo, & Rahila, 2019). Abdallah & Omar (2019) remarked on the antimicrobial activity of water, ethanol and methanol extracts of the aerial parts of *V. fruticulosum* against an *E. coli* clinical isolate. The results showed that water and methanol extracts did not inhibit *E. coli*; however, the ethanol extract repressed growth of *E. coli*. In another study by Dülger & Dülger (2018), it was reported that the methanol extract obtained from *V. antinori* Boiss. et Heldr. has an antibacterial effect against Gram (+) and Gram (-) bacteria. In asimilar study by Fares (2018), the antimicrobial activity of the methanol, acetone, n-hexane and aqueous extracts of the aerial parts of *V. fruticulosum* were examined against microorganisms. Methanol, acetone and n-hexane extracts of plant has higher activity than the water extract against *S. aureus*, *E. coli*, *P. aeruginosa*, *E. faecium*, *S. sonnei*, and methicillin-resistant *S. aureus* (MRSA).The methanol and acetone extracts of plant indicated the strongest inhibition against *P. aeruginosa* (1.56 mg/mL), *E. coli* and *S. aureus* (6.25 mg/mL). The aqueous extract of plant has effect against *S. aureus* (MRSA) (3.125 mg/mL) while the methanol and n-hexane extracts of plant have effect against *E. faecium* and *S. sonnei* (3.125 mg/mL). The methanol, acetone and n-hexane extracts had activity against *C. albicans* and *E. floccosum*. The highest effect (1.56 mg/mL) was obtained with the n-hexane extract against *E. floccosum*. However, the aqueous extract did not have any activity. An antibacterial effect was seen on *S. aureus* ATCC 6538P (22 mm), *B. cereus* ATCC 7064 (20 mm), *L. monocytogenes* ATCC 15313 (14 mm), and *M. luteus* CCM 169 (17mm). The methanol extract of *V. mucronatum* flowers was tested against *E.coli* ATCC 25922, *E. faecalis* ATCC 29212, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213, *C. albicans* ATCC 90028, *C. krusei* ATCC 6258, and *C. parapsilosis* ATCC 90018. It was found that *V. glabratum subsp. bosnense* (K. Maly) Murb. includes quercetin and rosmarinic acid, 4-hydroxybenzoic acid, salicylic acid, morin, and apigenin as bioactive compounds. In addition, the ethanol extracts of *V. glabratum subsp. bosnense* (K. Maly) Murb. had a moderate effect with MIC values of 600µg/mL – 1200µg/mL against *E. coli, S. aureus,* and *C. albicans* (Karalija, Parić, Dahija, Bešta-Gajević, & Zeljković, 2018). The phenolic profiles and endogenous hormone levels in embryogenic and nonembryogenic calli of *C. tomentosa* were analyzed, but an antimicrobial activity study of this endemic plant species has not been presented in the records (Coşkun, Gemici, &Yildirim, 2017). In another study, the methanolic extracts of *Verbascum cheiranthifolium Boiss. var. asperulum* (Boiss.) Murb. Monorg., *V. pynostachyum* Boiss. & Heldr and *V. orgyale* Boiss. & Heldr.were tested against *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. utilis*, *C. glabrata*, and *C. krusei*. It was remarked that *V. pynostactum* and *V. orygale* extracts indicated a higher effect than *V. cheriantifolium var. asperulum*. Particulary, *V. pycnostachyum* extract inhibited *C. krusei* at the concentration of 62.5 µg/mL (Küçük, Özdemir, İşcan, & İncesu, 2016). In a similar study, the antibacterial activity of the methanol and acetone leaf extracts of *V. thapsus* were examined against *E. coli*, *Y. pestis*, *B. cereus*, *P. aeruginosa*, *L. monocytogenes* and *S. aureus*. It was reported that the methanol extract of this plant has stronger activity than an acetone extract against the tested pathogenic bacteria (Prakash, Rana, & Sagar, 2016). The antimicrobial effect of the methanol extract of *V. speciosum* leaves was investigated against *S. aureus*, *L. monocytogenes*, *B. anthracis*, *B. cereus*, *S. typhimurium* and *E. coli*. It was found that the extract has remakable activity against *L. monocytogenes*, *B. cereus*, *S. aureus* and *S. thyphimurium*; even more than penicillin (Nofouzi, Mahmudi, Tahapour, Amini, &Yousefi, 2016). The antifungal activity of a methanol extract of the aerial part of *V. speciosum* was tested against *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. dublinii-ensis*, *A. flavus*, *A. niger*, *Penicillium* sp. and *Alternaria* sp. The highest activity was seen against *C. parapsilosis* and *Alternaria* sp. (Nofouzi, 2015). In another study, the antibacterial activity of some Turkish plants was screened against fish pathogens by Türker & Yıldırım (2015). The ethanol and aqueous crude extracts of *C. glomerata L. subsp. hispida* (Witasek) Hayek and *C. olympica* Boiss. were used for the antibacterial activity. It was reported that the ethanol extracts of *C. glomerata subsp. hispida* and *C. olympica* have a slight effect (11 mm and 8 mm). However, aqueous crude extracts of the plants did not inhibit fish pathogens. In a similar study, the antibacterial effects of ethanol, methanol and water extracts of *C. glomerata* and *C. olympica* flowers, leaves and stems were researched against *S. pyogenes* ATCC 19615, *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *K. pneumoniae* ATCC 13883. It was remarked that the ethanol extract of *C. olympica* has a strong effect (20 mm) against *K. pneumoniae* ATCC 13883 and the extracts of *C. glomerata* and *C. olympica* have high activity against at least one of the tested Gram (-) bacteria. However, the plant extracts had no effect against *S. aureus*, *S. epidermidis* and *P. aeruginosa* (Usta, Yildirim, & Turker, 2014). The antibacterial effect of the aqueous extract of *V. thapsus* was tested against *S. aureus* PTCC1431 and *E. coli* HP101BA 7601c. It was shown that the extract has an effect against Gram (+) and Gram (-) bacteria (Sepahi, Ghorani-Azam, Sepahi, Asoodeh, & Rostami, 2014). In another study, the antimicrobial activity of volatile oil and aqueous extracts of *C. portenschlagiana* Roem.et Schult were evaluated. It was reported that the results of the *C. portenschlagiana* volatile oil have a more powerful antimicrobial activity than the aqueous extract. The volatile oil had very strong activity (19.6-28.3 mm) against Gram (+) and Gram (-) bacteria and its MIC values were 7.8-125.0 mg/mL. However, the aqueous extract of *C. portenschlagiana* indicated considerable effect (10.8-21.5 mm) against the tested bacteria and its MIC values were 125.0-500 mg/mL. The volatile oil of the plant had the most effect (28.3 mm - 7.8 mg/mL) against *P. aeruginosa* FNSST 014 while the aqueous extract of *C. portenschla-*

giana had the most effect (21.5 mm - 125.0 mg/mL) against *S. aureus* ATCC 25923 (Politeo, Skocibusic, Burcu, Maravic, Carev, Ruscic, & Milos, 2013). In another study, the aerial parts of *V. lagurus* Fisch. & C.A.Mey., *V. gnaphalodes* M. Bieb., and *V. xanthophoeniceum* Griseb. were extracted with methanol, chloroform, ethyl acetate and water. The ethyl acetate extract of *V. lagurus* demostrated higher antimicrobial activity among the other *V. lagurus* extracts. The methanol, chloroform, ethyl acetate and water extracts of *V. lagurus* had an effect against *S. aureus* and the MIC values were 156-625mg/L. The methanol extract of *V. lagurus* showed only against *P. aeruginosa*. In addition, the methanol, ethyl acetate and aqueous extracts of *V. lagurus* had a moderate effect against *C. albicans* (Şen, Döşler, & Meriçli, 2012). In a similar study, the antibacterial activity of the aqueous and ethanol extracts of *V. speciosum* flowers were evaluated against *B. subtilis*, *E. aerogenes*, *P. vulgaris* and *S. paratyphi*. It was shown that the ethanol extract had a slight effect (10-11 mm) against *E. aerogenes and S. paratyphi* and the aqueous extract did not inhibit the selected bacteria (Noori, Malayeri, Moosaei, Pakzad, & Piriye, 2012).

When the results of our study are compared with the previous study results, the antimicrobial activities of *C. tomentosa* extracts (especially ethyl acetate and methanol extracts) indicated a higher effect than *C. portenschlagiana*, *C. glomerata*, *C. olympica*, *C. latifolia*, *C. retrorsa*, and *C. lyrata subsp. lyrata* extracts. Also, antimicrobial activities of *V. mykales* extracts were found to be more effective than the antimicrobial activity of other *Verbascum* species.

CONCLUSION

In this study, we investigated the antimicrobial activity of *C. tomentosa* and *V. mykales* endemic plant extracts against some microorganisms. It was determined that ethyl acetate and methanol extracts of plants showed high activity against the tested microorganisms. The results obtained will contribute to the pharmaceutical industry as a novel drug discovery.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of study- E.P.Ç.; Data Acquisition- E.B.; Data Analysis/Interpretation- E.P.Ç., E.B.; Drafting Manuscript- E.P.Ç.; Critical Revision of Manuscript- E.P.Ç.; Final Approval and Accountability- E.P.Ç.; Technical or Material Support- E.P.Ç., E.B.

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

Financial Disclosure: This study was funded by Scientific Research Projects Coordination Unit of Aydin Adnan Menderes University (Project number: FEF-17039).

Acknowledgement: This work was carried out by Aydin Adnan Menderes University Biology Department, Microbiology Laboratory. We thank Assoc. Prof. Dr. Özkan EREN for tlıe support provided to us in the collection and identification of plants.

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Istanbul J Pharm 51 (3): 357-364
DOI: 10.26650/IstanbulJPharm.2021.934461
Original Article

Inhibitory effects of aqueous extract of *Eremurus spectabilis* M. Bieb. on diabetes mellitus and skin related enzymes

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Cite this article as: Bayrak, B. B., & Yanardag, R. (2021). Inhibitory effects of aqueous extract of Eremurus spectabilis M. Bieb. on diabetes mellitus and skin related enzymes. *İstanbul Journal of Pharmacy, 51*(3), 357-364.

ABSTRACT

Background and Aims: Nutritional bioactive and natural ingredients isolated from wild edible plants, with promising effects against diabetes and/or diabetes-related diseases continue to be investigated. There is increasing interest in these substances due to their protective/therapeutic potential. The purpose of the present study is to investigate the inhibitory potential of aqueous extract of *Eremurus spectabilis* M. Bieb. on diabetes mellitus and skin-related enzymes.
Methods: Fresh *E. spectabilis* leaves were obtained from a local market in Eyup/İstanbul, Turkey and their aqueous extract was prepared. Inhibitory activities of the aqueous extract of this plant on α-amylase, α-glucosidase, elastase, hyaluronidase, and tyrosinase were examined.
Results: The aqueous extract of *E. spectabilis* showed effective inhibitor activity against both α-amylase and α-glucosidase as compared to acarbose. On the other hand, the aqueous extract exhibited a moderate inhibitory activity on elastase. Whereas, it exhibited a weak inhibitory effect on the activities of hyaluronidase and tyrosinase when compared to their respective standard inhibitors.
Conclusion: The current findings suggest that the consumption of *E. spectabilis* leaves may be of benefit for regulation of postprandial blood glucose, as shown by the strongly inhibiting action of its extract on both α-amylase and α-glucosidase. In addition, it might be beneficial against skin-related disorders, because of its inhibitory action on elastase activity.

Keywords: *Eremurus spectabilis*, Inhibitory activity, Diabetes mellitus, Skin-related enzymes

INTRODUCTION

Plants are an indispensable food source, due to their nutritional and medicinal properties, as well as being the primary sources of biochemically active phytochemicals. Besides, they are used as an alternative treatment option to modern medicine. Therefore, investigation of various plants and plant product-based inhibitors from nature have been of great interest to researchers. From this point of view, screening natural sources-based potent inhibitors of enzymes that play key roles in diabetes and many other diseases have attracted immense attention, due to the toxic and/or adverse/unwanted effects of chemically synthesized compounds (Li & Ma, 2017; Marrelli, Statti, & Conforti, 2020). The genus *Eremurus* (an important genus of *Xanthorrhoeaceae* that comprises approximately 60 species) is widespread in Central Asia and Middle East (including Turkey) (Beiranvand & Beiranvand, 2021; Salehi et al., 2017). *E. spectabilis* is naturally grown in Eastern and Southeastern Anatolia regions of Turkey. It is popularly referred to as "Çiriş otu" in these regions (Aysu, Demirbas, Bengü, & Küçük, 2015; Cinar et al., 2017; Tosun et al., 2012). Besides its nutritional features, the *Eremurus* species also possesses medicinal potential. The leaves and roots are traditionally used to cure diseases

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Submitted: 07.05.2021
Revision Requested: 31.05.2021
Last Revision Received: 02.06.2021
Accepted: 03.06.2021
Published Online: 00.00.0000

such as hemorrhoids, eye inflammation, jaundice, fungal infection, hypertension, and eczema (Dashti, Tavakoli, Zarif Ketabi, & Paryab, 2005; Gaggeri et al., 2013; Gaggeri et al., 2015). In addition, this wild edible plant is used to manage or cure gastrointestinal and liver disorders (Vala, Asgarpanah, Hedayati, Shirali, & Bejestani, 2011). Owing to the aforementioned traditional use of this plant, the phenolic compounds (major secondary metabolites) in some *Eremurus* species were isolated and their biological activities were investigated (Abubaker & Hidayat, 2015; Karaoglan Esen et al., 2018; Mottaghipisheh et al., 2021).

Diabetes mellitus (DM) is an endemic multifactorial endocrine disease characterized by insulin deficiency or resistance, which gives rise directly to hyperglycemia (Bonamonte & Filoni, 2021). The most important underlying factor in the development of DM is the deterioration of the regulatory effect of pancreatic β-cells on glucose metabolism, thus disrupting the balance between insulin production and secretion (Zafar et al., 2021). Coupled to this, the hydrolytic activities of two catabolic enzymes (e.g. α-amylase and α-glucosidase) on carbohydrate polymers (such as glycogen and starch) give rise to elevated blood glucose levels.

α-Amylase is an enzyme responsible for the conversion of complex carbohydrates (e.g. starch) into simple oligosaccharides (Pant et al., 2021). α-Glucosidase on the other hand is a hydrolytic enzyme catalyzing the cleavage of α-glycosidic bonds of both endogenous and exogenous carbohydrate polymers. The inhibition of these aforementioned enzymes is as important as dietary restriction in controlling hyperglycemia (Lankatillake et al., 2021; Papoutsis et al., 2021).

Skin disorders have been associated with prevalent complications of DM. Also, hyperglycemia leads to deterioration of cells in the dermis layer of skin. Thus, altering the flexibility and solubility of collagen as a consequence of altered advanced glycation end products (de Maceto, Nunes, & Barreto, 2016; Lai, Nor, Kamaruddin, Jamil, & Safian, 2021). Accumulated evidence shows that skin diseases such as scleredema, diabetic dermopathy, necrobiosis lipoidica, and acanthosis nigricans are strongly associated with DM (Demirkesen 2015; Mendes et al., 2017; Svoboda & Shields, 2021). For this reason, natural compounds that can inhibit α-amylase and α-glucosidase, and as well regulate postprandial glucose may have great significance in managing DM and DM-related skin disorders.

Elastases are proteolytic enzymes that mainly hydrolyze proteins of the connective tissue in lungs, arteries, ligaments, and skin. These enzymes also participate in a controlled proteolysis of elastic fibers during normal growth and tissue remodeling. However, under certain pathological conditions, the activity of these enzymes can lead to both major and uncontrolled destruction of structural proteins, thereby triggering severe diseases like pulmonary emphysema, acute pancreatitis, rheumatoid arthritis, thrombosis, stroke, and skin-aging (Desmiaty et al., 2020). To delay aging, inhibitors of elastase enzymes can be useful tools via preventing loss of skin elasticity and skin sagging (Apraj & Pandita, 2016).

The hydrolysis of glycosaminoglycans such as hyaluronic acid (HA) of the extracellular matrix (ECM) especially of the skin and other connective tissues is carried out by hyaluronidases. They play a crucial role in controlling the size and concentration of HA chains. Elevated hyaluronidase activity causes tissue injury, due to an increase in decomposition of HA (Buhren et al., 2020). Altered activities of these enzymes are implicated in cases of cancer, liver diseases, allergy, wound healing, and dermatological disorders, including skin-wrinkling and skin-aging (Gangadharan, Jacob, & Densely Jose, 2014; Girish & Kemparaju, 2007).

Tyrosinase, a multifunctional membrane oxidase (or oxidoreductase), catalyzes the first step of both the catecholamine and melanin biosynthesis (Pillaiyar, Manickam, & Navasivayam, 2017). Melanin accumulation may cause serious skin diseases such as melasma, freckles, senile lentigo, age spots, and sites of actinic damage (Zolghadri et al., 2019). Therefore, many tyrosinase inhibitors find application in cosmetics and pharmaceutical products, for preventing the overproduction of melanin in the epidermis (Ullah et al., 2019).

Inhibitions of these enzymes mentioned above have gained great important in DM and DM-associated skin diseases for many years. There are insufficient published reports about the effects of *E. spectabilis* on inhibitory effects of these enzymes. Therefore, the objective of the present study was to investigate the inhibition activities of aqueous extract of *E. spectabilis* on the α-amylase, α-glucosidase, elastase, hyaluronidase, and tyrosinase.

MATERIALS AND METHODS

Plant material and chemicals

Fresh *E. spectabilis* leaves were obtained from a local market in Eyup/İstanbul, Turkey. The plant was inspected and identified by Prof. Dr. Emine Akalin (Faculty of Pharmacy, İstanbul University). A specimen was deposited at the Faculty of Pharmacy Herbarium of İstanbul University (ISTE 93132). The plant material was washed twice with distilled water, and thereafter dried at room temperature. The dried plant was stored in -20°C until they were required for use.

The enzymes and their respective substrates (i.e., α-amylase, α-glucosidase, elastase, hyaluronidase, and tyrosinase; soluble starch, *p*-nitrophenyl-α-D-glucopyranoside (α-PNPG), N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide (STANA), hyaluronic acid sodium salt, and L-tyrosine) were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Merck (KGaA, Darmstadt, Germany). All chemicals used in the experiment were of analytical grade.

Preparation of extract

Aqueous extract was prepared by refluxing 20g of dried *E. spectabilis* leaves in 200mL of distilled water for 6 hours in reflux condenser. The extract was then filtered at room temperature and lyophilized to dryness. The resultant lyophilized aqueous extract of the plant was stored in deep freeze (at -20°C) until use.

In vitro α-amylase inhibitory activity

α-Amylase inhibitory activity was examined according to Al-Dabbas et al., (Al-Dabbas, Kitahara, Suganuma, Hasimato, &

Tadera, 2006). In brief, a soluble starch solution (substrate, in 0.25 M phosphate buffer, pH 7.0) was mixed with varying concentrations of the extract, then the mixture was allowed to stand at 37ºC for 5 min. Equal aliquots of α-amylase solution and phosphate buffer (0.25 M pH 7.0) were added to the reaction medium and then further incubated at 37ºC for 7.5 min. Appropriate aliquots of 0.01 N iodine solution and distilled water were pipetted into the solution, then the absorbance of resulting mixture was recorded at 660nm by using a spectrophotometer. Acarbose was used as a standard inhibitor. Results were expressed as the average of triplicate trials.

Percentage inhibition was determined using the following formula:

$$\text{α-Amylase inhibitory activity (\%)} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100$$

A_o is the enzyme activity without inhibitor.

A_1 is the activity in presence of inhibitor.

***In vitro* α-glucosidase inhibitory activity**

α-Glucosidase inhibitory activity was assessed by the method of Tao et al., with a slight modification using α-PNPG as a substrate (Tao, Zhang, Cheng, & Wang, 2013). Briefly, a phosphate buffer (0.1 M, pH 7.40), appropriate aliquot of different concentrations of the extract, and α-glucosidase (in phosphate buffer pH 7.40) were mixed in 96-well plate. After pre-incubation (at 37°C for 10 min), α-PNPG (in phosphate buffer pH 7.40) was pipetted into the reaction mixture, and then incubated at 37°C for another 10 min. At the end of the incubation period, the absorbance was recorded at 410nm by using a microplate reader. Acarbose was used as the standard inhibitor instead of the plant extract. Results were expressed as the average of triplicate trials.

Percentage inhibition was calculated using the following formula:

$$\text{α-Glucosidase inhibitory activity (\%)} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100$$

A_o is the enzyme activity without inhibitor.

A_1 is the activity in presence of inhibitor.

***In vitro* elastase inhibitory activity**

Elastase inhibitory activity was determined by spectrophotometric method of Moon et al., (Moon, Yim, Song, Lee, & Hyun, 2010). The assay mixture, containing enzyme solution, plant extract (or standard inhibitor) and Tris-HCl buffer (200 mM, pH 7.8), was pre-incubated at 37ºC for 15 min. The reaction was initiated by adding a substrate (STANA) solution to the test tube and the mixture was incubated at the same temperature for 30 min. At the end of the incubation period, the absorbance was measured at 410nm. Ursolic acid was used as the standard compound instead of the plant extract. Results were expressed as the average of triplicate trials.

The percentage of elastase inhibitory activity was determined according to the following equation:

$$\text{Elastase inhibitory activity (\%)} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100$$

A_o is the enzyme activity without inhibitor.

A_1 is the activity in presence of inhibitor.

***In vitro* hyaluronidase inhibitory activity**

Hyaluronidase inhibitory activity was assayed by the spectrophotometric method developed by Lee et al., in which sodium hyaluronate is formed from *N*-acetylglucosamine (Lee, Kim, Cho, & Choi, 1999). The reaction mixture, containing suitable aliquot of bovine testes hyaluronidase dissolved in 0.1 M acetate buffer (pH 4.0) was mixed with different concentrations of the extract, and then incubated in a water bath at 37°C for 20 min. For the control experiment, solvent from which the extracts were dissolved was used. The absorbance of the reaction mixture was then measured by using the spectrophotometer at 585nm. Rutin was used as the standard inhibitor instead of the plant extract. Results were expressed as the average of triplicate trials.

The percent inhibition of hyaluronidase was calculated using the following equation:

$$\text{Hyaluronidase inhibitory activity (\%)} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100$$

A_o is the enzyme activity without inhibitor.

A_1 is the activity in presence of inhibitor.

***In vitro* tyrosinase inhibitory activity**

Tyrosinase inhibitory activity was determined according to the procedure described by Vanni et al., (Vanni, Gastaldi, & Giunata, 1990). Briefly, tyrosinase solution, L-tyrosine, and different concentrations of the extract were added to a solution of phosphate buffer (0.1 M, pH 6.5). The test mixture was incubated for 10 min at 37°C and the absorbance was monitored at 475nm. Kojic acid was used as the standard inhibitor instead of the plant extract. Results were expressed as the average of triplicates.

The percent inhibition of tyrosinase was determined according to the following equation:

$$\text{Tyrosinase inhibitory activity (\%)} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100$$

A_o is the enzyme activity without inhibitor.

A_1 is the activity in presence of inhibitor.

For α-amylase, α-glucosidase, elastase, hyaluronidase, and tyrosinase inhibitory activities, the extract (or standard) concentration resulting in a 50% inhibition (IC_{50}) was calculated by regression equations (by plotting the extract solution concentration versus percentage of inhibition). Lower IC_{50} values indicate higher inhibitory potential of the tested plant extract.

RESULTS

***In vitro* α-amylase inhibitory activity of extract**

The inhibition effects of aqueous extract of *E. spectabilis* as well as that of acarbose against α-amylase activity are summarized in Table 1. According to the results, both the aqueous extract and the standard inhibitor exerted an α-amylase inhibition in a dose-dependent manner with IC_{50} values of 0.043±0.003 µg/mL and 91.84±0.94 µg/mL, respectively. Considering the high

inhibitory activities (associated with the lower IC_{50} values), the aqueous extract demonstrated a higher inhibitory activity against α-amylase in comparison to acarbose. The order for an inhibitory effect is as follows: aqueous extract > acarbose (Table 1).

In vitro α-glucosidase inhibitory activity of extract

The inhibitory activities and IC_{50} values of an aqueous extract and acarbose on α-glucosidase are given in Table 2. It was found that α-glucosidase inhibition increased in a concentration-dependent manner for all tested samples. According to the results, the IC_{50} values of aqueous extract and the acarbose were calculated as $1.27x10^{-3}±7.78x10^{-5}$ µg/mL and 98.71±1.13 µg/mL, respectively. As a result, the aqueous extract had a much lower IC_{50} value than that of the acarbose. The α-glucosidase inhibitory activity of *E. spectabilis* extracts and the standard decreased in the order of: aqueous extract > acarbose (Table 2).

In vitro elastase inhibitory activity of extract

The inhibitory activities of aqueous extract of *E. spectabilis* and ursolic acid against elastase are shown in Table 3. The elastase

Table 1. α-Amylase inhibitory activity of *E. spectabilis* extract.

Extract/Standard	Concentrations (µg/mL)	Inhibition (%)*	IC_{50} (µg/mL)*
Aqueous Extract	0.0001	17.39±2.90	0.043±0.003
	0.001	25.99±2.25	
	0.005	33.31±2.42	
	0.01	45.51±2.29	
	0.05	51.19±2.06	
Acarbose	0.1	19.71±1.26	91.84±0.94
	1	32.44±1.73	
	10	43.37±2.01	
	100	54.44±0.56	
	1000	64.32±2.80	

*Mean±SD, Results were expressed as the average of triplicate trials.

Table 2. α-Glucosidase inhibitory activity of *E. spectabilis* extract.

Extract/Standard	Concentrations (µg/mL)	Inhibition (%)*	IC_{50} (µg/mL)*
Aqueous Extract	$5x10^{-5}$	4.91±0.66	$1.27x10^{-3}±7.78x10^{-5}$
	$1x10^{-4}$	7.65±0.96	
	$2x10^{-4}$	13.37±1.54	
	$4x10^{-4}$	21.11±0.80	
	$6x10^{-4}$	23.68±1.44	
Acarbose	0.1	16.58±1.12	98.71±1.13
	1	31.91±1.78	
	10	39.26±0.73	
	100	50.67±0.59	
	1000	63.78±1.38	

*Mean±SD, Results were expressed as the average of triplicate trials.

Table 3. Elastase inhibitory activity of *E. spectabilis* extract.

Extract/Standard	Concentrations (µg/mL)	Inhibition (%)*	IC_{50} (µg/mL)*
Aqueous Extract	0.01	14.83±1.32	60.30±2.00
	0.1	18.00±1.15	
	0.5	21.70±1.75	
	1	34.28±2.92	
	50	41.48±1.36	
Ursolic Acid	0.01	21.09±3.86	38.58±3.29
	0.1	35.01±0.38	
	1	39.92±1.88	
	10	48.21±3.15	
	100	73.40±1.86	

*Mean±SD, Results were expressed as the average of triplicate trials.

inhibition by the aqueous extract and the standard were exhibited in a dose-dependent manner. At 1 µg/mL concentration, it was observed that both aqueous extract of *E. spectabilis* and ursolic acid had very close inhibition values (34.28±2.92% and 39.92±1.88%, respectively). IC_{50} values of aqueous extract and ursolic acid were diminished in the following order: Ursolic acid (38.58±3.29 µg/mL) > aqueous extract (60.30±2.00 µg/mL) (Table 3).

In vitro hyaluronidase inhibitory activity of extract

In Table 4, the aqueous extract of *E. spectabilis* and rutin are depicted. A comparison shows that the aqueous extract had a lower inhibition effect than that of rutin (standard inhibitor). Whereas, rutin had the lower IC_{50} value (0.68±0.01 µg/mL) in comparison to the aqueous extract (2517.50±59.09 µg/mL) of *E. spectabilis* (Table 4).

In vitro tyrosinase inhibitory activity of extract

In this study, the inhibitory activities of aqueous extract of *E. spectabilis* and kojic acid on tyrosinase are presented in Table 5. The aqueous extract and kojic acid exhibited tyrosinase inhibitor activities in a dose-dependent manner. The IC_{50} values of *E. spectabilis* and kojic acid increased in the order of: Kojic acid (74.14±4.76 µg/mL) > aqueous extract (9391.02±180.54 µg/mL) (Table 5).

DISCUSSION

Diabetes mellitus is multifactorial and the most prevalent endocrine disorder and, is increasing in prevalence. Not only multiorgan (e.g. eyes, kidneys, and skin). but also blood vessels are unavoidably affected. Moreover, DM leads to an enormous financial burden and a low quality of life. Long-term detrimental effects of DM may impair skin homeostasis resulting in skin diseases such as infections, diabetic foot syndrome, lichen planus, pruritus, and vitiligo in at least one-third of all diabetics (Behm, Schreml, Landthaler, & Babilas, 2012).

Since plants consumed as food are easily accessible and contain natural ingredients, their effects against DM and DM-related diseases are constantly being investigated. For an antidiabetic effect, the focus is mostly on the inhibition of α-amylase and α-glucosidase- the enzymes associated with digestion of carbohydrates. Therefore, inhibitors of these two catabolic enzymes can decrease the release of D-glucose from dietary carbohydrates, slow down glucose absorption, lower blood glucose levels and reduces hyperglycemia (Etsassala et al., 2019; Gong et al., 2020). In this study, both α-amylase and α-glucosidase inhibitory activities of aqueous extract of *E. spectabilis* were found to be remarkable lower (IC_{50} values) than that of acarbose. It has been reported that *E. persicus* extracts had less than 10% in-

Table 4. Hyaluronidase inhibitory activity of *E. spectabilis* extract.

Extract/Standard	Concentrations (µg/mL)	Inhibition (%)*	IC_{50} (µg/mL)*
Aqueous Extract	750	22.17±2.92	2517.50±59.09
	1000	30.44±1.00	
	2000	52.87±2.43	
	4000	70.46±1.40	
	5000	75.98±2.31	
Rutin	0.001	16.94±1.33	0.68±0.01
	0.01	32.72±1.53	
	0.1	45.04±2.03	
	1	72.46±1.03	
	5	78.44±1.52	

*Mean±SD, Results were expressed as the average of triplicate trials.

Table 5. Tyrosinase inhibitory activity of *E. spectabilis* extract.

Extract/Standard	Concentrations (µg/mL)	Inhibition (%)*	IC_{50} (µg/mL)*
Aqueous Extract	100	6.17±2.14	9391.02±180.54
	1000	12.45±2.45	
	5000	39.20±2.45	
	10000	59.03±0.47	
	15000	66.46 ±1.27	
Kojic Acid	25	13.28±2.01	74.14±4.76
	50	42.21±5.27	
	75	50.74±3.33	
	100	80.87±2.34	
	250	89.26±1.32	

*Mean±SD, Results were expressed as the average of triplicate trials.

hibitory activity on α-glucosidase enzyme *in vitro* (Gholamhoseinian, Hossein, Fariba, & Mirtaj Aldini, 2008). It has been shown that ethyl acetate, methanol, and aqueous extracts of *E. himalaicus* (at a dose of 500 mg/kg body weight) administered to alloxan-induced diabetic rats provided a significant decrease in fasting blood glucose levels compared to diabetic rats. These effects have been suggested to be a mechanism independent on insulin secretion or inhibition of endogenous glucose production (Tramboo, 2013). Sacan, Akev, & Yanardag (2017) revealed that *Aloe vera* (L.) Burm. f. extracts were potent *in vitro* α-amylase inhibitors. In addition, it was demonstrated that administration of *Aloe vera* (L.) Burm. f. leaf extract to diabetic rats led to a decrease in blood glucose levels (Okyar, Can, Akev, Baktir, & Sutlupinar, 2001). Meanwhile, the inhibitory effects of *E. spectabilis* against both α-amylase and α-glucosidase may be attributed to its richness in varied amount of active constituents and antinutritional factors, which are reported to include sterols such as β-sitosterol and ergosterol; flavonoids (e.g. rutin, morin, and quercetin) and resveratrol (Bircan & Kırbağ, 2015).

Elastase and hyaluronidase are the group of dermal enzymes belonging to matrix metalloproteinases (MMPs). They are known to be ECM hydrolyzing enzymes (Genc, Guragac Dereli, Saracoglu, & Kupeli Akkol, 2020). It has been shown that the elevation of these enzyme activities causes degradation of ECM which leads to loss of skin integrity (Acıkara, Ilhan, Kurtul, Šmejkal, & Küpeli Akkol, 2019). Uncontrolled and excessive hydrolysis of these proteins brings about an increase in wrinkle formation, and eventual skin aging (Buhren et al., 2020; Deniz et al., 2020). On the other hand, tyrosinase, a melanogenic enzyme, participates in melanogenesis (i.e. formation of melanin, which is one of the basic constituents of hair, eye, and skin colour). Additionally, elevated melanin levels and its uncontrolled synthesis can give rise to skin disorders such as acanthosis nigricans, melasma, and senile lentigines (Zolghadri et al., 2019). Hence, the search for new inhibitors of these enzymes so as to preserve the natural structure and maintain a healthy skin continues. In a comprehensive study by Chiocchio et al., (2018), elastase inhibitory activities of a hundred plants were screened and eleven of these plants were shown to strongly inhibit elastase activities. Celik Onar, Yusufoglu, Turker, & Yanardag (2012) revealed that the aqueous extract of *Epilobium angustifolium* L. leaves exerted strong antielastase activity with a low IC_{50} value. It was also reported that the total phenolic and flavonoid contents of the plants were positively correlated with their elastase inhibitory activities. In our study, the aqueous extract of *E. spectabilis* had moderate inhibitory activity against elastase as compared to ursolic acid. These effects of *E. spectabilis* may be attributed to its richness in varied amounts of phytochemical factors, which possibly possess inhibitory properties (Kumud & Sanju, 2018). Moreover, the number of hydroxyl groups of phenolics found in *E. spectabilis* tends to form stronger hydrogen bonds with the enzyme's functional side chain groups. This may have led to the higher inhibitory activity of the extracts on the enzymes (Wittenauer et al., 2015). However, the aqueous extract of *E. spectabilis* had a very weak inhibitory effect against both hyaluronidase and tyrosinase enzymes when compared with their respective standard inhibitors (rutin and kojic acid). The possible explanation for the low inhibitory activities of this plant extract might be associated with secondary metabolites found in *E. spectabilis* which are poorly interacting with the active site of both enzymes.

CONCLUSION

Considering the outcomes, the aqueous extract of *E. spectabilis* clearly exhibited inhibitory activities against both α-amylase and α-glucosidase enzymes. Moreover, the potent inhibitory effect of this herb on these catabolic enzymes in a carbohydrate metabolism may be promising for diabetics in the regulation of postprandial blood sugar. On the other hand, the extract of *E. spectabilis* showed a better inhibitor effect on elastase than that of both hyaluronidase and tyrosinase. More so, it could be suggested that this plant might be of benefit for usage as a raw extract form for skin-related disorders, because of its good inhibitory action on elastase activity. Thus, more supporting researches against antidiabetic effects of *E. spectabilis* both *in vitro* and *in vivo* should be conducted.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- B.B.B., R.Y.; Data Acquisition-B.B.B.; Data Analysis/Interpretation- B.B.B., R.Y.; Drafting Manuscript- B.B.B., R.Y.; Critical Revision of Manuscript- B.B.B., R.Y.; Final Approval and Accountability- B.B.B., R.Y.

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

Financial Disclosure: This study was supported by İstanbul University-Cerrahpaşa Scientific Research Projects Units with the grant number 52245.

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Istanbul J Pharm 51 (3): 365-371
DOI: 10.26650/IstanbulJPharm.2021.1016208
Original Article

Total phenolic, total flavonoid contents, and *in vitro* biological activities of *Cephalaria procera* Fisch. & Ave-Lall.

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Cite this article as: Yazici Bektas, N., Barut, B., Mataraci Kara, E., & Yesil Canturk, Y. (2021). Total phenolic, total flavonoid contents, and *in vitro* biological activities of *Cephalaria procera* Fisch. & Ave-Lall. *İstanbul Journal of Pharmacy, 51*(3), 365-371.

ABSTRACT

Background and Aims: This study aims to determine total phenolic, total flavonoid contents and *in vitro* biological activities of methanol (CEP-1), *n*-butanol (CEP-2), water (CEP-3), *n*-hexane (CEP-4) extracts obtained from *Cephalaria procera*.
Methods: The total phenolic and flavonoid content analysis, *in vitro* DPPH radical scavenging activities, cholinesterase, and tyrosinase inhibitory properties of the extracts were evaluated using spectrophotometric assays. DNA-damage and DNA-damage protective effects of the extracts were examined using agarose gel electrophoresis method. Antimicrobial activities of the extracts were determined by microdilution method.
Results: CEP-3 had the best total phenolic content (79.64±1.11 mg GAE/g dry weight), and CEP-1 had the highest total flavonoid content (15.33±0.27 mg QEE/g dry weight) among tested extracts. CEP-1 showed the highest radical scavenging activity with 83.21±3.20 µg/mL of IC_{50} value. CEP-3 exerted the highest AChE and BuChE inhibitory action with 134.63±4.49 µg/mL and 62.76±0.63 µg/mL of IC_{50} values, respectively. CEP-3 showed significant tyrosinase inhibitory action with 51.95±0.35 µg/mL IC_{50} value compared to kojic acid (58.26±0.25 µg/mL). CEP-1 and CEP-3 were tested, and the both extracts did not damage supercoiled DNA at studied concentrations. Incidentally, results indicated that CEP-1 and CEP-3 protected supercoiled DNA against Fenton's reagents. CEP-4 exhibited the highest antimicrobial activity on *C. tropicalis* with the MIC value of 156.2 µg/mL.
Conclusion: The results showed that crude and subextracts of *C. procera* exerted several moderate activities on tested systems. It suggested that the species might be a promising medicinal plant for the treatment or prevention of several diseases associated with skin damage and wounds.

Keywords: Antioxidant, antimicrobial, anticholinesterase, *Cephalaria procera*, DNA protective, tyrosinase

INTRODUCTION

Natural products are used extensively in drug research, and it is known that many active substances of herbal origin are used use today in modern pharmacotherapy directly or indirectly. According to the World Health Organization, approximately 20.000 plants are still used for treatment today, and approximately 80% of the world population primarily resorts to herbal drugs to eliminate their health problems. In addition, 1881 compounds of natural origin have been approved by the FDA for medical use since 1981, and 25% of pharmaceutical preparations contain active ingredients of plant origin (Faydaoğlu & Sürücüoğlu, 2011; Newman & Cragg, 2020).

The genus *Cephalaria* Schrad. ex Roem. & Schult. is a member of the Caprifoliaceae family. South Africa and the Holarctic Kingdom (from Balkans to West China and from South Ukraine to Middle East) are the main centers of distribution of the genus. It has been

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Submitted: 01.10.2021
Revision Requested: 10.10.2021
Last Revision Received: 25.10.2021
Accepted: 01.11.2021
Published Online: 04.11.2021

determined that 94 species are grown in the world and 39 species in Turkey (Göktürk and Sümbül, 2014). Fresh stems of *Cephalaria procera* Fisch. & Avé-Lall. are used for wound healing and as antihemorrhagic, traditionally. The species is called as Ganteper, Gulinga, Cevrük and Cipreş in Turkey (Özgen, Kaya, & Houghton, 2012; Kahraman et al., 2019).

It has been reported that *Cephalaria* is a rich genus in terms of saponins (Boke Sarikahya, Goren, Sumer Okkali, & Kirmizigul, 2021), phenolic compounds (Chrzaszcz, Krzeminska, Celinski, & Szewczyk, 2021), flavonoids (Godjevac et al., 2004), lignans (Pasi, Aligiannis, Skaltsounis, & Chinou, 2002), triterpene glycosides (Top, Sarikahya, Nalbantsoy, & Kirmizigul, 2017; Böke Sarıkahya & Kirmizigul, 2010), iridoid glycosides (Mustafaeva et al., 2011) as phytochemicals. Studies about biological activities showed that *Cephalaria* species have antioxidant (Böke Sarıkahya, Pekmez, Arda, Kayce, Karabay Yavaşoğlu, & Kirmizigul, 2011; Godjevac et al., 2004), antimicrobial (Böke Sarıkahya, Pekmez, Arda, Kayce, Karabay Yavaşoğlu, & Kirmizigul, 2011, Böke Sarıkahya, & Kirmizigul S, 2010), hemolytic (Top, Sarikahya, Nalbantsoy, & Kirmizigul, 2017), immunomodulatory (Celenk, Boke Sarikahya, & Kirmizigul, 2020; Top, Sarikahya, Nalbantsoy, & Kirmizigul, 2017), and cytotoxic activities (Celenk, Boke Sarikahya, & Kirmizigul, 2020; Pasi, Aligiannis, Skaltsounis, & Chinou, 2002).

In this study, it was aimed to test total phenolic, total flavonoid contents and investigate *in vitro* biological activities of *Cephalaria procera* extracts. To the best of our knowledge, there has not been any study conducted to investigate the cholinesterase and tyrosinase inhibitory activities, antimicrobial activity on yeasts, and supercoiled DNA damage and damage protective effects of *Cephalaria procera*.

MATERIALS AND METHODS

Plant material

The aerial parts of *Cephalaria procera* were collected from Erzurum (Eastern Turkey) by Dr. Yeter Yeşil, Nurdan Yazıcı Bektaş and Burak Bektaş in July 2017. Voucher specimens were authenticated by Dr. Yeter Yeşil. These specimens were deposited at the Herbarium of İstanbul University (ISTE 115 326, ISTE 115 327).

Extraction

Air dried and powdered aerial parts of *Cephalaria procera* were extracted at room temperature with methanol for overnight three times. The methanol extract was concentrated to dryness under reduced pressure. The crude methanol extract (CP-1) dissolved with distilled water and extracted with *n*-butanol using partition method. By this way water extract (CP-3) was obtained. Then the *n*-butanol phase was concentrated and extracted with *n*-hexane. Finally, *n*-butanol (CP-2) and *n*-hexane (CP-4) were obtained, concentrated to dryness, and stored at refrigerator (Top, Sarikahya, Nalbantsoy, & Kirmizigul, 2017).

Total phenolic content analysis

The total phenolic content analyses of the extracts were evaluated utilizing the Folin-Ciocalteu colorimetric assay according to study of Barut & Şöhretoğlu (Barut & Şöhretoğlu, 2020). The results were expressed as mg gallic acid equivalent (GAE) per g of dry weight of the extracts.

Total flavonoid content analysis

The total flavonoid content analyses of the extracts were evaluated aluminium nitrate colorimetric assay (Barut et al., 2017). The results were expressed as mg quercetin equivalent (QEE) per g of dry weight of the extracts. The extracts, 10% aluminium nitrate and 1 M ammonium acetate were added to a tube. The mixtures were incubated for 40 min at room temperature. Afterwards, the absorbance was measured at 415 nm.

In vitro Biological activities

DPPH radical scavenging effects of the extracts

The DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging effects of the extracts were determined using spectrophotometric assay according to previous study conducted by (Barut & Şöhretoğlu, 2020). The results were expressed as IC_{50} (Half-maximal inhibitory concentration) values. Gallic acid (GA) was used as a positive control.

AChE/BuChE inhibitory effects of the extracts

The acetylcholinesterase (AChE) and buthyrylcholinesterase (BuChE) inhibitory effects of the extracts were evaluated using the previously reported method (Barut & Şöhretoğlu, 2020). The results were expressed as IC_{50} values. Galantamine was used as a positive control.

Tyrosinase inhibitory effects of the extracts

The tyrosinase (Tyr) (Sigma, T3824) inhibitory effects of the extracts were evaluated using previous reported method (Barut & Şöhretoğlu, 2020). The results were expressed as IC_{50} values. Kojic acid was used as a positive control.

DNA damage effects of CEP-1 and CEP-3

The supercoiled pBR322 plasmid DNA damage effects of CEP-1 and CEP-3 was determined using agarose gel electrophoresis according to the previous study (Şöhretoğlu, Barut, Sari, Özel, & Arroo, 2020). In this study, Tris-HCl (50 mM, pH 7), plasmid DNA, extracts at various concentrations (50, 100, and 200 µg/mL) was mixed at 37 ºC for 1 h. Afterwards, loading buffer (bromophenol blue, sodium dodecyl sulphate, xylene cyanol, glycerol) was added and the mixtures were loaded on gel (0.8% (m/v)) with ethidium bromide staining for 90 min at 100 V in Tris-acetic acid-EDTA (TAE) buffer. After electrophoresis, gel was visualized and calculated using BioRad Gel Doc XR system and Image Lab Version 5.0.1 software.

DNA damage protective effects of CEP-1 and CEP-3 on Fenton reagents

The supercoiled pBR322 plasmid DNA damage protective actions of CEP-1 and CEP-3 on Fenton's reagents were evaluated using agarose gel electrophoresis (Şöhretoğlu, Barut, Sari, Özel, & Arroo, 2020). In this study, Tris-HCl (50 mM, pH 7), plasmid DNA, H_2O_2 (2%), $FeSO_4$ (1 mM), extracts at various concentrations (50, 100, and 200 µg/mL) was mixed at 37 ºC for 1 h. The electrophoresis studies were performed according to the above method.

Antimicrobial effects of the extracts

The antimicrobial activities of CEP-1, CEP-2, and CEP-3 extracts were determined against a set of microorganisms including *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 14153, *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019, and *Candida tropicalis* ATCC 750 using the broth microdilution technique approved by Clinical Laboratory Standards Institute (CLSI) (CLSI, 1997, 2020)

Cefuroxime, cefuroxime-Na, amikacin, ceftazidime and fluconazole were used as positive control; RPMI-1640 medium for the yeast strain and Mueller-Hinton broth for bacteria were used as negative control.

Statistical analysis

The results were expressed as the mean±SD and were analysed using GraphPad Prism 5.0. In this work, two-way analysis of variance (ANOVA) followed by Bonferroni tests were used as statistical analysis.

RESULTS

Total phenolic and total flavonoid contents of the extracts

In this work, total phenolic and total flavonoid contents of the extracts were investigated and the results were given in Table 1. CEP-3 had the highest total phenolic content with 79.64±1.11 mg GAE/g dry weight among tested extracts. Also, the total phenolic contents of the extracts (CEP-1, CEP-2, and CEP-4) were 68.81±4.11, 13.38±0.82, and 50.05±5.14 mg GAE/g dry weight, respectively. On the other hand, the total flavonoid contents of CEP-1, CEP-2, CEP-3, and CEP-4 were calculated as 15.33±0.27 mg QEE/g dry weight, 2.14±0.50 mg QEE/g dry weight, 11.27±2.21 mg QEE/g dry weight, and 5.25±0.88 mg QEE/g dry weight, respectively.

DPPH radical scavenging effects of the extracts

In this study, DPPH radical scavenging effects of the extracts were determined using a spectrophotometric method. The results were presented in Table 1. CEP-1 showed the highest radical scavenging effect with 83.21±3.20 µg/mL of IC_{50} value among the tested extracts as shown in Table 1. However, CEP-1 found to have less scavenging properties than gallic acid (GA) (IC_{50}=68.25±0.35 µg/mL) which used as a positive control. In addition, the IC_{50} values of CEP-2, CEP-3, and CEP-4 were determined as 264.05±6.52 µg/mL, 89.91±0.13 µg/mL, and 179.02±0.23 µg/mL, respectively.

AChE and BuChE inhibitory effects of the extracts

In this paper, the AChE obtained from *Electrophorus electricus* (electric eel) and BuChE from equine serum inhibitory properties of the extracts were investigated, and the results were shown in Table 2. The IC_{50} value of CEP-3 was 134.63±4.49 µg/mL on AChE, as shown in Table 2. Other extracts have IC_{50}

Table 1. Total phenolic and flavonoid content, and DPPH radical scavenging effects of *C. procera* extracts.

Extracts	Total phenolic content (mg GAE/g extract)	Total flavonoid content (mg QEE/g extract)	DPPH (µg/mL, IC_{50})
CEP-1	68.81±4.11	15.33±0.27	83.21±3.20
CEP-2	13.38±0.82	2.14±0.50	264.05±6.52
CEP-3	79.64± 1.11	11.27±2.21	89.91±0.13
CEP-4	50.05± 5.14	5.25±0.88	179.02±0.23
GA	-	-	68.25±0.35

GAE: Gallic acid equivalent, QEE: Quercetin equivalent, DPPH: 2,2-Diphenyl-1-picrylhydrazyl, GA: Gallic acid, CEP-1: Methanol extract of *C. procera*, CEP-2: *N*- butanol extract of *C. procera*, CEP-3: Water extract of *C. procera*, CEP-4: *N*-hexane extract of *C. procera*

Table 2. AChE, BuChE, and Tyr inhibitory effects of *C. procera* extracts.

Extracts	AChE (µg/mL, IC_{50})	BuChE (µg/mL, IC_{50})	Tyr (µg/mL, IC_{50})
CEP-1	>200	73.16±1.94	$56.13±1.17^{ns}$
CEP-2	>200	87.07±1.88	100.19±2.00
CEP-3	134.63±4.49	62.76±0.63	51.95±0.35**
CEP-4	>200	78.32±3.58	63.55±2.75
Galantamine	20.30±0.25	36.05±0.18	-
Kojic acid	-	-	58.26±0.25

AChE: Acetylcholinesterase, BuChE: Buthyrylcholinesterase, Tyr: Tyrosinase, CEP-1: Methanol extract of *C. procera*, CEP-2: *N*- butanol extract of *C. procera*, CEP-3: Water extract of *C. procera*, CEP-4: *N*-hexane extract of *C. procera*
*Values expressed are means±standard deviation of three parallel measurements, **p<0.001
ns: not significant vs positive control.

values higher than 200 µg/mL. On the other hand, CEP-3 had the highest BuChE inhibition with 62.76±0.63 µg/mL of IC_{50} value followed by CEP-1 with 73.16±1.94 µg/mL. However, galantamine (IC_{50}=20.30±0.25 for AChE, 36.05±0.18 for BuChE) which was used as a positive control, had higher inhibition than CEP-3 on AChE and BuChE.

Tyrosinase inhibitory effects of the extracts

In this study, the *in vitro* tyrosinase inhibitory properties of the extracts were determined using a spectrophotometric assay. The results were tabulated in Table 2. The IC_{50} value of CEP-3 was 51.95±0.35 µg/mL on Tyr. CEP-3 showed significant inhibitory action when compared to kojic acid (58.26±0.25 µg/mL) against Tyr ($p<0.001$). CEP-1 had similar inhibitory effect with kojic acid according to the their IC_{50} values.

Supercoiled DNA damage effects of CEP-1 and CEP-3

Supercoiled pBR322 plasmid DNA damage effects of CEP-1 and CEP-3 which were the most potent radical scavenging extracts, were evaluated using agarose gel electrophoresis. The results were given in Figure 1. It is well-known that plasmid DNA has three forms on gel: form I (supercoiled form moves the fastest); form II (nicked form); form III (linear form moves the slowest). As shown in Figure 1 (lane 1), the percentage of form I was about 75%. At increasing concentrations CEP-1 and CEP-3, the amounts of form I did not change significantly, and they were determined as about 70-75%. These results showed that both extracts did not show any damage effects on supercoiled pBR322 plasmid DNA at studied concentrations.

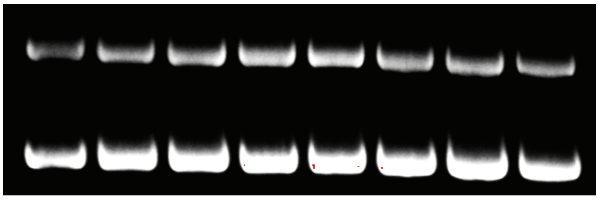

Figure 1. DNA damage effects of the extracts. Lane 1: DNA control; lane 2-4: DNA+ (50, 100, and 200 µg/mL of CEP-1); lane 5-7: DNA+ (50, 100, and 200 µg/mL of CEP-3).

Supercoiled DNA damage protective effects of CEP-1 and CEP-3 on Fenton's reagents

Supercoiled pBR322 plasmid DNA damage protective effects of CEP-1 and CEP-3 against Fenton's reagents were investigat-

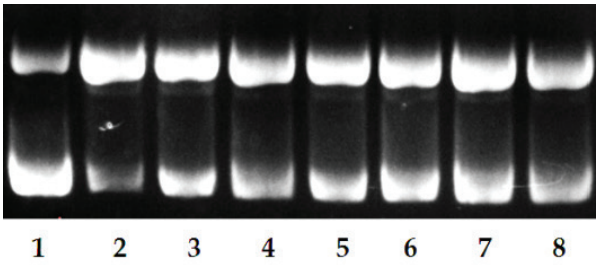

Figure 2. DNA damage protecitve effects of the extracts against Fenton reagents. Lane 1: DNA control; lane 2: DNA+$FeSO_4$+H_2O_2; lane 3-5: DNA+$FeSO_4$+H_2O_2+ (50, 100, and 200 µg/mL of CEP-1); lane 6-8: DNA+$FeSO_4$+H_2O_2+ (50, 100, and 200 µg/mL of CEP-3).

Table 3. The antimicrobial activities of the extracts (MIC values µg/mL).

	Microorganisms											
	Gram-negative Bacteria					Gram-positive Bacteria				Fungi		
	P. aeruginosa	*E. coli*	*K. pneumoniae*	*P. mirabilis*	*E. faecalis*	*S. epidermidis*	*S. aureus*	*C. albicans*	*C. parapsilosis*	*C. tropicalis*
CEP-1	-	-	-	-	-	-	-	-	-	-
CEP-2	-	-	-	-	-	-	-	-	-	-
CEP-3	-	312.5	-	-	-	-	-	-	-	-
CEP-4	-	312.5	-	-	-	-	-	-	-	156.2
Reference antimicrobials	2.4 Ceftazidime	4.9 Cefuroxime-Na	4.9 Cefuroxime-Na	2.4 Cefuroxime-Na	128 Amikacin	9.8 Cefuroxime	1.2 Cefuroxime-Na	1 Fluconazole	2 Fluconazole	4 Fluconazole

CEP-1: Methanol extract of *C. procera*, CEP-2: *N*- butanol extract of *C. procera*, CEP-3: Water extract of *C. procera*, CEP-4: *N*-hexane extract of *C. procera*, *P. aeruginosa*: *Pseudomonas aeruginosa* ATCC 27853, *E. coli*: *Escherichia coli* ATCC 25922, *K. pneumoniae*: *Klebsiella pneumoniae* ATCC 4352, *P. mirabilis*: *Proteus mirabilis* ATCC 14153, *E. faecalis*: *Enterococcus faecalis* ATCC 29212, *S. epidermidis*: *Staphylococcus epidermidis* ATCC 12228, *S. aureus*: *Staphylococcus aureus* ATCC 29213, *C. albicans*: *Candida albicans* ATCC 10231, *C. parapsilosis*: *Candida parapsilosis* ATCC 22019, *C. tropicalis*: *Candida tropicalis* ATCC 750.
-: No Activity

ed using agarose gel electrophoresis. The results were presented in Figure 2. Supercoiled pBR322 plasmid DNA in buffer (including %0.1 DMSO) was used as a negative control and forms I and II was determined as 77.40% and 22.60%, respectively (Figure 2, lane 1). As shown in Figure 2, when Fenton's reagent was added, supercoiled DNA was damaged (Form I: %74.80; Form II: %25.20). When increasing concentrations of CEP-1 and CEP-3 are added into mixture, supercoiled pBR322 plasmid appears to be preserved. Addition of 200 µg/mL of CEP-1 and CEP-3, the amounts of Form I increased from 22.90% to 43.80% and 45.60%, respectively (Figure 2, lanes 5 and 8). These results indicated that CEP-1 and CEP-3 protected supercoiled pBR322 plasmid DNA on Fenton's reagents.

Antimicrobial activities of the extracts

All the *in vitro* antimicrobial activity results of the tested extracts are given in Table 3. Different concentrations from 1.22 to 2500 µg/mL concentrations of CEP-1 and CEP-2 were tested and none of them showed any activity. However, CEP-3 and CEP-4 exhibited moderate *in vitro* antibacterial activity against *E. coli*. Moreover, CEP-4 displayed intense antifungal activity against *C. tropicalis*. According to antifungal screening results, only CEP-4 was active extract against tested *Candida* species.

DISCUSSION

In recent years, natural antioxidants, especially polyphenols, have been notable agents for the treatment of many chronic diseases such as cancer, cardiovascular diseases, diabetes mellitus etc (AlFaris et al., 2021). To the best of our knowledge, there has not been any study conducted to investigate the anti-cholinesterases, anti-tyrosinase, DNA damage, and DNA damage protective activities of *C. procera*. In this paper, total phenolic contents of the extracts ranged from 79.64±1.11 to 13.38±0.82 mg GAE/g dry weight. The results showed that *n*-butanol extract has the highest total phenolic content among the tested extracts. Sarikahya et al. reported that the total phenolic content of *n*-hexane extract was found to be 1.561±0.042 mg GAE/g extract (Sarikahya et al. 2015). These results showed that extracts of this study had higher total phenolic contents than Boke Sarikahya's reports. On the other hand, CEP-1 had the best total flavonoid content than other extracts according to the Table 1.

The DPPH assay is a low cost, short time, and simple spectrophotometric method to understand scavenging effects of natural or synthetic compounds (Akar, Küçük & Doğan, 2017). This assay is based on single electron transfer and hydrogen atom transfer that produces a violet solution (Liang & Kitts, 2014). In this work, CEP-1 had the best radical scavenging properties following by CEP-3, shown as Table 1. The results of total phenolic/flavonoid contents and DPPH radical scavenging studies were found to be compatible. Godjevac and co-authors reported the DPPH radical scavenging activity of the flavonoids isolated from the flowers of *C. pastricensis* (Godjevac et al., 2004). Sarikahya et al. reported that the *n*-hexane extract of DPPH radical scavenging from *C. procera* determined as 6.938±2.56 mg/mL of IC_{50} value (Sarikahya et al., 2015). According to the literatures, *C. procera* contains kaempferol, astragalin, tiliroside, quercimeritrin, gigantoside A, hyperoside, quercitrin, apigenin, luteolin, cynaroside, cyanidin-3-O-glucoside (Sarıkahya & Kirmizigul, 2012; Sarıkahya, Goren & Kirmizigul, 2019). These compounds can be responsible for the antioxidant activities of this plant.

Alzheimer's disease (AD), a type of dementia, is the most common form of neurodegenerative disease. Although the pathophysiology of AD has not been clearly established, the cholinergic hypothesis is one of the most accepted causes. According to the cholinergic hypothesis, AD is associated with alterations of cholinergic markers such as cholinesterases (Tuğrak, Gül & Gülçin, 2020; Kahraman et al., 2019). IC_{50} values of the extracts were above 200 µg/mL on AChE for all extracts, while IC_{50} values for BuChE were determined as below 100 µg/mL. CEP-3 showed the highest AChE and BuChE inhibitory effects. The results demonstrated that the extracts showed moderate inhibition on BuChE and low inhibition against AChE.

Tyrosinase contains two copper atoms in its active site, and it is a metalloenzyme belonging to the oxidoreductase. It commonly is found in mammals, plants, insects, fungi, and bacteria (Şöhretoğlu, Sari, Barut & Özel, 2018). Tyrosinase forms melanin pigment from monophenols with many reactions. The excessive formation of melanin pigment causes various problems such as hyperpigmentation, age spots, melanoma etc. (Şöhretoğlu, Sari, Barut & Özel, 2018). CEP-3 showed more significant tyrosinase inhibitory effects than kojic acid as a positive control ($p<0.001$) in this study. Studies in the literature show that antioxidant compounds have tyrosinase inhibitory properties (Wang et al., 2018; Morais et al., 2018; Sun, Guo, Zhang & Zhuang, 2017). In this study, we determined that extracts with a high antioxidant effect showed high tyrosinase inhibition.

The supercoiled DNA damage actions of CEP-1 and CEP-3 were determined by agarose gel electrophoresis. In this paper, CEP-1 and CEP-3 extracts were used due to their antioxidant potentials. As presented in Figure 1, the amounts of form I were similar percentages. The results showed that both extracts did not damage supercoiled plasmid DNA at increasing concentrations.

When $FeSO_4$ and H_2O_2 are mixed, a hydroxyl radical is formed, and the resulting hydroxyl radical could trigger biological damage such as DNA damage (Barut, Barut, Engin, Özel & Sezen, 2019). In this work, when Fenton's reagent was added to supercoiled DNA, the amount of form II was determined as 74.80%. On the increasing concentrations of extracts, the amount of form II decreased, and form I increased. The obtained results pointed that both extracts preserved supercoiled pBR322 plasmid DNA against Fenton's reagents.

In antimicrobial activity studies, the antimicrobial potential of the tested extract was examined by MIC method. While CEP-3 and CEP-4 showed antibacterial activity against *E. coli*, CEP-1 and CEP-2 did not show *in vitro* activity against all the studied strains. In contrast to our results, Sarıkahya et al. (Böke Sarıkahya, Pekmez, Arda, Kayce, Karabay Yavaşoğlu, & Kirmizigul, 2011) found strong *in vitro* antibacterial activity against a panel of bacteria including *S. aureus*, *S. epidermidis*, *E. coli*, *E. fae-*

calis, *P. aeruginosa* and *K. pneumoniae* with the pure chemical constituents of *Cephalaria* species in Anatolia. The differences between these results could be explained by using different *Cephalaria* species and using total or pure contents of the prepared different extracts. On the other hand, although only CEP-4 exhibited excellent antifungal activity against *Candida tropicalis*, this is the first report on the *in vitro* antifungal activity of the *Cephalaria* procera total extracts.

CONCLUSION

Cephalaria procera is used for wound healing, and as antihemorrhagic in Anatolia, traditionally. This study investigated total phenolic and flavonoid content, DPPH radical scavenging, supercoiled DNA damage/damage protective effects, AChE/BuChE, tyrosinase inhibitory, antimicrobial activities of CEP-1, CEP-2, CEP-3 and CEP-4 obtained from *Cephalaria procera*. The investigations showed that CEP-1 and CEP-3 had better activities on DPPH radical scavenging, tyrosinase enzyme inhibition, DNA damage/DNA damage protection test systems, while they had higher contents of total phenolic, and flavonoid compared to other extracts. These results suggested that crude methanol and water extracts of *C. procera* might have a promising potential for the treatment of several disorders associated with skin damage, and further studies are required to confirm these used test systems and mechanisms of action.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- N.Y.B., B.B., E.M.K., Y.Y.C.; Data Acquisition- N.Y.B., B.B., E.M.K., Y.Y.C.; Data Analysis/Interpretation- N.Y.B., B.B., E.M.K., Y.Y.C.; Drafting Manuscript- N.Y.B., B.B., E.M.K..; Critical Revision of Manuscript- N.Y.B., B.B., E.M.K., Y.Y.C.; Final Approval and Accountability- N.Y.B., B.B., E.M.K., Y.Y.C.

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

Financial Disclosure: Authors declared no financial support.

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Istanbul J Pharm 51 (3): 372-377
DOI: 10.26650/IstanbulJPharm.2021.846741
Original Article

Chemical profiling and cytotoxic activity of aqueous extract of *Veronica peduncularis* M.Bieb.: A chemotaxonomical approach

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Cite this article as: Dogan, Z., Genc, Y., Harput, U. S., Karadeniz Pekgoz, A., & Saracoglu, I. (2021). Chemical profiling and cytotoxic activity of aqueous extract of *Veronica peduncularis* M.Bieb.: A chemotaxonomical approach. *İstanbul Journal of Pharmacy, 51*(3), 372-377.

ABSTRACT

Background and Aims: Genus *Veronica* (Plantaginaceae) is represented by 79 species in Turkish flora, 26 of which are endemic. *Veronica* species have a variety of uses including diuretic, anticancer, and rheumatic pains, wounds, and respiratory problems. According to phytochemical studies, *Veronica* species contain predominantly iridoid glucosides with some phenylethanoid and flavonoid glycosides.
Methods: The aqueous extract of *Veronica peduncularis* M.Bieb. was tested for its cytotoxic activity on human rhabdomyosarcoma (RD) and human epidermoid carcinoma (HEp-2) cell lines using the MTT method. Chemical profile of the extract was determined by HPLC-DAD, and isolation studies were conducted.
Results: The extract was found to show concentration-dependent cytotoxicity against tested cell lines. In addition, a comparison of the iridoid fraction of *Veronica peduncularis* with previously isolated iridoid glucosides on the HPLC-DAD system, showed the presence of aucubin, amphicoside, veratroyl catalpol, and veronicoside in this fraction. On the other hand, isolation and structure elucidation of plantamajoside and 4′-*O*-methylisoscutellarein-7-O-2″-O-(6‴-O-acetyl-*ß*-*D*-allopyranosyl)-*ß*-*D*-glucopyranoside from the phenolic fractions were performed by serial chromatographic and spectroscopic methods.
Conclusion: To the best of our knowledge, this is the first cytotoxic activity and phytochemical study on the titled plant. The presence of iridoid glucosides and 8-hydroxyflavone glycosides is important for the chemotaxonomy of the genus *Veronica*.

Keywords: 4′-*O*-methylisoscutellarein-7-*O*-2″-*O*-(6‴-*O*-acetyl-*ß*-*D*-allopyranosyl)-*ß*-*D*-glucopyranoside, HPLC, iridoid glucosides, phenolics, plantamajoside, *Veronica*

INTRODUCTION

Genus *Veronica* formerly a member of the Scrophulariaceae family, was moved to Plantaginaceae after phylogenetic and chemotaxonomic studies (Olmstead, 2002). The genus is represented by 79 species in the flora of Turkey, 26 of which are endemic (Fischer, 1978).

Veronica species have been used as a diuretic, expectorant, antiscorbutic, and in the treatment of rheumatic pains, wounds, cough, and influenza in Turkish traditional medicine (Baytop, 1999; Fujita et al., 1995; Harput, Genc, Khan, & Saracoglu, 2011).

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Submitted: 25.12.2020
Revision Requested: 24.07.2021
Last Revision Received: 23.08.2021
Accepted: 16.09.2021
Published Online: 00.00.0000

Additionally, there are various uses worldwide as an anticancer agent (Graham, Quinn, Fabricant, & Farnsworth, 2000), against hemoptysis, laryngopharyngitis, and hernia (Salehi et al., 2019), irregular menstruation, and embolism (Xue, Chen, Zhang, & Li, 2019). Phytochemical studies on the genus *Veronica* showed the presence of many iridoid glycosides, most of which are benzoic and cinnamic acid esters of catalpol, in addition to the presence of phenylethanoids and flavonoid glycosides (Harput et al., 2011; Saracoglu, Oztunca, Nagatsu, & Harput, 2011; Saracoglu, Varel, Harput, & Nagatsu, 2004). Biological activity studies on *Veronica* extracts showed their antimicrobial, antioxidant, cytotoxic, anti-tumour, anti-inflammatory activities via *in vitro* and *in vivo* experiments (Harput, Saracoglu, Inoue, & Ogihara, 2002; Salehi et al., 2019). Additionally, it is thought that *Veronica* can be used as a natural food preservative, considering its antimicrobial effects (Salehi et al., 2019).

In our previous studies, water extract from *V. peduncularis* was tested for its radical scavenging activity against different radicals such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), superoxide (SO) and nitric oxide (NO) radicals, spectroscopically. Concentration dependent radical scavenging activity was observed and the results were found to be comparable to reference compounds; ascorbic acid, quercetin and BHA. According to this study, IC_{50} values of aqueous fraction were found as 54.2, 186.3, and 770 µg/mL for DPPH, NO and SO, respectively. *V. peduncularis* showed the highest activity against DPPH radical among the tested free radicals. In addition, gallic acid equivalent total phenolic contents of the plant were also determined using Folin-Ciocalteau reagent, and found as 139.9 mg/g dry extract (Harput, Karadeniz, Genc, & Saracoglu, 2009).

It has been shown that free radicals damage DNA and this damage can cause many diseases, including cancer (NCI, 2020). Based on the moderate radical scavenging effect of this genus and their usage against cancer in traditional medicine, in the present study *V. peduncularis* was investigated for its cytotoxic effects. On the other hand, it is known that some of the iridoid glucosides, flavonoids, and phenyletanoid glycosides may be responsible for the anticancer properties of plants (Saracoglu & Harput, 2012; Saracoglu, Inoue, Calis, & Ogihara, 1995; Yin et al., 2016). Furthermore, iridoid glucosides are characteristic of the genus *Veronica* and play an important role in the reclassification of the genera in the families of Scrophulariaceae and Plantaginaceae (Jensen, Albach, Ohno, & Grayer, 2005). Therefore, in the current study, the cytotoxic effect of aqueous extract of *Veronica peduncularis* was evaluated in addition to the determination of the chemical profile of the extract by HPLC-DAD for iridoid glucosides and isolation studies for phenolic compounds.

MATERIALS AND METHODS

Plant materials

Veronica peduncularis Bieb. (Plantaginaceae) was collected from Macka, Trabzon, Turkey. A voucher specimen (HUEF 09012) was deposited in the Herbarium of the Faculty of Pharmacy. The authentication of plant specimens was made by Serdar Aslan, Duzce University, Duzce, Turkey (previous address: Gazi University, Ankara, Turkey).

General

Polyamide (50–160 µm, Fluka, Seelze, Germany), Sephadex LH-20 (GE Healthcare, Chicago, IL, USA), and thin-layer chromatography (TLC) plate (Kieselgel 60 F254, 0.20mm, Merck, Darmstadt, Germany) were used in chromatography system. In vacuum liquid chromatography (VLC), samples were chromatographed on LiChroprep C18 (40–63 µm, Merck, Darmstadt, Germany). Minimum essential medium Eagle with Earl's salts (MEM) and Dulbecco's phosphate-buffered saline (DPBS) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Antibiotics (penicillin and streptomycin) and fetal bovine serum (FBS) and were purchased from Biochrom AG (Berlin, Germany).

Preparation of extract and isolation of compounds

The air-dried aerial parts of *V. peduncularis* (185 g) were extracted with 1 L MeOH for four times at 40°C. The combined extracts were evaporated under vacuum to give 45.8 g of crude MeOH extract. MeOH extract was dissolved in water and partitioned with petroleum ether to remove chlorophylls and other lipophilic compounds. The aqueous fraction (36.2 g) was subjected to polyamide column chromatography eluting with increasing concentrations of MeOH. Fraction A (Fr. A, 9.8 g) eluted with H_2O was determined as iridoid fraction according to TLC characteristics. Fr. A was dissolved in water and partitioned with *n*-butanol to remove the sugar part of the fraction. The refined iridoid fraction was subjected to analytical HPLC analysis to determine the iridoid composition of the plant using previously isolated iridoid glucosides as reference compounds. Fr. B (0.9 g) eluted with 25% methanol was subjected to vacuum liquid chromatography (45 g using CH_3OH: H_2O, 0%→70%) to get 6 sub-fractions, Frs. B1-B6. Fr. B5 was applied to Sephadex LH 20 column (100% methanol) to get plantamajoside (10.2 mg). Fr. D (0.5 g) eluted with 75% methanol was subjected to vacuum liquid chromatography (45 g using CH_3OH: H_2O, 20%→70%) to obtain 15 sub-fractions, Frs D1-D15. For purification of 4′-*O*-methylisoscutellarein-7-*O*-2″-*O*-(6‴-*O*-acetyl-*β*-*D*-allopyranosyl)-*β*-*D*-glucopyranoside (7.8 mg), Fr. D12 was chromatographed on Sephadex LH 20 column (100% methanol).

Plantamajoside (5): White amorphous powder. UV λ_{max} (MeOH) nm: 291, 330. NMR data are consistent with the literature (Kawada, Yoneda, Asano, Kan-No, & Schmid, 2006; Ravn, Nishibe, Sasahara, & Li, 1990; Zou et al., 2008).

4′-*O*-Methylisoscutellarein-7-*O*-2″-*O*-(6‴-*O*-acetyl-*β*-*D*-allopyranosyl)-*β*-*D*-glucopyranoside (6): Pale yellow amorphous powder. UV λ_{max} (MeOH) nm: 276, 303, 325. NMR data are consistent with the literature (Albach, Grayer, Jensen, Ozgokce, & Veitch, 2003; Lenherr, Lahloub, & Sticher, 1984; Saracoglu, Harput, & Ogihara, 2004).

Determination of cytotoxicity by MTT method

In the determination of the cytotoxic activity of the extract, MTT [3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide] method developed by Mossman was used (Moss-

man, 1983). Activity studies were performed on HEp-2 (Human Epidermoid Carcinoma) and RD (Human Rhabdomyosarcoma) series, which are cancer cells of human origin. Cells were incubated in MEM containing 10% fetal bovine serum and 1% penicillin/streptomycin solution. 100 µL cell suspensions at a concentration of $1x10^5$ cells/mL for RD and HEp-2 were transferred to 96 well plates. After incubating for 24 hours in an incubator containing 5% CO_2, 95% humidity at 37°C, supernatant in the wells was aspirated and 100 µL of extract solutions (200 and 400 µg/mL) in the medium was added and kept in the incubator for another 48 hours. At the end of the period, the wells were washed with 100 µL of medium and then 100 µL of fresh medium was added to each well. 10 µL of MTT solution in PBS (phosphate-buffered saline) at a concentration of 5 mg/mL was added to each well and the wells were incubated for another 4-6 hours. During the incubation, the dehydrogenase enzyme in living cells reduced MTT to the purple formazan crystals. After incubation, 100 µL of 10% SDS (Sodium dodecyl sulfate) solution was added to the wells and incubated for 20 hours to dissolve the formazan crystals. The absorbance was measured at 577/655 nm (Harput, Genc, & Saracoglu, 2012; Saracoglu & Harput, 2012).

HPLC analysis

HPLC analyses were carried out on a Dionex HPLC instrument system (Thermo Fisher Scientific, Waltham, MA, USA): P680 HPLC pump, Dionex ASI-100 autosampler, and Dionex Photodiode Array Detector. The column was Hichrom-Nucleosil 100-5 C18 (5 µm, 250 mm X 4.6 mm, Berks, UK) and the column temperature was maintained at 27°C. 20 µL injection volume and 1 mL/min flow rate were used for each experiment. Samples were passed through a 0.45 µm filter and injected into the HPLC system. The mobile phase consisted of phosphoric acid (1%) in water (solution A), and acetonitrile (solution B). The gradient system developed by authors was used for elution of samples as 95% A, 5% B for 0–15 min; 80% A, 20% B at 20th min; 70% A, 30% B at 45th min, and then 67% A, 33% B at 52nd min.

RESULTS AND DISCUSSION

In this study, the cytotoxic activity of *V. peduncularis* was evaluated on two cancer cell lines; HEp-2 and RD. The aqueous extract was tested at different concentrations, 200 and 400 µg/mL. While the extract showed slight cytotoxicity on HEp-2 cells with the cell viability value of 89.87% ± 3.6 and 36.66% ± 2.7, it showed moderate cytotoxicity with 85.62% ± 3.8 and 8.43% ± 3.2 against RD cells at 200 and 400 µg/mL, respectively (Figure 1). According to Saracoglu et al. (2011), cytotoxic activities of *V. cuneifolia* subsp. *cuneifolia* D. Don and *V. cymbalaria* Bodard aqueous extracts were tested on the same cell line, IC_{50} values of extracts were found between 250.4-546.5 µg/mL with similar results to our results (IC_{50}: 230 and 390 µg/mL for RD and HEp-2, respectively). According to Harput et al. (2002), five different *Veronica* species; *V. polita* Fries, *V. persica* Poiret, *V. hederifolia* L., *V. pectinata* L. var. *glandulosa* Riek ex M.A., *V. cymbalaria* were tested for their cytotoxic effects on KB epidermoid carcinoma and B16 melanoma cells. In that study, it was found that chloroform fractions of methanolic extracts were more potent than the main methanolic extracts, although water-soluble portions didn't exhibit cytotoxic activity on the two tested cell lines.

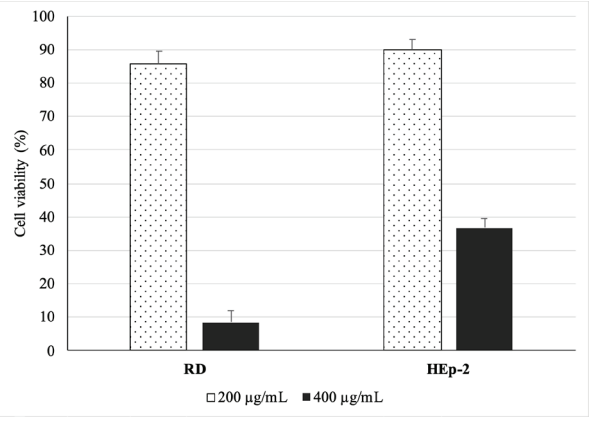

Figure 1. Cytotoxic activity of *V. peduncularis* aqueous extract.

Main iridoid glucosides of the iridoid fraction of the extract were determined with a specific HPLC-DAD method. The previously isolated iridoid glucosides from *V. cymbalaria* and *V. cuneifolia subsp. cuneifolia*, whose structures were elucidated by advanced NMR techniques, were used as reference (Saracoglu et al., 2011) (Figure 2). The comparison of the iridoid fraction with the reference compounds showed the presence of aucubin (**1**), amphicoside (**2**), veratroyl catalpol (**3**), and veronicoside (**4**) in this fraction (Figure 3). Their presences were confirmed by comparing their retention time and UV spectra with those of reference compounds (Figure 4). Iridoid glucosides are the most abundant constituents in *Veronica* species (Xue et al., 2019).

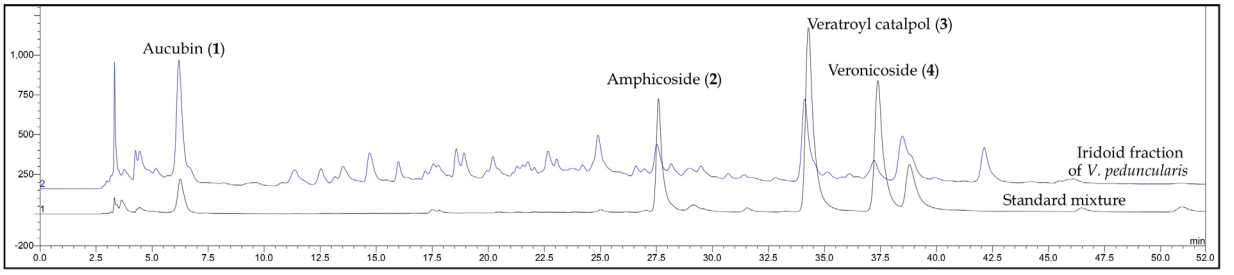

Figure 2. HPLC chromatogram of iridoid fractions from *V. peduncularis* and the mixture of reference iridoid glucosides (aucubin, amphicoside, veratroyl catalpol and veronicoside) at 200 nm.

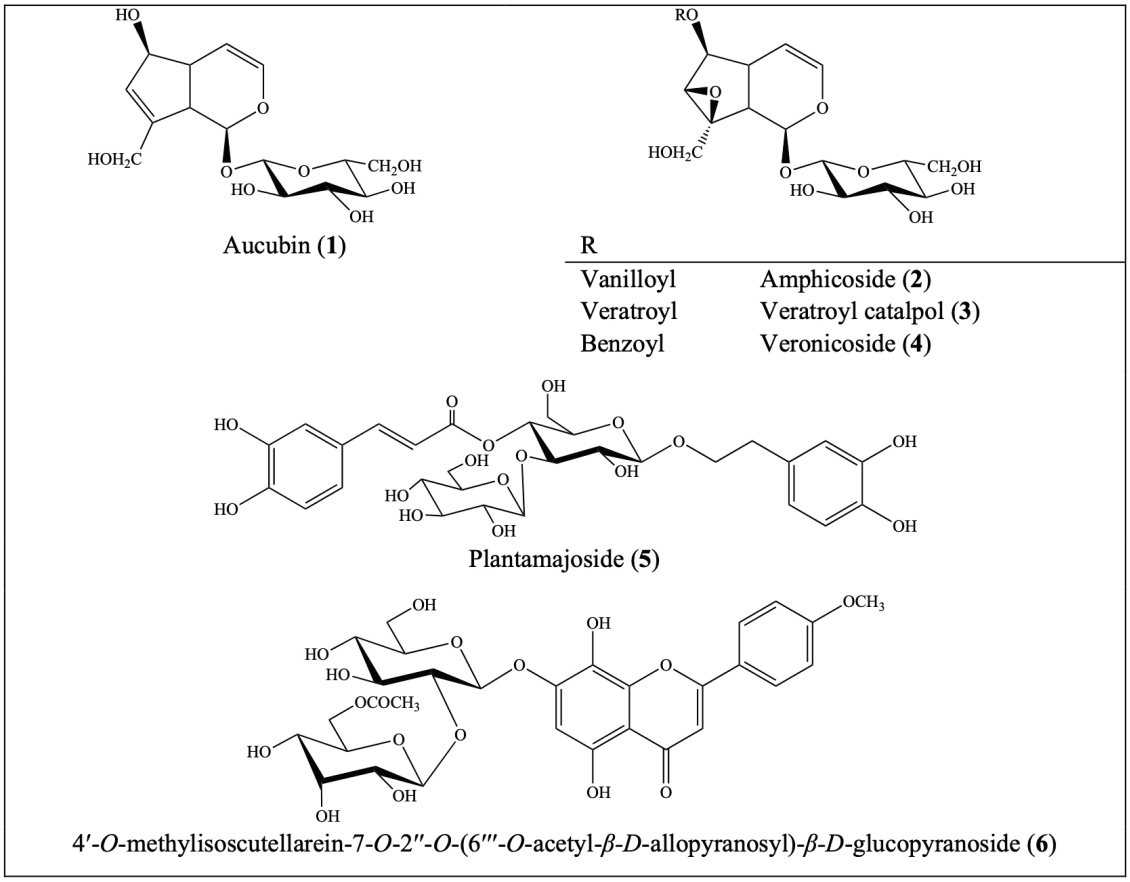

Figure 3. Structures of the compounds **1**-**6** determined and isolated from *V. peduncularis*.

Veronica species are classified into 4 sections; Chamaedrys, Alsinebe, Beccabunga, and Veronicastrum. *V. peduncularis* belongs to section Chamaedyrs (Fischer, 1978). It is known that 4-substituted iridoid glucosides are absent in section Chamaedrys (Saracoglu et al., 2011; Taskova, Albach, & Grayer, 2004). In the present study, the HPLC results also support this knowledge about the lack of such molecules in section Chamaedrys.

Besides the chemotaxonomic importance of iridoid glucosides, they have also various biological effects as antitumor, anti-inflammatory, hepatoprotective, neuroprotective, hypoglycemic, hypolipidemic (Saracoglu & Harput, 2012; Wang et al., 2020). In the current study, the cytotoxic activity of the aqueous extract was studied and the iridoid glucosides profile of the extract was determined. Iridoid glycosides may contribute to the cytotoxic effect of the extract. According to Saracoglu and Harput (2012), while aucubin had no activity on RD and HEp-2 cells, veratroyl catalpol showed cytostatic activity. Veronicoside exhibited a cytotoxic effect on RD and HEp-2 cell lines with IC_{50} values of 153.3 and 355 µM, respectively. Moreover, amphicoside showed cytotoxic effect only on HEp-2 cell line with IC_{50} values of 340 µM. In another study, amphicoside had stronger anti-hepatocarcinoma activity on the Hep-G2 cell line than the reference compound, 5-fluorouracil. On the other hand, veronicoside showed strong cytotoxic activity on the proliferation of Hep-G2 cells (Yin et al., 2016).

Phenolics naturally occurring in plants are potential antioxidant compounds and important for the chemotaxonomy of genus *Veronica*. They may possess cytotoxic activity via their radical scavenging or prooxidant effects (Harput et al., 2012). For that reason, it is important to identify the phenolic profile of *V. peduncularis*. As a result of our isolation studies on phenolic fractions, two compounds; plantamajoside (**5**) and 4′-*O*-methylisoscutellarein-7-*O*-2″-O-(6‴-*O*-acetyl-*β*-*D*-allopyranosyl)-*β*-*D*-glucopyranoside (**6**) were isolated from aqueous extract of *V. peduncularis* (Figure 3). Compound **6** is an acylated 8-hydroxyflavone glycoside. It has been previously reported that allose-containing acylated 8-hydroxyflavone glycosides are important as chemotaxonomic markers, especially for section Alsinebe and section Chamaedyrs (Albach, Jensen, Ozgokce, & Grayer, 2005; Tomas-Barberan, Grayer-Barkmeijer, Gil, & Harborne, 1988). The presence of these types of compounds in *V. peduncularis* (Section Chamaedyrs) was detected chromatographically previously, however, this is the first isolation study of compounds (**5** and **6**) from *V. peduncularis* (Albach et al., 2005; Tomas-Barberan et al., 1988). Compound **5** was previously isolated from *V. orsiniana* Ten. (Sin: *V. fuhsii*) (Ozipek, Saracoglu, Kojima, Ogihara, & Calis, 1999), and *V. beccabunga* L. (Jensen, Opitz, & Gotfredsen, 2011). Compound **6** was isolated from another *Veronica* species; *V. pectinata* var. *glandulosa* (Saracoglu, Harput, et al., 2004) and *V. orientalis* (Albach et al., 2003).

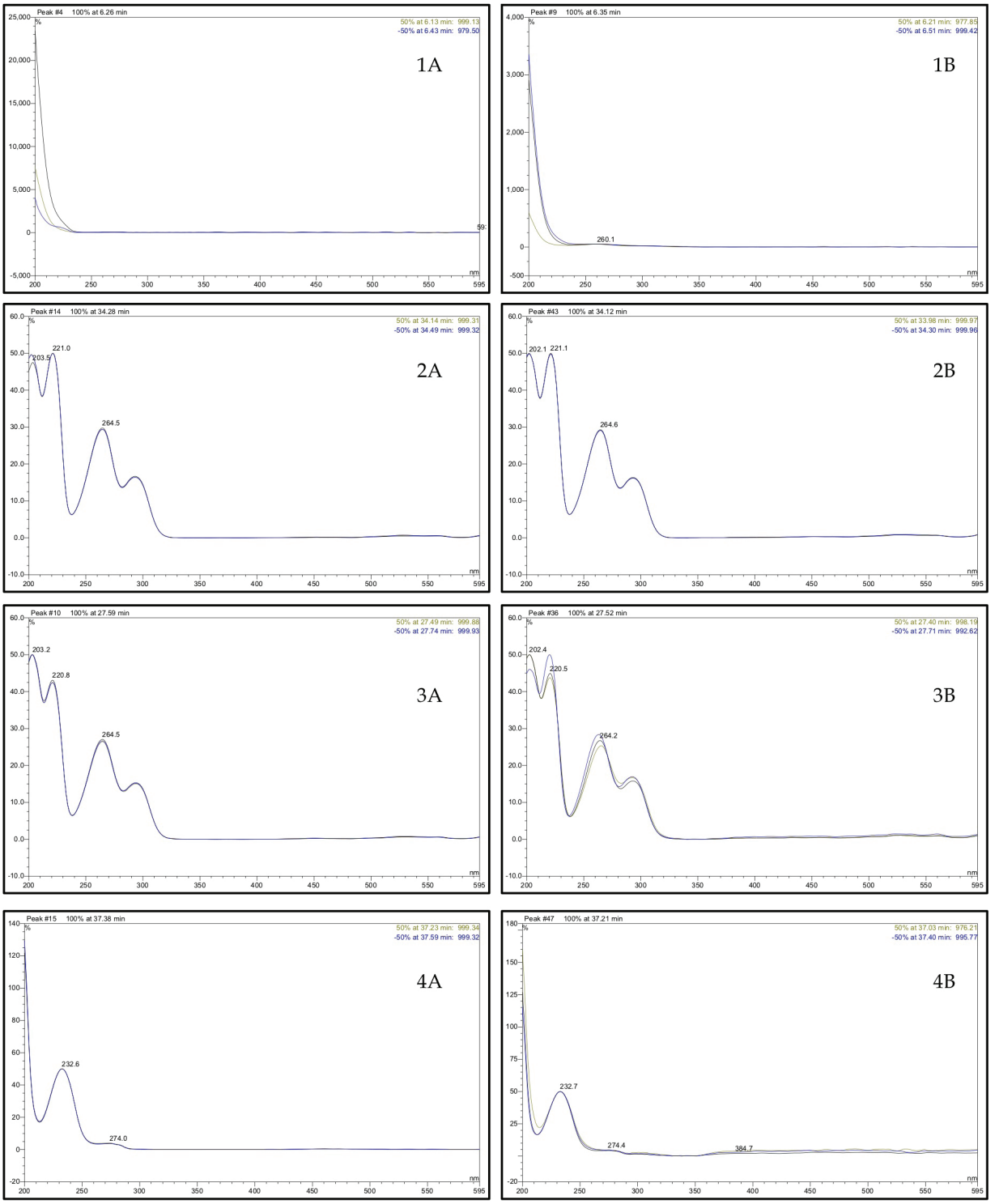

Figure 4. UV spectra of reference compounds (1A-4A) and iridoid glucosides in iridoid fraction of *V. peduncularis* (1B-4B).

CONCLUSIONS

Cytotoxic activity of *V. peduncularis* was performed on HEp-2 and RD cancer cell lines. Four iridoid glucosides, aucubin (**1**), amphicoside (**2**), veronicoside (**3**), and veratroyl catalpol (**4**) were determined from iridoid fraction of *V. peduncularis* by the HPLC-DAD method. Isolation of plantamajoside (**5**) and 4′-*O*-methylisoscutellarein-7-*O*-2″-*O*-(6‴-*O*-acetyl-*β*-*D*-allopyranosyl)-*β*-*D*-glucopyranoside (**6**) were also performed. To the best of our knowledge, this is the first cytotoxic activity and phytochemical study on the titled plant. The current study contributed to chemotaxonomic studies on *Veronica* species in terms of iridoid glucosides and 6-hydroxyflavone glycosides.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- Ü.Ş.H., İ.S.; Data Acquisition- Z.D., Y.G., Ü.Ş.H., A.K.P.; Data Analysis/Interpretation- Z.D., Ü.Ş.H., A.K.P., İ.S.; Drafting Manuscript- Z.D., Ü.Ş.H., İ.S.; Critical Revision of Manuscript- Z.D., Y.G., Ü.Ş.H., A.K.P., İ.S.; Final Approval and Accountability- Z.D., Y.G., Ü.Ş.H., A.K.P., İ.S.

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

Financial Disclosure: This study was supported by by The Scientific and Technological Research Council of Turkey TUBITAK with the grant number 108T518.

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Istanbul J Pharm 51 (3): 378-385
DOI: 10.26650/IstanbulJPharm.2021.908035
Original Article

Chemical compositions of *Sideritis albiflora* Hub. – Mor.

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Cite this article as: Kirici, D., Saltan, N., Goger, F., Kose, Y. B., & Demirci, B. (2021). Chemical compositions of *Sideritis albiflora* Hub. – Mor. *İstanbul Journal of Pharmacy, 51*(3), 378-385.

ABSTRACT

Background and Aims: *Sideritis* species (Lamiaceae) which are quite widespread in the Mediterranean region and represented by 46 species and 53 taxa in Turkey, are often used as a antirheumatic, anti-inflammatory, antimicrobial and diuretic remedies.
Methods: In this study, the infusion (INF), essential oil (EO) and methanolic extract (ME) of chemical compounds prepared from aerial parts of *Sideritis albiflora* Hub.-Mor. (endemic) were investigated.
Results: The presence of chlorogenic acid, verbascoside, forsythoside, apigenin glucoside, and isoscutellarein derivatives in the infusion and methanol extract of the plant was determined by LC-MS / MS analysis. In addition, the main constituents of the essential oil were found as germacrene D (23.5%), *ß*-caryophyllene (13.6%) and caryophylene oxide (8.0%) by GC / MS and GC / FID simultaneously.
Conclusion: In this study, the essential oil, the methanolic extract and the infusion of S. albiflora species were prepared and their chemical compositions elucidated. It was determined that the essential oil chemical compound composition is rich in terms of the sesquiterpene group.
Infusions of aerial parts of some *Sideritis* species have traditionally been used in the treatment of many diseases. Accordingly, the fact that the phenolic compounds of *S. albiflora* infusion have not been studied before increases the importance of this study. In addition, the therapeutic effect was shown to be related to major *S. albiflora* compounds, and the correlation between *in vitro* activity and ethnobotanical use was evaluated. The effective phenolic compounds contained in *S. albiflora* are thought to support the traditional uses of the plant.

Keywords: *Sideritis albiflora*, infusion, polyphenolic compounds, methanolic extract, essential oil

INTRODUCTION

There are over 10,000 species of wild flowering plants in Turkey and one third of them are aromatic (Karahüseyin & Sarı, 2019). The consumption of herbal tea prepared from wild plants is very common, especially in rural areas, and one of the most used herbs asherbal tea is the *Sideritis* genus, which is generally found in the Aegean and Mediterranean regions. As of the most recent taxonomic classification, *Sideritis* genus includes over 150 species distributed in the Western Palearctic region. Around 90% of all *Sideritis* species are found in Turkey, with around 80% of them being endemic to the country (Aneva, et al., 2019). *Sideritis* (*Lamiaceae*) species are represented in Turkey by 46 species and 53 taxa, 39 of which are endemic (Kirimer, Tabanca, Özek, Tümen, & Baser, 2000).

Sideritis species are generally known as "Dağ çayı" in the regions where they grow in Turkey. Infusions of aerial parts of some species of *Sideritis* are used as diuretic, anti-inflammatory agent, antispasmodic remedyies, and as a carminative tonic. In ad-

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Submitted: 02.04.2021
Revision Requested: 11.05.2021
Last Revision Received: 25.05.2021
Accepted: 24.06.2021
Published Online: 00.00.0000

dition, they are used in the treatment of colds and digestion (Ezer, Vila, Canigueral, & Adzet, 1996; Yeşilada, & Ezer, 1989).In addition, *Sideritis* species, which are common in our country, are poor in diterpenes, phenylethanoid glycosides, flavonoids and essential oils. *Sideritis albiflora* Hub.- Mor, belonging to the section *Empedoclia*, is endemic in Turkey and, locally known as "Akçiçek çayı, Yayla çayı, Bozlan çayı" (Guner, Aslan, Ekim, Vural, & Babaç, 2012; Türkmenoğlu, & Duman, 2015) . In folk medicine, herbal tea prepared from inflorescence and leaves is used as an antimicrobial, anti-inflammatory, analgesic, nerve stimulative, sedative, antitussive, anticonvulsant, antispasmodic, carminative, and cold and cough suppressant. It is also used in the treatment of digestive system diseases (González-Burgos, Carretero, & Gómez-Serranillos, 2011; Türkmenoğlu, & Duman, 2015; Sarac, & Ugur, 2007).

While over 15 *Sideritis* species have been investigated for their non-volatile components in studies to date, about 50 *Sideritis* species have been investigated for their essential oil compositions, and most of these have been reported to contain *α* or *β*-pinene or both (Topçu et al., 2008; Deveci, Tel-Çayan, Usluer, & Duru, 2019a). *Sideritis* essential oils were classified by Başer and Kırımer according to their main components, and *S. albiflora* was included in those rich in sesquiterpenes and it was reported that the main component was *β*-caryophyllene (Başer, & Kırımer, 2018). Although *Sideritis* species are poor in essential oil, they have a pleasant aroma and fragrance (Kirimer, Baser, Demirci, & Duman, 2004; Deveci et al., 2019a).

Since the compounds with polyphenolic structure have important biological activities, it is necessary to determine their presence in plants. Rosmarinic acid, carvacrol, caffeic acid, apigenin, luteoline were detected in the methanol extract of *S. albiflora* by Askun et al, 2009. In a study by Deveci et al., 2019, Rosmarinic acid and caffeic acid were identified as the most abundant phenolic compounds, and acetone extract of *S. albiflora* was found to be the best reducing agent in the copper reducing antioxidant capacity (CUPRAC) test (Askun, Tumen, Satil, & Ates, 2009; Deveci, et al., 2019a).

Owing to the importance of the genus *Sideritis* in herbal remedies, it is necessary to investigate its chemical compounds and its biological activities in detail. This study aimed to elucidate the chemical compositions of the volatile and non-volatile compounds of *S. albiflora,* which is traditionally used in the treatment of many diseases in Turkey.

Therefore, in this study, the chemical composition of methanolic extract (ME), essential oil (EO) and infusion (INF) of *Sideritis albiflora*, an endemic species, was investigated by GC-MS and GC-FID, LC-MS/MS. Thus, the relationship between their chemical composition and biological effect has been elucidated.

MATERIAL AND METHODS

Plant material and essential oil (EO)

*The aerial parts of S. albiflora were collected in July 2018 from Muğla, Turkey. The plant material was diagnosed by Dr. Y. B. Köse and voucher specimens are kept at the Herbarium of the Faculty of Pharmacy of Anadolu University in Eskisehir, Turkey (*ESSE 15497).

The EO was obtained by hydrodistillation using a Clevenger type apparatus for 3h. The yield of S. albiflora herba was 0.07% on moisture-free basis and the oil was analyzed by GC-FID and GC-MS, simultaneously.

Infusion and methanol extract

The dried aerial parts of S. albiflora were weighed to prepare 10% of the infusion extract. The sample was added to boiled water at 70-80 °C. Then it was brewed for 10 minutes. Infusion extract was lyophilized in a freeze dryer (FreeZone 2.5 Liter Benchtop Freeze Dryer, Labconco) after it was analyzed for non-volatile compounds with LC-MS/MS.

To prepare a methanolic extract of S. albiflora aerial parts, 10g of the aerial parts were weighed and powdered. The plant material was put through maceration process for 48 hours in a dark container at room temperature and then filtered. This process was repeated, after which the two methanolic extracts obtained because of the process were combined and evaporated to dryness using an evaporator and then stored at -20°C.

Essential oil composition with GC-MS and GC-FID methods

The hydrodistilled essential oil of S. albiflora was analyzed by GC-MS and GC-FID (Demirci et al., 2019). The results of the analyses are given in Table 1.

*Identification of the essential oil compounds was performed by comparison of their relative retention indices (RRI) with those of authentic samples. Computer matching against commercial (MassFinder 3 Library, Wiley GC-MS Library) (Tabanca et al., 2014) and in-house "Başer Library of Essential Oil Constituents" built up by genuine compounds of known essential oils, as well as MS published in the literature (*Joulain, & Koenig, 1998; ESO, 2000) *were used for the identification.*

LC-MS/MS analysis

LC-MS/MS analysis of the methanolic extract of Sideritis albiflora was assessed using a previously described process (Gürbüz et al., 2019). LC-ESI-MS/MS data were collected and processed by Analyst 1.6 software.

RESULTS AND DISCUSSION

Extraction yield and composition of essential oil

The aerial parts of *S. albiflora* were subjected to Clevenger type apparatus to obtain the EO and the EOyield was found to be 0.07%. While *Sideritis* is a member of the *Lamiaceae* family, it does not contain much essential oils (Żyżelewicz, Kulbat-Warycha, Oracz, & Żyżelewicz, 2020).

The EO was analyzed by both GC-FID and GC-MS, simultaneously. 88 compounds representing 88.2% of the EO was characterized with germacrene D (23.5%), *β*-caryophyllene (13.6%), caryophyllene oxide (8.0%) and hexadecanoic acid (3.8%) as major constituents. The results are given Table 1.

Terpenoids are included in the major class of natural components, with a few thousands known compounds. The terpenoids that are originated from plant and marine organisms are classified as monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes and meroterpenes (Gozari, Alborz,

Table 1. The Composition of the Essential Oil of *Sideritis albiflora*.

RRI	Compound	%
1032	α-Pinene	2.0
1035	α-Thujene	0.1
1118	β-Pinene	3.5
1132	Sabinene	0.1
1151	δ-3-Carene	0.1
1174	Myrcene	0.1
1176	α-Phellandrene	0.1
1203	Limonene	1.8
1218	β-Phellandrene	1.0
1225	*(Z)*-3-Hexenal	0.2
1244	2-Pentyl furan	0.1
1255	γ-Terpinene	0.1
1280	*p*-Cymene	tr
1290	Terpinolene	tr
1400	Nonanal	1.0
1452	1-Octen-3-ol	0.4
1466	α-Cubebene	tr
1495	Bicycloelemene	tr
1497	α-Copaene	0.5
1506	Decanal	0.2
1528	α-Bourbonone	0.1
1535	β-Bourbonone	1.3
1553	Linalool	0.2
1572	α-Bergamotene	0.1
1583	Longifolene (=*Junipene*)	0.1
1586	Pinocarvone	0.2
1589	β-Ylangene	0.2
1597	β-Copaene	0.2
1600	β-Elemene	0.3
1612	β-Caryophyllene	13.6
1638	β-Cyclocitral	0.1
1648	Myrtenal	0.2
1655	*(E)*-2-Decenal	0.2
1661	Alloaromadendrene	0.1
1668	*(Z)*-β-Farnesene	0.1
1670	*trans*-Pinocarveol	0.3
1683	*trans*-Verbenol	0.3
1687	α-Humulene	0.8
1704	γ-Muurolene	0.3
1706	α-Terpineol	0.3
1726	Germacrene D	23.5
1740	α-Muurolene	0.2
1755	Bicyclogermacrene	2.6
1758	*(E,E)*-α-Farnesene	tr
1764	*(E)*-2-Undecenal	0.2
1773	δ-Cadinene	0.1
1776	γ- Cadinene	0.9
1784	*(E)*-α-Bisabolene	0.1
1785	7-*epi*-α-Selinene	0.3
1804	Myrtenol	0.3
1808	Nerol	0.1
1868	*(E)*-Geranyl acetone	0.2
1945	1,5-Epoxy-salvial-4(14)-ene	0.1
1957	Cubebol	0.1
1958	*(E)*-β-Ionone	0.4
2001	Isocaryophyllene oxide	0.6
2008	Caryophyllene oxide	8.0
2037	Salvial-4(14)-en-1-one	0.3
2050	*(E)*-Nerolidol	0.3
2071	Humulene epoxide II	0.5
2104	Viridiflorol	0.3
2130	Salviadienol	1.3
2131	Hexahydrofarnesyl acetone	1.0
2144	Spathulenol	1.8
2179	3,4-Dimethyl pentyliden-2(5H)-furanone	0.1
2186	Eugenol	0.2
2192	Nonanoic acid	0.1
2202	Germacrene D-4-ol	0.2
2209	*T*-Muurolol	0.3
2214	ar-Turmerol	0.1
2219	Dimyrcene II-a	0.2
2247	*trans*-α-Bergamotol	0.1
2255	α-Cadinol	1.2
2264	Intermedeol	1.0
2312	9-Geranyl-*p*-cymene	0.4
2324	Caryophylladienol II	0.4
2369	Eudesma-4(15)7-dien-1-β-ol	1.3
2380	8α-13-Oxy-14-en-epi-labdane	0.3
2389	Caryophyllenol I	0.3
2392	Caryophyllenol II	0.9
2503	Dodecanoic acid	0.2
2567	14-Hydroxy-α-muurolene	0.1
2607	14-Hydroxy-δ-cadinene	0.3
2622	Phytol	0.4
2670	Tetradecanoic acid	0.4
2700	Heptacosane	0.6
2900	Nonacosane	1.8
2931	Hexadecanoic acid	3.8
	Total	**88.2**

Note: %: calculated from FID data; tr: Trace (<0.1 %)

El-Seedi, & Jassbi, 2020). In this research, the chemical structure of germacrene D, which is determined as one of the major components of the EO of *S. albiflora* herb, is a sesquiterpene and this component constitutes approximately one quarter of the chemical composition of the EO obtained from the plant.

β-Caryophyllene is a sesquiterpene compound with a bicyclic structure. It is found in the content of food and nutrition supply. That compound is very important due to its biological activities such as antiinflammatory, antipruritic, lavricidal, anticolitis, antimicrobial, neuroprotective, gastroprotective and nephroprotective activities. *β*-Caryophyllene is considered to be safe for safe to consuming, due to its low toxicity level, and a wide therapeutic index (Russo & Marcu, 2017).

Caryophyllene oxide, which is a sesquiterpenoid oxide, is commonly included in the plants. It is of great importance due to its insecticidal and broad-spectrum antifungal biological activities and this compound shows antiplatelet aggregation properties. Also, caryophyllene oxide is non-sensitizing and non-toxic (Russo & Marcu, 2017).

Kırımer and co-workers were first to report the main constituents of the essential oil of *S. albiflora* as *β*-caryophyllene (35.0%) (Kirimer, Baser, Demirci, & Duman, 2004). Topçu and co-workers determined *γ*-cadinene (12.8%; 12.1%), *trans*-caryophyllene (14.8%; 17.4%), *β*-pinene (15.4%; 13.5%) and *α*-pinene (16.3%; 15.4%) as major compounds by headspace analysis (Topçu et al., 2008). Deveci et al., 2019 recently identified *β*-caryophyllene (21.2%), palmitic acid (12.3%), *τ* -gurjunene (13.6%), caryophyllene oxide (9.0%), carvacrol (6.0%) and viridiflorol (6.0%), as major components (Deveci et al., 2019a). Carvacrol (24.82%), *β*-caryophyllene (17.32%) and *γ*-elemene (14.13%) were found as major components in the essential oil of *S. albiflora* by Usluer et al., 2005 (Usluer, Duru, & Öztürk, 2005). In the previous studies, *S. albiflora* was collected from Fethiye and Muğla (Topçu et al., 2008; Deveci et al., 2019a). Although the results are compatible with our study, there are also some differences in the quantity and quality of *S. albiflora* essential oils. This can be attributed to the climatic conditions, soils and extraction methods. When *Sideritis* genus is classified according to essential oil compositions, monoterpene hydrocarbons are widely available. *β*-Caryophyllene is the most detected major compound (Başer, & Kırımer, 2018).

Infusion and methanolic extract composition

In LC-MS/MS analysis, chlorogenic acid, verbascoside, forsythoside, apigenin glucoside and isoscutellarein derivatives were determined in the methanolic extract and infusion of the plant. The other *Sideritis* species have also been shown to contain these compounds as main constituents (Jaiswal, Kiprotich, & Kuhnert, 2011; Petreska et al., 2011; Stanoeva et al., 2015; Axiotis, Petrakis, Halabalaki, & Mitakou, 2020; Żyżelewicz et al., 2020). The isoscutellarein derivatives are very characteristic for *Sideritis* species and verified in literature studies (Żyżelewicz et al., 2020). The compounds have phenolic structure and, they have very important biological activities. All of the compounds found in this study have antimicrobial and anti-inflammatory properties (Xing, Peng, Wang, Chen, & Li, 2014; Smiljkovic et al., 2017; Kim, & Park, 2019; Kubica et al., 2020; Żyżelewicz et al., 2020). Traditional use is consistent with the literature studies.

The results are given in Table 2. MS spectra of the identified compounds are given in Figures 1 and 3. Compound 1 showed the molecular ion peak at m/z 353 [M-H]- and base peak at m/z 191 and the small amount product ion at m/z 179. Compound 1 was determined as 5-caffeoylquinic acid (Deveci et al., 2019a; Clifford, Knight, & Kuhnert, 2005) as previously determined in the genus *Sideritis* (Petreska et al., 2011; Stanoeva, Bagashovska, & Stefova, 2012).

The molecular ion peak of Compound 2 was peak at m/z 755 [M-H]- which fragmented to the base peak ion at m/z 593 due to the loss of a caffeoyl unit. A pentose loss was due to m/z 461 (M-H-132). Rhamnose loss was due to the ion at m/z 315 (M-H-132-146). m/z 297 ion was observed due to the loss of H_2O. Caffeic acid related ions at m/z 179, 161 and 135 were also observed. This fragmentation behavior led us to believe that compound 2 is a caffeoyl phenylethanoid glycoside. MS fragmentation agreeing with Forsythoside B was reported by Mitreski and Kırmızıbekmez (Stanoeva et al., 2012; Mitreski et al., 2014; Kırmızıbekmez et al., 2005).

Compound 3 showed the molecular ion peak at m/z 623 [M-H]- which presented fragment ions at m/z 461, 305, 179 and 161. The fragmentation pattern was like that of forsythoside B. Verbascoside was previously identified in several *Sideritis* species. It gives the same fragments, therefore, compound 3 was determined as verbascoside (Petreska et al., 2011; Ah-

Table 2. The phenolic composition of *S. albiflora* ME and INF.

Compound No	Rt	M-H	Base peak	MS^2	Identification
1	7,8	353	191	179	5-Caffeoylquinic acid
2	9,6	755	593	461, 315, 297, 179, 161, 135	Forsythoside B
3	10,1	623	161	461, 315, 179,133	Verbascoside
4	11,2	623	299	461, 284	4'-O-Methylisoscutellarein 7-O-allosyl(1-2) glucoside
5	12,7	431	295	363, 269	Apigenin glucoside
6	13,2	651	285	609, 591, 447, 429, 379	Isoscutellarein 7-O-[6'''-O-acetyl]-allosyl(1-2) glucoside

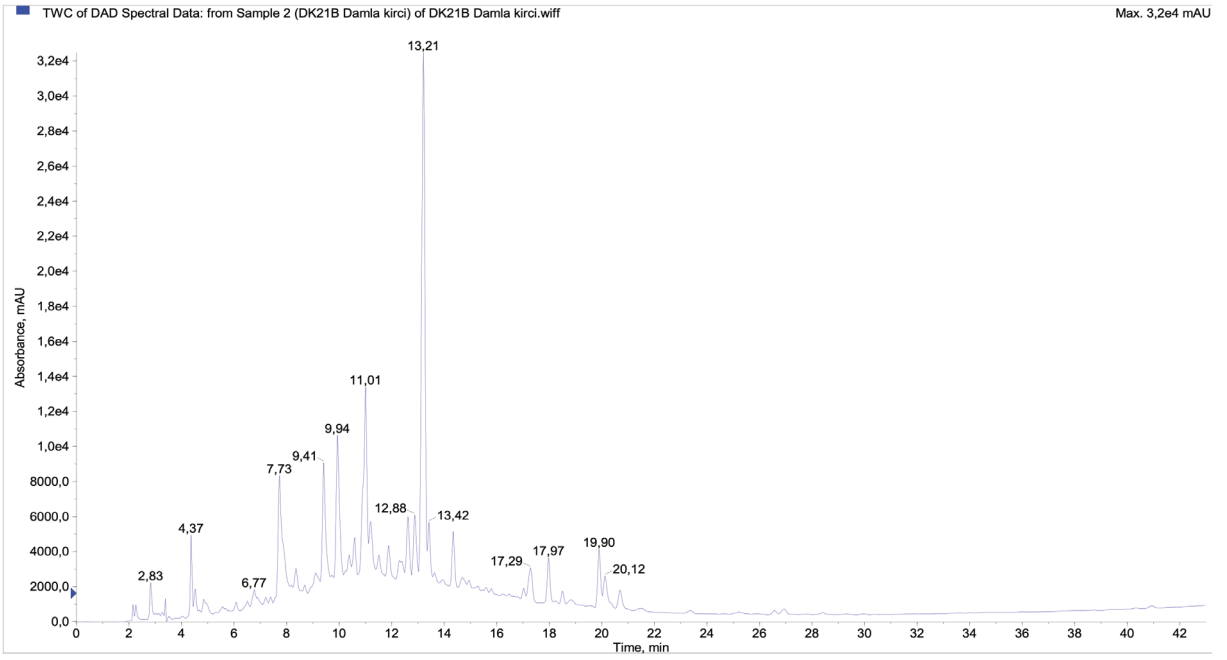

Figure 1. 70% Methanol extract LC-MS/MS.

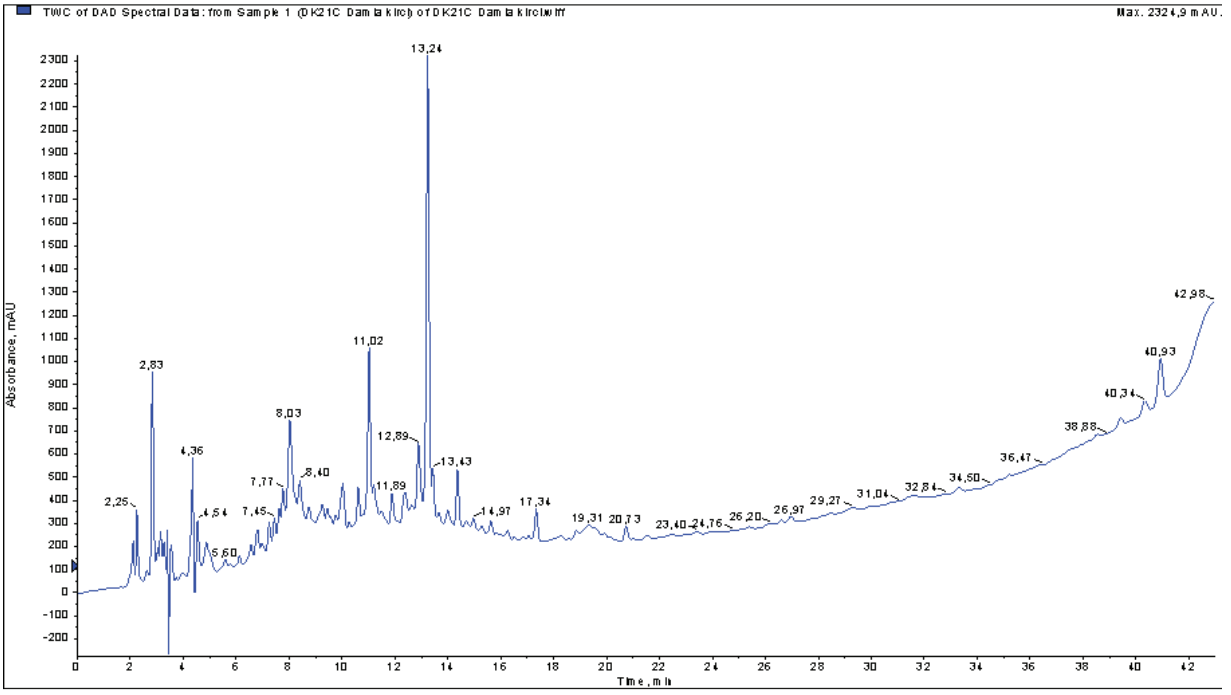

Figure 2. Infusion LC-MS/MS.

mad et al., 2006; Karioti, Bolognesi, Vincieri, & Bilia, 2010; Asnaashari et al., 2010).

Compound 4 showed the molecular ion peak at m/z 623 [M-H]- Ion at m/z 299 was fragmented to m/z 284 due to the weakness of a 15 amu methyl unit. Loss of 324 amu from molecular ion peak indicates a dihexoside of methylisoscutellerain. 366 amu (dihexose +acetyl) indicates that sugar part was acetylated. Methylisoscutellerain is a flavonoid which is found in many *Sideritis* species (Karioti et al., 2010). Compound 4 was therefore characterized as 4'-O-methylisoscutellarein 7-O-allosyl (1→2) glucoside as previously determined in *Sideritis* species (Petreska et al., 2011).

Compound 5 was identified as apigenin glucoside which presented a molecular ion peak at m/z 431 M-H]- and fragmented to base peak ion at m/z 269 (apigenin) after the loss of a glucose unit (-162). Apigenin glucoside and derivatives

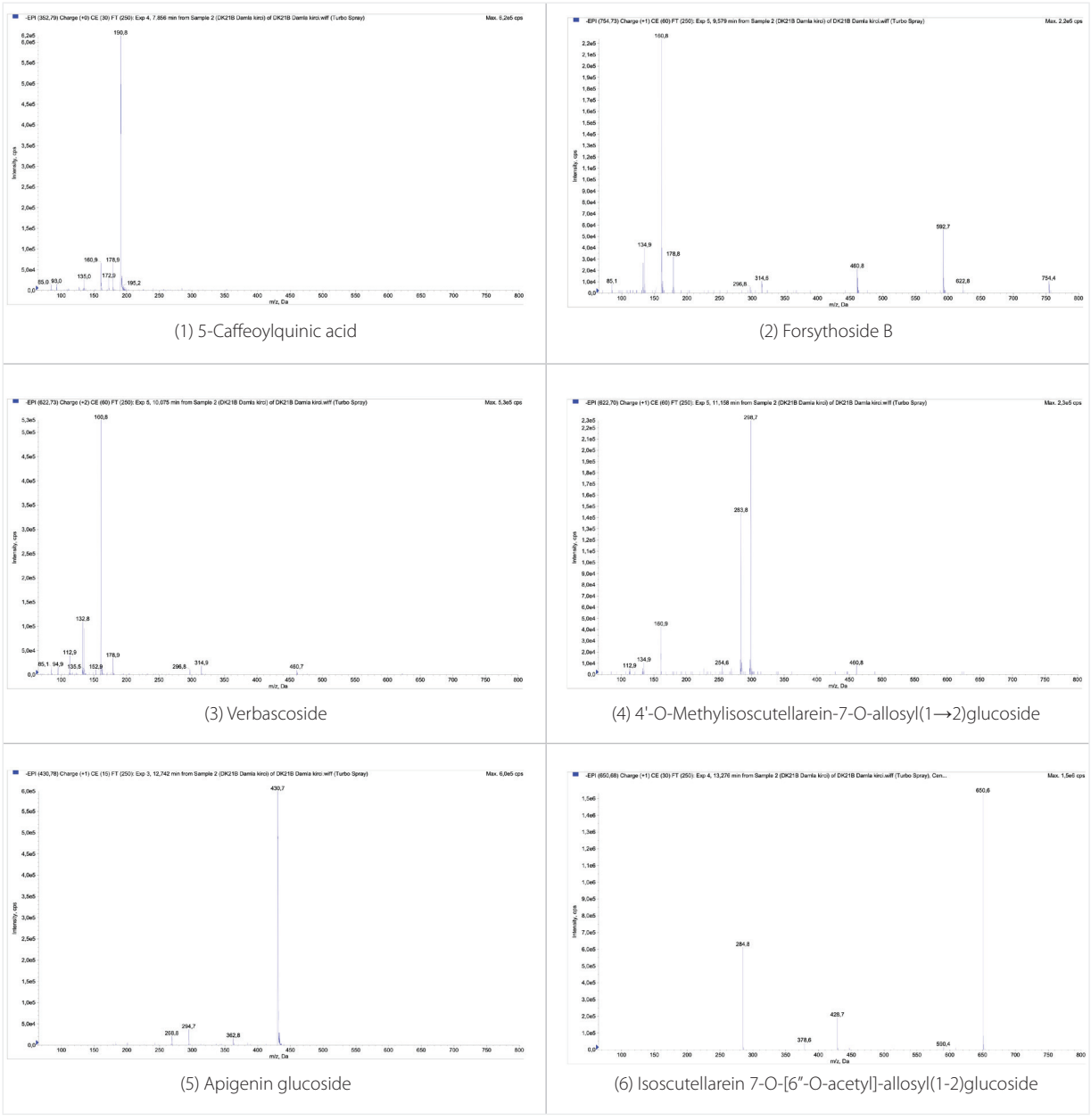

Figure 3. LC-MS/MS analysis of *S. albiflora* ME and INF compounds: (1) 5-Caffeoylquinic acid, (2) Forsythoside B, (3) Verbascoside, (4) 4'-O-Methylisoscutellarein-7-O-allosyl(1→2)glucoside, (8) Apigenin glucoside, (9) Isoscutellarein 7-O-[6"-O-acetyl]-allosyl(1-2)glucoside.

were previously determined in *Sideritis* species (Ulubelen, Topcu, & Kolak, 2005).

Compound 6 showed a [M-H]- ion at m/z 651 and its MS2 spectrum showed a base peak ion at m/z 285 due to the loss of 324 amu, probably a diglucose moiety. The presence of an ion at m/z 429 (M-H-180) indicates that glycosidation occurred in position 1-2 between the sugars (Petreska et al., 2011). Fragmentation behavior of compound 6 was identified as similar to that of isoscutellarin 7-O-allosyl (1-2) glucoside but 42 amu higher molecular ion peak than this compound indicated that compound 6 was an acetylated derivative of this compound, which led us to characterize the compound 6 as isoscutellarein 7-O-[6‴-O-acetyl]-allosyl(1-2) glucoside (Petreska et al., 2011; Pereira, Domingues, Silva, & Cardoso, 2012).

Deveci and co-workers investigated the phenolic compounds of the methanolic extract (ME) of the aerial parts of *S. albiflora* and identified carvacrol and rosmarinic acid (Deveci, Tel-Çayan, Duru, & Öztürk, 2019b). In another study, rosmarinic acid, caffeic acid and carvacrol were found as major phenolic compouns in S. albiflora. . Askun et al. 2009, determined the phenolic compounds of five *Lamiaceae* family members including *S. albiflora* by HPLC (Askun, et al., 2009).They reported that the methanol extract prepared from the aerial parts of *S. albiflora* contained caffeic acid, rosmarinic acid, carvacrol, apigenin, luteolin, naringin and it exhibited antibacterial effect in some Gram posi-

tive and Gram negative bacteria species. In another study, the antioxidant effect of *S. albiflora* aqueous extract was revealed by Güvenç et al. (2005) without reporting the chemical composition of the extract (Güvenç, Houghton, Duman, Coşkun, & Şahin, 2005).

In previous studies, no research was found on the chemical constituents of infusion of the herbal parts of *S albiflora*. Considering the widespread use of the herbal tea of *S. albiflora* species as infusion in our country, a proper determination of the chemical composition of the infusion of this plant was deemed necessary.

CONCLUSIONS

Sideritis genus is widely available in Turkey, which are among it is an important group of medical and economic plants. In this study, essential oil, methanolic extract and infusion of *S. albiflora* species were prepared and their chemical compositions were defined. It has been determined that the essential oil composition is rich in terms of the sesquiterpene group.

Infusions of aerial parts of some *Sideritis* species have traditionally been used in the treatment of many diseases. Accordingly, the fact that the phenolic compounds of *S. albiflora* infusion have not been studied before increases the importance of this study. In addition, in this study, the therapeutic effect was shown to be related to major *S. albiflora* compounds, and the correlation between *in vitro* activity and ethnobotanical use was evaluated. The effective phenolic compounds contained in *S. albiflora* are thought to support the traditional uses of the plant.

Peer-review: Externally peer-reviewed.

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

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

Financial Disclosure: This study was supported by Anadolu University Scientific Research Projects Units with the grant number BAP1905S077.

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Istanbul J Pharm 51 (3): 386-391
DOI: 10.26650/IstanbulJPharm.2021.909411
Original Article

Capsaicin determination from pain patch for the calculation of Scoville heat units by gas chromatography-mass spectrometry

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Cite this article as: Daniş, I., & Unal, D. O. (2021). Capsaicin determination from pain patch for the calculation of Scoville heat units by gas chromatography-mass spectrometry. *İstanbul Journal of Pharmacy, 51*(3), 386-391.

ABSTRACT

Background and Aims: A sensitive, accurate and precise method has been developed for the determination of Capsaicin from pain patches by Gas chromatography Mass Spectrometry (GC-MS). Capsaicin has irritant effects in high concentrations, so these effects can be minimized by knowing the amount present in pain patches for the efficacy and safety of patches.
Methods: Capsaicin was extracted by using liquid-liquid extraction from patches. The Gas Chromatographic separation was performed by using 5% diphenyl 95% dimethylpolysiloxane column with high a purity 2 mL/min flow rate helium gas. The separation was made with a gradient oven temperature program. The oven temperature started at 250°C and was increased to 275°C at 10°C.min^{-1} ramp rate and held at 275°C for 2.5 min. The injection port was adjusted at 300°C and a split injection mode was used. The analysis was carried out in a split mode of 5:1. MS ionization potential was determined at 70 eV.
Results: The calibration curve was found to be linear in the range 5 - 50 µg/mL. The limits of detection and quantification for capsaicin was found to be 3.46 µg/mL and 5 µg/mL, respectively. The method developed was validated and successfully applied to the patch analysis.
Conclusion: This method is simple, reproducible, and can be used safely for the routine analysis of Capsaicin without derivatization. This study has the potential how to calculate the Scoville Heat Units (SHU) of pain patches that contain Capsaicin. The amount of Capsaicin in the pain patch, its irritant effects, and its efficacy and safety appear to be low when evaluated by the SHU.

Keywords: Capsicum, Capsaicin, pain patch, gas chromatography-mass spectrometry, Scoville

INTRODUCTION

Capsaicin is an alkaloid, derived from hot chilli pepper plants. It is an active component of the plants belonging to the Capsicum (pepper) genus. Capsaicin ($C_{18}H_{27}NO_3$), E-N-(4-hydroxy-3-methoxybenzyl)–8 –methylnon–6 enamide) has analgesic and antioxidant properties (Figure 1) (Lu, Ho, & Huang, 2017). Certain capsicum preparations have been used for the treatment of postherpetic neuralgia pain in recent years that are however, a strong irritant to skin and mucous membranes. Topical Capsaicin therapy may be a benefit in providing pain relief. Capsaicin patches are applied to the most painful areas of the skin.

Capsaicin and other members of the group of Capsaicinoids produce a large number of physiological and pharmacological effects such as effects on the gastrointestinal tract, the cardiovascular, and the respiratory system, as well as the sensory and thermoregulation systems. These effects result principally from the specific action of Capsaicinoids on primary afferent neurons of the C-fiber

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Submitted: 04.04.2021
Revision Requested: 24.08.2020
Last Revision Received: 13.09.2021
Accepted: 13.09.2021
Published Online: 00.00.0000

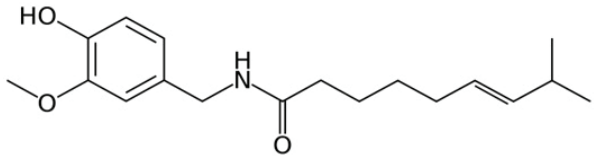


Figure 1. Chemical structure of Capsaicin.

type. This provides the rationale for their use to treat some peripheral painful states, such as rheumatoid arthritis (Surh & Lee, 1995). In addition, Capsaicinoids are powerful irritants, causing burning and pain at low concentrations on the skin and mucous membranes. Given orally, they induce an increase of salivation and gastric secretion, a rapid change of sensation, warm to intolerable burning, and gastrointestinal disorders depending on the dose (Govindarajan & Sathyanarayana, 1991).

Capsaicin was first isolated by John Clough Thresh in 1876 (Tresh, 1876) and the structure determined by E.K Nelson, and was first synthesized by E Spath and S.F Darling. Besides the analgesic properties of Capsaicin, in recent years, law enforcement has been using Capsaicin based pepper sprays against lawbreakers (Benzon2013).

Capsaicin helps to relieve chronic soft tissue pain, back pain, and neuropathic pain. Topical Capsaicin products are commonly used for pain relief. They have been available in various formulations such as lotions, creams, gels, or patches in low concentrations of Capsaicin. They have been in clinical use for many years to relieve pain, however, the effectiveness of Capsaicin in pain relief also has some adverse side effects, like allergies and irritation at specific concentrations causing burning and pain on the skin and mucous membranes. So the concentration of Capsaicin in topical formulations is important in avoiding side effects. A topical application of gel and cream contains 2.5% and 8% concentration of Capsaicin (Christo & Cauley, 2009). Because of the irritant and burning effect, the concentration of Capsaicin is reduced in strengths of 0.025% and 0.075% (Moon 2017). Capsaicin patches are used to treat patients with postherpetic neuralgia or neuropathy (especially HIV associated) and non-diabetic adults. The FDA and EU approved the use of the Capsaicin 8% patch in 2009 (Baranidharan et al., 2013; Anand et al., 2011; Laklouk et al., 2016). The amount of Capsaicin in the patches were important to the efficacy, safety, and tolerability of the patches (Anand et al., 2011; Laklouk et al., 2016). In this study, the methods were developed and validated for the determination of Capsaicin from patch formulations.

In the literature, various chromatographic methods were reported for the analysis of Capsaicinoids from natural products including High-Performance Liquid Chromatography (HPLC) with the detection of flourimetric (Daood et al., 2015), ultraviolet (Ciulu-Costinescu et al., 2015; Kuzma et al., 2015; Ashwini et al., 2015; Barbero et al.,2016) and mass spectrometric analysis (Barbero et al., 2016). A GC-MS determination of Capsaicin was also used for its analysis from pepper (You et al., 2013; Bononi et al., 2012; Pena-Alvarez et al., 2012; Peña-Alvarez et al., 2009; Ha et al., 2008). The Ultra-Fast Liquid Chromatographic method was developed by Usman et al. For analyzing multiple samples in a short time, the total run time was about 12 min (Usman et al., 2014). As a result of a full literature review, Capsaicin and DihydroCapsaicin (DHC) determination from pharmaceutical preparations by liquid chromatographic (LC) method was found only in the topical cream formulation. Sample preparation involves liquid-liquid extraction prior to LC analysis (Kaale et al., 2002).

This study aimed to develop and validate a sensitive and straightforward GC-MS method, then to analyze the Capsaicin level in patches to evaluate the irritant and burning effect. This is the first time in the literature that the SHU for the patch are determined and calculated. The chromatographic peak area of DihydroCapsaicin is used to calculate the Scoville Heat Units from the formula (Usman et al., 2014). A GC-MS method has been developed and validated for the calculation of SHU, which are important for the quantitation of Capsaicin from the pain patch and the control of the effectiveness and irritation properties of the patches.

MATERIAL AND METHODS

Chemicals and reagents

Methanol MS grade was purchased from Merck (Darmstadt, Germany). Capsaicin was supplied from Medigen (Medigen Pharma, Turkey). The Capsicum Oleoresin patch (53 mg Capsicum Oleoresin / 4.6 mg Capsaicin, 17 × 12 cm^2) was used for analysis. The Capsicum Oleoresin patch was purchased from the pharmacy.

Preparation of standard solutions

The standard stock solution of Capsaicin was prepared by dissolving with MeOH to obtain a final concentration of 1 $mg.mL^{-1}$. Capsaicin calibration curve solutions (5, 10, 20, 40, 50 µg/mL) and quality control samples (5; 25; 50 µg/mL) were prepared from stock solution by diluting with MeOH. All standard solutions were kept at +4°C.

Extraction procedure from patches

A 51.47 cm^2 patch containing 4.6 mg of Capsaicin was cut into four equal parts, each was placed in 50 mL falcon tubes, and each portion of the patch was extracted separately with 50 mL of MeOH (final concentration: 23 µg/mL). Samples were extracted with a rotary shaker for 5 hours. The alcoholic extract was taken by filtration and injected directly into the GC / MS system. The patches were stored at 4°C until assayed for volatile components such as menthol and camphor contained to keep the formulation stable.

Instrumentation and conditions

An Agilent 7890B Gas Chromatographic system equipped with a split or splitless injector and a 5977A MSD (Mass Spectrometer Detector) was used for the determination (Darmstad, Germany). HP-5MS (30 m X 0.25 mm) 0.25 µm film thickness (Agilent Technologies) analytical column was used in the separation process. The separation was made with a gradient oven temperature program. The oven temperature started at 250°C and was increased to 275°C at 10°C.min^{-1} ramp rate and held at 275°C for 2.5 min. The injection port was adjusted at 300°C and a split injection mode was used. The analysis was carried out in a split mode of 5:1 and the MS ionization potential was determined at 70 eV. The ion source and GC-MS transfer line temperature was selected as 300°C. The Scan mode spectra

of compounds are needed in mass spectrometric analysis. When examining the spectrum to determine candidate SIM ions to be used in the analysis, the compound-specific m/z ion or ions must be selected. SIM ions are important in a mass spectrometric analysis for precision. An analysis performed in a scanning mode in the range of 40-350 m/z and a value of m/z: 137, which is specific to the molecule and has the highest ion abundance, was selected for Capsaicin quantitation.

RESULTS

Development and optimization of the GC-MS method

Total ion chromatograms (TIC's) were obtained by using a standard solution of Capsaicin. When using an MS detector in scan mode, quantitation is usually done by monitoring a response for a specific ion in an analyte's mass spectrum. In many cases, this ion, termed the "quantitation ion", is the most abundant in the spectrum. Other lesser abundant ions may also be monitored to aid in proper identification of the analyte. These are often termed "qualifier" ions, and are not used in quantification of the peak. It is common practice to monitor 3 ions per compound. One ion signal is used to quantitate, and the others are used for qualitative information. The *m/z*: 137 ion was chosen for the quantification of Capsaicin (Figure 2). DihydroCapsaicin also has the same m/z: 137 fragment ion.

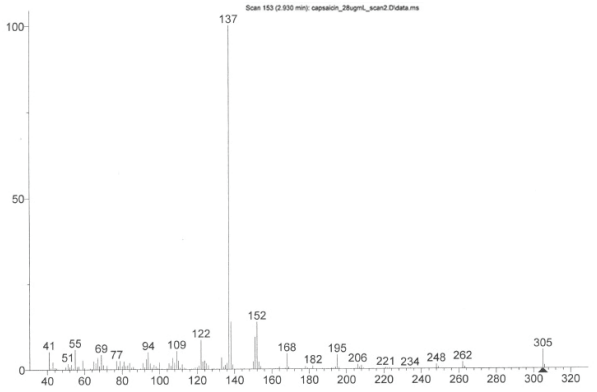


Figure 2. Mass spectrum of Capsaicin Standard.

Fragmentation of Capsaicin to m/z 137 is shown in Figure 3. In the method, developed retention time of Capsaicin and DihydroCapsaicin 2.9 and 3.0 min, respectively.

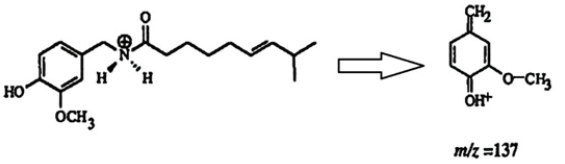


Figure 3. Fragmentation of m/z: 137.

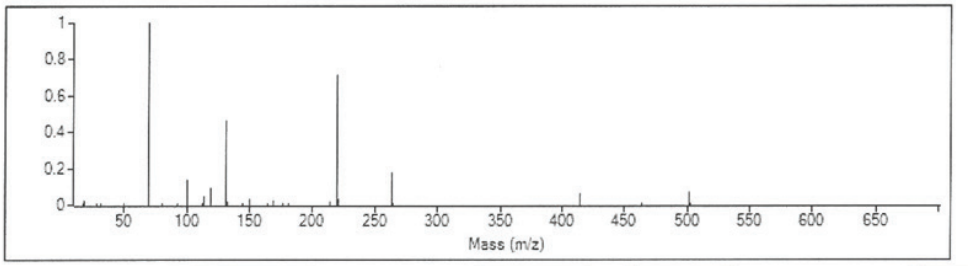
Strength and robustness studies aim to examine the effect of potential sources of variation in the response of the method. According to the ICH guidelines, the effect of flow rate and oven temperature in GC analyses is examined for the robustness of the method. When analyses were performed between flow rate ± 0.05 mL.min^{-1} and temperature ± 0.5°C, it was observed that it had no effect on the peak shape and area and retention time. Therefore the method developed is robust and rugged.

System suitability and tuning mass spectrometer

Tuning and calibration is performed to ensure that the mass spectrometer is working correctly, or that mass assignment and relative abundance of spectral signals resemble a previously determined standard. The tuning process will check that spectrometer contamination or degraded electronic components have not changed the assigned calibration of the mass axis. The MS system is tuned with a perfluorotributylamine (PFTBA) which is a known mass spectrum. The Autotune uses three ions from the PFTBA spectrum for tuning m/z: 69, 219 and 502. Before the analysis, systems were checked by tuning. Autotune provides information about the mass spectrometer; their sensitivities and responses change with time and usage. During tuning, the relative and absolute abundances of fragments of a known tuning compound are established, and the mass assignment, resolution, and spectral peak width generated by the mass analyzer are also adjusted and set. (Table 1). All the tuning parameters were found in acceptable limits (Figure 4).

Table 1. Autotune acceptance limits.

m/z	Relative abundance %	Isotop m/z	Isotop Ratio %
69.0	100	70.0	0.5-1.6
219.0	>35	220.0	3.2-5.4
502.0	>1	503.0	7.9-12.3



Target m/z	Actual m/z	Abund	Rel Abund	Iso m/z	Iso Abund	Iso Ratio
69.00	69.00	399,296	100.0%	70.00	4,503	1.1%
219.00	219.00	284,224	71.2%	220.00	12,325	4.3%
502.00	502.00	27,520	6.9%	503.00	2,847	10.3%

Figure 4. Autotune Results.

Linearity/Limit of quantification

A set of 5 calibration standards were prepared and analyzed in duplicate in three days. The calibration curve was constructed by plotting the area against the concentration at Capsaicin using linear regression analysis. The linearity of the method was demonstrated by the calibration equation and correlation coefficient (Table 2). The linearity of the method was found satisfactory $r^2 \geq 0.990 \pm 0.002$. The detection limit and quantification limit of the method are determined according to the signal / noise ratio. The LOD value was found to be 3.46 µg/mLand LOQ as 5 µg/mL (signal-noise>10).

Table 2. The Linearity data of the method (n=6).

Parameters	
Calibration Equation	y= 56158x -141036
Correlation Coefficient (r^2)	0.9973
Linear range (µg.mL^{-1})	5-50
LOQ (µg.mL^{-1})	5
LOD (µg.mL^{-1})	3.46

Selectivity

The selectivity of the method was performed by preparing the analyte and solvent that was used. It was observed that the signal was represented only by the analyte and chromatogram showed a very fine peak of analyte. There were no considerable changes in the area under curve or retention time evidently indicated the selectivity of the proposed method (Figure 5). There is no carry over seen during analysis.

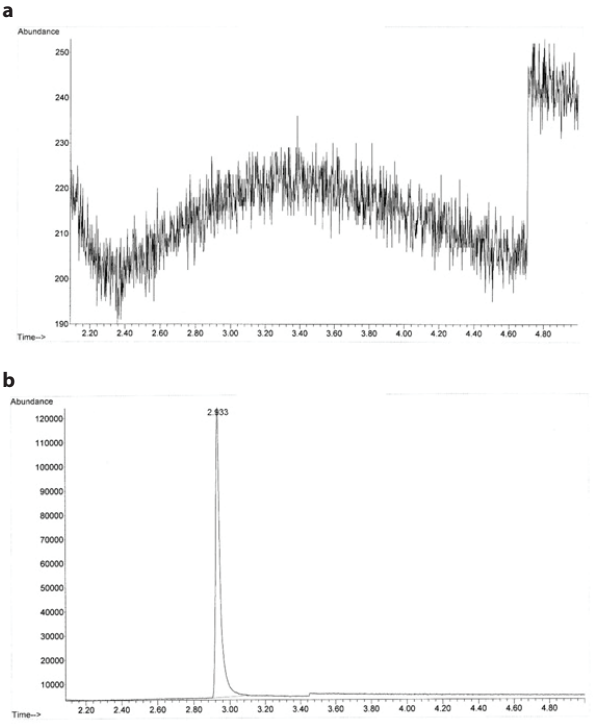


Figure 5. Blank sample chromatogram (a) and 5 µg.mL^{-1} Capsaicin chromatogram (b).

Accuracy and precision

The accuracy and precision was demonstrated by preparing low, high and medium concentrations samples according to calibration samples. The precision and accuracy of intra-day were settled by an analysis of six replicates of 3 concentrations including low, medium and high concentrations of quality control samples. Inter-day precision and accuracy were examined by the analysis of these quality control samples on three separate batches. The precision of the method was shown as the percentage of the coefficient of variation and the accuracy of the method was shown in terms of relative errors. The intra-day and inter-day accuracy as indicated by the standard deviation (SD) ranged from 0.1914 to 3.8383 (Table 3, 4). Intra-day

Table 3. Intra-day Accuracy and precision results.

Sample	Concentration (µg.mL^{-1})	Mean	Mean%	Standard Deviation (SD)	Relative Standard Deviation RSD	n
QC1	5	5.5369	110.7385	0.2877	5.1963	12
QC2	25	23.3753	93.5012	1.7569	7.5160	12
QC3	50	50.2949	100.5898	2.7037	5.3756	12

Table 4. Inter-day Accuracy and precision results (3 days for every concentration).

Sample	Concentration (µg.mL^{-1})	Mean	Mean%	Standard Deviation (SD)	Relative Standard Deviation RSD	n
QC1	5	5.1526 5.5547 5.4790	103.0526 111.0934 109.5801	0.5310 0.1914 0.3191	10.3048 3.4458 5.8237	18
QC2	25	23.6233 22.8733 24.1378	94.4933 91.4932 96.5513	2.3606 1.8400 2.0638	9.9925 8.0445 8.5501	18
QC3	50	49.3284 50.3633 50.3638	98.6567 100.7226 100.7276	3.4207 3.8383 3.6894	6.9345 7.6215 7.3256	18

and inter-day precision expressed by relative standard deviation (RSD) ranged from 3.4458 to 10.3048. The method developed was found to be accurate and precise.

Patch analysis

Capsaicin topical is used for the temporary relief of muscle or joint pain. Capsaicin can cause a burning sensation wherever it is applied. The method developed was applied for the determination of Capsaicin content in the pain patches which contain Capsaicine to avoid sensation. The patches were prepared as mentioned before in an extraction procedure from patch. The patch extracts were analyzed. Capsaicin and dihydroCapsaicin were separated by the developed method (Figure 6).

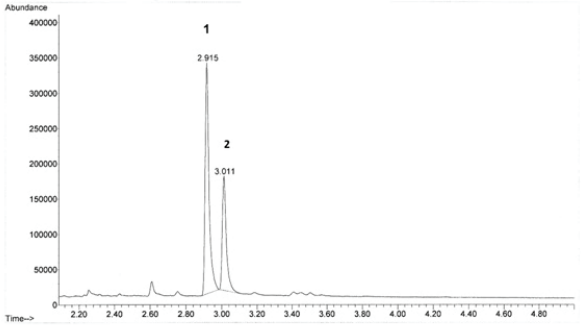


Figure 6. Chromatogram of Capsaicin and DihydroCapsaicin from the patch.

Calculation of Scoville heat units of patch

SHU was used to calculate the heat level of samples such as pepper. The units are calculated in parts per million of heat (ppmH) based on sample weigth (Usman et al., 2014). SHU is calculated by multiplying ppmH by a factor of 15. ppmH formula was converted to calculate the patch heat unit. The calculation of SHU of a Capsaicin pain patch is the first original study.

ppmH = [(peak area of Capsaicin in patch + 0.82) × (peak area of DHC in patch)] (Standart Capsaicin ppm in mL solution) / (total peak area of Standart Capsaicin) × (g Capsaicin in patch)

Capsaicin content is expressed in grams of Capsaicin per cm^2 of patch. For the conversion of the Scoville Heat Unit, the Capsaicin content in the patch is multiplied by a coefficient corresponding to the heat value for pure Capsaicin and calculated from the formula using the amounts of dyhydroCapsaicin. The Capsaicin Patch Scoville Heat Unit was calculated as 323.9 per cm^2 according to the equation given above.

DISCUSSION

In this study, the amount of Capsaicin from the pain patch was made by a gas chromatography-mass spectrometry and its irritating effects were determined by the SHU value. There are several methods to determine Capsaicin in the literature review. With the developed method, the retention times of Capsaicin and DihydroCapsaicin are 2.9 and 3.0 minutes, respectively. Compared to the methods found in the literature, it is one of the advantages of the method as it has a shorter analysis time compared to the UPLC method and other chromatographic methods. (Barbero et al., 2016). For compounds affected by temperature and light, it would be appropriate to reduce the analysis time to complete the analysis to reduce the risk of degradation of the compound. Capsaicin solution stability test results showed that protection from light at +4 ^oC increases the stability of the solution. (Kopec et al., 2002). This method can be applied to samples that take a long time to analyze and also in the case of a need for re-analysis for any reason. This investigation can also be used to determine the level of Capsaicin for Quality Control and the stability of a pharmaceutical preparation containing Capsaicin.

The calculated SHU of the Capsaicin patch was calculated as 323.9 per 1 cm^2 patch. Pure Capsaicin was rated between 15 and 16000000 SHU accordingly the SHU value of the Capsaicin patch calculated on the patch was found to be quite low. The amount of Capsaicin in the pain patch, its irritant effects, and its efficacy and safety appear to be low when evaluated by the SHU. Topical Capsaicin patch treatment can be beneficial in relieving pain without side effects.

CONCLUSION

The literature survey revealed that no Chromatographic determination of Capsaicin from pain patches is reported. This method was successfully applied to the analysis of Capsaicin from patches. The procedure is also accurate and precise, so recommended for routine quality control analysis. This study has the potential to be able to calculate the SHU of pain patches that contain Capsaicin.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- İ.D., D.Ö.Ü.; Data Acquisition- İ.D., D.Ö.Ü.; Data Analysis/Interpretation- İ.D., D.Ö.Ü.; Drafting Manuscript- İ.D., D.Ö.Ü.; Critical Revision of Manuscript- İ.D., D.Ö.Ü.; Final Approval and Accountability- İ.D., D.Ö.Ü.

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

Financial Disclosure: This study was supported by İstanbul University, Scientific Research Projects (Project Number: 55974).

Acknowledgement: We would like to thank Prof. Dr. Süleyman Patır for providing us Capsaicine standard.

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Istanbul J Pharm 51 (3): 392-397
DOI: 10.26650/IstanbulJPharm.2021.1000200
Original Article

Assessment of toxic metals in commonly used herbs and spices in Turkey

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Cite this article as: Ozden, H. (2021). Assessment of toxic metals in commonly used herbs and spices in Turkey. *İstanbul Journal of Pharmacy, 51*(3), 392-397.

ABSTRACT

Background and Aims: The levels of cadmium (Cd) and lead (Pb) were analyzed in frequently used herbs including ginger, liquorice, nutmeg and turmeric in Turkey.
Materials and Methods: The levels of Cd and Pb in commonly used herbs were analyzed by inductively coupled plasma optical emission spectrometry after a closed microwave-assisted digestion.
Results: The concentration ranges for the Cd and Pb were found to be 0.25-0.78 mg/kg and 3.04-6.45 mg/kg, respectively. While Pb levels were below the maximum permissible limits, high Cd levels were detected in herbs which would not pose any health risk for consumers regarding an exposure assessment.
Conclusion: It is important to implement regular monitoring of heavy metal content in herbs, including medicinal plants, to assess their potential risks to human health in consideration of the potential for multiple exposure via other sources.

Keywords: Cadmium, lead, ginger, liquorice, nutmeg, turmeric

INTRODUCTION

Herbs are commonly used as flavoring agents, spices, and confectionery additives in the food industry. Moreover, their use as remedies in traditional medicine has increased dramatically worldwide. Thus, a critical evaluation of the safety and quality of herbs and herbal products is important for health authorities (WHO, 2007). It is well known that herbs may be contaminated by natural and chemical contaminants, including pesticides and heavy metals, which may be harmful to consumers (WHO, 2007). In addition to the natural existence in water and soil, the common sources of heavy metal pollution in the environment are anthropogenic activities such as industrial production processes, household waste and waste materials (Järup, 2003). Heavy metals are considered to be significant potential hazards to human, animal and plant health due to their widespread presence, toxicity and persistence in the environment (Järup, 2003). The accumulation of heavy metals in the harvestable parts of plants occurs by root uptake, foliar absorption, and decomposition of specific compounds (Haider, Naithani, & Barthwal, 2004; Kishan, Bhattacharya, & Sharma, 2014; Sarma, Deka, & Deka, 2011). While heavy metals such as copper, iron, zinc, and manganese play an essential role in the structural and biochemical function of the plants, toxic heavy metals such as lead (Pb) cadmium (Cd), mercury and arsenic can cause harmful effects in plants (Nagajyoti, Lee, & Sreekanth, 2010). In addition, an accumulation of toxic heavy metals in plants could also cause adverse effects for consumers (WHO, 2007).

Cd and Pb are two of the major heavy metals that are biologically non-essential and exhibit toxicity, according to health authorities (WHO, 2007, WHO 2011, EFSA 2011). Pb causes significant alterations in various biological processes including cell adhesion, intra- and inter-cellular signaling, apoptosis, ionic transportation, enzyme regulation, calcium homeostasis and oxidative stress re-

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Submitted: 24.09.2021
Revision Requested: 22.11.2021
Last Revision Received: 26.11.2021
Accepted: 02.12.2021
Published Online: 00.00.0000

sulting in toxic effects (Jaishankar, Tseten, Anbalagan, Mathew, & Beeregowda, 2014). Cd binds to metallothionein, cysteine-rich protein, which is accumulated in the renal tissue causing nephrotoxicity (Jaishankar et al., 2014). Available data indicates cause for concern regarding these heavy metals because they are known human carcinogens (Järup, 2003). On the basis of occupational studies, Cd has been classified as a human carcinogen-group 1 (IARC, 1993) and inorganic Pb has been classified as probably carcinogenic to humans-group 2A (IARC, 2006) by the International Agency for Research on Cancer. The World Health Organization (WHO) established the maximum permissible limits for Cd and Pb as 0.3 mg/kg and 10 mg/kg in herbal materials, respectively (WHO, 2007).

Different parts of plants are rich in phytochemicals and antioxidants and have been used as food commodities, medicinal plant, and dietary supplement. Liquorice, roots and extracts of *Glycyrrhizia glabra L*., have been commonly used as medicinal plant, dietary supplement and food commodities. It has been shown to be beneficial in the treatment of respiratory diseases, gastrointestinal diseases, endocrine disorders, skin diseases, immunodeficiency and cancer (Karahan, Avsar, Ozyigit, & Berber, 2016; Sharma, Katiyar, & Agrawal, 2018). Ginger, the root of *Zingiber officinale*, has been widely used as a food condiment and dietary supplement all over the world due its high content of minerals, vitamins, and phytochemicals. Ginger has been used in medicine as anti-microbial, anti-pyretic, analgesic, anti-inflammatory, hypoglycaemic, anti-ulcer, anti-emetic, anti-hypertensive, hypolipidemic effects (Shahrajabian, Sun & Cheng, 2019). Nutmeg, the dried seed kernel of *Myristica Fragrans*, has been used as spice which exhibits many pharmacological activities such as anti-inflammatory, analgesic, antioxidant, antibacterial, antidiabetic and anticancer activities (Ha, Vu, Tran, Kim, Woo, & Min, 2020). Turmeric, the rhizome of *Curcuma longa*, has been used as a spice and medical herb due to its antioxidant, anti-inflammatory, antimutagenic, antimicrobial, and anticancer properties (Hewlings, & Kalman, 2017).

In order to avoid toxicity in humans, it is important to monitor the levels of toxic metals present in every step of the food chain (Jeong et al., 2012). Up to now, there have been a number of studies on the heavy metal contamination levels of herbal materials, including herbal medicines in Turkey (Başgel & Erdemoğlu, 2006; Bilgic Alkaya, Karaderi, Erdoğan, & Kurt Cücü, 2015; Karahan, Ozyigit, Saracoglu, Yalcin, Ozyigit & Ilcim, 2020; Leblebici, Bahtiyar, & Ozyurt, 2012; Ozcan, 2004; Ozcan, Ünver, Uçar, & Arslan, 2008; Ozcan & Akbulut, 2007; Ozturk, Altay, & Karahan, 2017; Ozyigit et al., 2018; Sekeroglu, Ozkutlu, Kara, & Ozguven, 2008; Tercan, Ayanoglu, & Bahadirli, 2016; Görür, Keser, Akçay, Dizman, & Okumuşoğlu, 2011; Divrikli, Horzum, Soylak, & Elci, 2006; Ozkutlu, Sekeroglu, & Kara, 2006; Ozden & Ozden, 2018; Tokalıoğlu, 2012). In the previous study, it was reported that Cd and Pb were found in the range of 0.324-0.524 mg/kg and 3.123-6.487 mg/kg, respectively, in linden, chamomile and sage teas (Ozden & Ozden, 2018). The aims of the present study were (i) to determine levels of Cd and Pb in frequently used herbs including ginger, liquorice, nutmeg, and turmeric (ii) to evaluate their potential hazards to human health.

MATERIAL AND METHODS

Reagents

Standard solutions of 1000 µg/mL of Cd and Pb in nitric acid were obtained from Merck (Darmstadt, Germany). Nitric acid (65%) was purchased from Merck (Darmstadt, Germany). All of the reagents used were of analytical grade.

Sample collection

A total of 54 herbs including liquorice, ginger, turmeric, and nutmeg samples were randomly collected from local markets in İstanbul and the botanical identification was carried out by Hakan Ozden. Scientific name and the part of the plant used were shown in Table 1.

Sample preparation

Each herb sample was milled using a Waring Blender (Conair Corp., Stamford, CT, USA) and stored in clean polyethylene packages at 2-8°C until analysis. The digestion and extraction processes of Pb and Cd were carried out using a closed microwave system (Berghof MWS-4 device, Berghof instruments, Eningen, Germany) equipped with Teflon containers. 0.1-0.3 g of dried and homogenized herbs were placed in the Teflon containers and wet-digested at 150-190ºC with 8 mL of 65% nitric acid. After cooling down to room temperature, the suspensions were diluted to 25 mL with deionized water. Blanks were also prepared using the same method. The samples were passed through syringe-type filters (Chromafil PET-45/25, Macherey Nagel, Düren, Germany), then analyzed by ICP-OES instrument.

ICP-OES analysis

Analyses of Cd and Pb were conducted in herb samples using the inductively coupled plasma optical emission spectrometry (ICP-OES, Pelkin Elmer, Waltham, MA, USA) with optima 7000

Table 1. Classification of herb samples by scientific name and used part.

Common name	Scientific name	Used part	No. of samples analyzed
Ginger	*Zingiber officinale*	Dried rhizome	15
Liquorice	*Glycyrrhizia glabra L.*	Dried root	12
Nutmeg	*Myristica fragrans*	Dried seed kernel	12
Turmeric	*Curcuma longa*	Dried rhizome	15

Table 2. Contamination of heavy metals in the analyzed herbs.

Herbs	No. of samples	Positive (%)[a]	Mean of contamination[b] (mg/kg)±SD*	
			Cd	Pb
Ginger	15	15 (100)	0.43±0.09	5.46±0.92
Liquorice	12	9 (75)	0.34±0.07	3.51±0.38
Nutmeg	12	9 (75)	0.51±0.07	4.25±0.72
Turmeric	15	14 (93.33)	0.67±0.08	5.64±0.81
Totally	54	47 (87.04)	0.47±0.13	4.89±1.09

*SD: standard deviation, [a]Percentage of samples tested that were contaminated by Cd and Pb, [b]Mean contamination of positive samples

DV model. The specifications of the instrument were as follows: RF generator power 1.3 kW, gas flow rate 0.2 L/min and nebulizer flow rate 0.8 L/min. The emission wavelengths were 226 nm for Cd and 220 nm for Pb.

Calibration procedure

To assess the linearity of the method, calibration curves were prepared at six different concentrations (0.25-5 mg/L for Cd and 0.4-10 mg/L for Pb) and each injected in triplicates. Blanks were also used during the analysis to check for any possible contamination. For the sensitivity of method, limit of detections (LOD; signal-to noise ratio = 3) and limit of quantifications (LOQ; signal-to-noise ratio = 10) were calculated for Cd and Pb in each of the matrixes. Recovery studies were performed utilizing certified reference materials and were done in triplicate.

RESULTS

Method validation

Cd and Pb recoveries were in the range of 86-104% with a relative standard deviation of 2.15-4.8%. A good linear relationship was observed with correlation coefficients of 0.999 for Cd (with calibration equation of y = 0.9326x - 0.053) and 0.9986 for Pb (with calibration equation of y = 0.9625x - 0.0079). The levels of LOD and LOQ in herbs were 0.08 mg/kg and 0.25 mg/kg for Cd and 0.13 and 0.4 mg/kg for Pb, respectively.

Pb and Cd levels in herbs

In total we analyzed 54 herbs, including liquorice (12), ginger (15), turmeric (15) and nutmeg (12) from unpackaged samples collected from local markets in İstanbul (Table 1). As shown in Table 2, 9 out of 12 liquorice, 15 out of 15 ginger, 14 out of 15 turmeric and 9 out of 12 nutmeg samples contained both Cd and Pb in the ranges of 0.25-0.78 mg/kg and 3.04-6.45 mg/kg, respectively. Among the Cd-positive samples, only one liquorice (0.25 mg/kg) and one ginger (0.25 mg/kg) sample did not exceed the maximum permissible level of Cd (0.3 mg/kg) set by WHO for herbal products (WHO, 2007), whereas 45 out of 54 herbs contained Cd above the maximum permissible level. In addition, the Pb levels in herbs were lower than the maximum permissible level (10 mg/kg) set by WHO (WHO, 2007). We also observed that all positive samples contained both Cd and Pb.

DISCUSSION

The levels of Pb and Cd were investigated in 54 herb samples. The LOD levels were observed as 0.08 for Cd and 0.13 for Pb by ICP-OES analysis. Alhusban, Ata, & Shraim (2019) reported that the LOD levels for Cd and Pb were 0.15 and 0.10 mg/kg, respectively by ICP-OES which are similar for Pb and higher for Cd compared with our results.

As shown in Table 2, 47 out of 54 herbs contained Cd and Pb in the ranges of 0.25-0.78 mg/kg and 3.04-6.45 mg/kg, respectively. Consistently, in a study from Turkey, the rhizomes of *Glycyrrhiza glabra* contained Cd and Pb at 0.4 and 7.725 mg/kg, respectively (Karahan et al., 2020). In India, Kumar et al. showed that nutmeg contained Cd at 0.46 mg/kg and Pb at 30.07 mg/kg, implicating risks associated with the ingestion of herbal medicines contaminated with high levels of Pb (Kumar et al., 2018). Siriangkhawut, Sittichan, Ponhong, & Chantiratikul (2017) reported different levels of Cd and Pb found in medicinal plants, but they did not detect any in turmeric samples in Thailand. In Pakistan, Idrees et al. (2018) and Alhusban et al. (2019) showed Pb at levels of 4.48 mg/kg and 3.74 mg/kg, respectively, in ginger samples, which are similar to the present results. Gasser et al. also reported Cd and Pb in the concentration ranges of < 0.07-0.64 and < 0.4-4.12 in ginger samples, < 0.07-0.18 and < 0.4-1.45 in liquorice samples and < 0.07-0.21 and < 0.4-0.8 in turmeric samples, respectively (Gasser, Klier Kuhn, & Steinhoff, 2009). In a study from North Carolina, Pb was found in the range of 0.7-1.6 in ginger and 0.1-740 mg/kg in turmeric samples (Angelon-Gaetz, Klaus, Chaudhry, & Bean, 2018). In Saudi Arabia, Pb was found at a rate of 1 mg/kg while Cd was not detected in turmeric samples (Seddigi, Kandhro, Shah, Danish, & Soylak, 2016). In a study reported in Latvia, Reinholds, Pugajeva, Bavrins, Kuckovska, & Bartkevics (2017) showed that mean levels of Cd and Pb were 0.04 and 0.13 mg/kg in nutmeg samples, respectively. In Italy, Bua et al. reported detecting Cd in the range of 0.033-0.294 mg/kg in nutmeg and 0.029-0.092 mg/kg in ginger, while Pb was found in the range of 0.164-1.402 mg/kg in nutmeg and 0.309-1.154 mg/kg in ginger samples (Bua, Annuario, Albergamo, Cicero, & Dugo, 2016). In a study from Ethiopia, Baye & Hymete (2010) showed Cd and Pb at mean levels of 0.34-0.42 mg/kg and 0.17-0.25 mg/kg in ginger samples, respectively, from three different places of collection. In a study from Malaysia, Cd and Pb were

found at mean levels of 1.08 mg/kg and 5.54 mg/kg in turmeric and 1.97 mg/kg and 3.15 mg/kg in ginger samples, respectively (Nordin & Selamat, 2013). In Zambia, high levels of Pb and Cd were found at the mean levels of 26.85 mg/kg and 2.39 mg/mg in ginger samples, respectively (Alolga, Chavez, & Muyaba, 2018). Olujimi et al. (2017) did not detect Pb or Cd in ginger samples in Nigeria. In Iran, commercial powder and root samples of turmeric were positive for Cd content (0.11–1.15 mg/kg) and were positive for Pb content (0.11–0.62 mg/kg) (Ahmed, Khaleeq, Huma, & Munir, 2017). Pb and Cd were detected at the levels of 0.093 mg/kg and 0.059 mg/kg in turmeric and 0.039 mg/kg and 0.105 mg/kg in nutmeg, respectively in the Republic of Korea (Shim, Cho, Leem, Cho, & Lee, 2019).

Limited studies have been conducted regarding heavy metal contamination of herbal materials in Turkey (Başgel & Erdemoğlu, 2006; Bilgic et al., 2015; Leblebici et al., 2012; Ozcan, 2004; Özcan et al., 2008; Ozcan & Akbulut, 2007; Ozyigit et al., 2018; Sekeroglu et al., 2008; Tercan et al., 2016; Görür et al., 2011; Divrikli et al., 2006; Ozkutlu et al., 2006; Ozden & Ozden, 2018; Tokalıoğlu, 2012). Among these, only a few studies reported on the heavy metal contamination of turmeric, ginger, nutmeg, and liquorice. Ozkutlu et al. (2006) reported Cd at the mean levels of 0.029 mg/kg in turmeric and 0.072 mg/kg in ginger, while Cd was not detected in nutmeg samples. Studies from Ozcan & Akbulut (2007) and Sekeroglu, Ozkutlu, Kara, & Ozguven (2008) found Cd at mean levels of 0.72 mg/kg and 0.043 mg/kg in liquorice, respectively. In another study, Pb was detected at a mean concentration of 3.01 mg/kg in ginger samples Tokalıoğlu (2012). In general, our data found levels of Cd and Pb in herbs very similar to the values reported in the literature from Turkey and elsewhere. It has been implied that variations in metal accumulation may be a result of differences in species, harvesting times, soil properties, locations and geographic conditions (Özcan et al., 2008; Kumar et al., 2018; Arpadjan, Celik, Taskesen, & Gucen, 2008). As shown in the studies above conducted worldwide, it has been observed that very different levels of Cd and Pb levels are detected in herbs. It is concluded that the result may be due to different geographic locations, agricultural input (fertilizer and pesticides) and industrial activities.

A tolerable weekly intake of Cd of 2.5 µg/kg bw was established by the European Food Safety Authority (EFSA) (EFSA, 2011). In the present study, the weekly intake of Cd (µg/kg bw) was calculated according to our results because the tested herbals contained Cd levels exceeding those proposed by WHO (WHO, 2007). It is considered a mean daily consumption of 2.3 g of herbal teas for the Middle Eastern diet (WHO, GEMS/Food Regional Diets) for an adult with a mean body weight of 70 kg (WHO, 2003). In the present study, we found the maximum Cd level to be 0.78 mg/kg in the turmeric sample, which gives us the highest estimated human weekly intake for Cd as 0.18 µg/kg. Thus, the intake of Cd represents 7.2% of the tolerable weekly intake set by EFSA (EFSA, 2011) which did not represent a risk to human health. Exposure assessment of Cd from the consumption of herbs (such as turmeric) used in tea was estimated for the first time for the Turkish population. Considering the different exposure sources, it is thought that it is important to monitor heavy metals in herbal products.

CONCLUSION

In summary, Cd and Pb were detected in the range of 0.25-0.78 mg/kg and 3.04-6.45 mg/kg in herbs, respectively. We revealed that Cd levels in herbals exceeded the permissible limits, whereas Pb levels fell under the limit recommended by WHO (WHO, 2007). According to the exposure assessment for Cd, the intake of herbs does not represent a risk to human health. However, taking herbs together with other foods contaminated with heavy metals may cause adverse health effects resulting from the accumulative effects of heavy metals. Considering the different factors in geographical conditions and industrial activities, metal levels in herbs should be carefully monitored. In conclusion, regular monitoring of herbal materials, including medicinal plants sold in local markets as well as imported products, is necessary, as is continued consideration of the risks of heavy metal contamination.

Peer-review: Externally peer-reviewed.

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

Financial Disclosure: Authors declared no financial support.

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Istanbul J Pharm 51 (3): 398-402
DOI: 10.26650/IstanbulJPharm.2021.880454
Original Article

Anatomy of *Consolida orientalis* (Gay) Schröd. (Ranunculaceae): Root, stem and leaf

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Cite this article as: Gumusok, S., & Hurkul, M. M. (2021). Anatomy of *Consolida orientalis* (Gay) Schröd. (Ranunculaceae): Root, stem and leaf. *İstanbul Journal of Pharmacy, 51*(3), 398-402.

ABSTRACT

Background and Aims: *Consolida orientalis* (J.Gay.) Schrödinger belonging to the Ranunculaceae family, which is called "morçiçek" in Turkey. It can be recognized by its laciniae linear leaves, intense violet flower, and follicle fruit. The aim of the present study is to elucidate the anatomical properties of *C. orientalis*, as one of the medically important plant species of Ranunculaceae family.

Methods: Plant material was collected from Kahramankazan (Ankara-Turkey). The samples were protected in 70% alcohol. The transverse and surface sections were investigated by light microscope and photographed.

Results: The root is characterized by a periderm, pericyclic sclerenchymatous ring, and vessels embedded in sclerenchymatous pith cells. It was observed that in the anatomical features of the stem, the adaxial walls of epidermis cells curved outward and were covered with a thick cuticle layer. Moreover, stomata were observed in the epidermis and the stem had hairs only at the above parts, while other parts were bare. These hairs consisted of both glandular (with a long stalk) and non-glandular (unicellular, silicified) types. The leaf is broadly v-shaped, monofacial and has a 1-layer epidermis. Ranunculaceous stomata and non-glandular (unicellular, silicified and slightly bowed) hairs were observed on both surfaces.

Conclusion: The root, stem, and leaf anatomy of *C. orientalis* was studied for the first time. The anatomical features described in detail with reference reports could be helpful in future taxonomic studies.

Keywords: Ranunculaceae, *Consolida orientalis*, plant anatomy, Turkey

INTRODUCTION

Ranunculaceae is a widely propagated and prolific family, spreading across all continents except Antarctica and includes 52 accepted genera (Heywood, Brummitt, & Culham, 2007; POWO, 2021). The family usually consists of perennial herbs, and rare woody climbers. The flowers are actinomorphic or zygomorphic. Achenes, follicles or baccate are fruit types of the family (Brummitt, 1992; Davis, 1965; Evans, 2002).

The most common chemical substances in this family are alkaloids, flavonoids, phenolic acids, phytosterols, fatty acids, and essential oils and also includes important medicinal plants with insecticidal, antiparasitic, antimicrobial, antiviral, antitumor, and antioxidant properties (Evans, 2002; Hao, 2018; Yin, Cai & Ding, 2020).

Consolida (DC.) S.F. Gray members are annual herbs, spread from the West Mediterranean to Central Asia, and Anatolia is accepted as the diversity centre of the *Consolida* genus (Davis, 1965). *Consolida orientalis* (Gay) Schröd. can be recognized by its laciniae linear leaves, intense violet flower, and follicle fruit (Davis, 1965). According to current reports, the plant named *Delphinium ajacis*

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Submitted: 16.02.2021
Revision Requested: 04.06.2021
Last Revision Received: 06.06.2021
Accepted: 24.06.2021
Published Online: 06.12.2021

L. and *C. orientalis* are synonymous (IPNI, 2021; POWO, 2021). According to Güner, Aslan, Ekim, Vural & Babaç, (2012), *C. orientalis* is named "*morçiçek*" in Turkey, and its synonyms are the *Delphinium orientale* Gay, *D. bithynicum* Griseb., *D. hispanicum* Willk., *D. orientale* subsp. *hispanicum* (Willk.) Batt., *D. orientale* var. *brevicalcaratum* Huth, *D. ajacis* var. *orientale* (Gay) Finet & Gagnep.

The *Consolida* and *Delphinium* species are morphologically mixed with each other (Yin et al., 2020). Researching the anatomical features and differences of plant organs is of great importance in distinguishing taxonomically mixed species. There have been no comprehensive anatomical studies done on the *Consolida* species until now. With other taxonomic features, the anatomical knowledge is required for *Consolida* taxa. The anatomical studies evaluated until now consisted of generalizations on the Ranunculaceae family by revealing the anatomical features of certain taxa (Maxwell, 1893; Metcalfe & Chalk, 1965). In this study, the anatomical features of *C. orientalis* root, stem, and leaf were examined in detail.

MATERIALS AND METHODS

The plant material was collected from Kahramankazan (Ankara/Turkey) and described by Safa Gümüşok. A voucher specimen was deposited in the Ankara University Faculty of Pharmacy Herbarium (AEF 29964) in Turkey. The samples for anatomical studies were protected in 70% alcohol. The transverse and surface sections were cut by hand with a razor blade into microscopic preparation form. The *Sartur* solution (Türk Farmakopesi, 2017) was used in microscopic examinations. A Leica DM 4000B microscope was used for anatomical analysis and micro photographing.

RESULTS AND DISCUSSION

Root anatomy

The transverse section of the root consists of the periderm, pericyclic derivatives, and vascular tissues. The root consists of phellem layers outward and 1-2 layered phellogen and multi layered phelloderm inward. The phellem is composed of 1-5 layers of cells having an isodiametric or elongated shape. Cortex parenchyma cells are almost absent. Sometimes, under the pit walled single-row endodermis, there is a pericycle forming a sclerenchyma cap. The vessels of the root are embedded in lignified cells. A sclerenchymatous cap are present immediately on the outside of the phloem. Polyarch xylem is branched into 10-12 radial multiples (Figure 1).

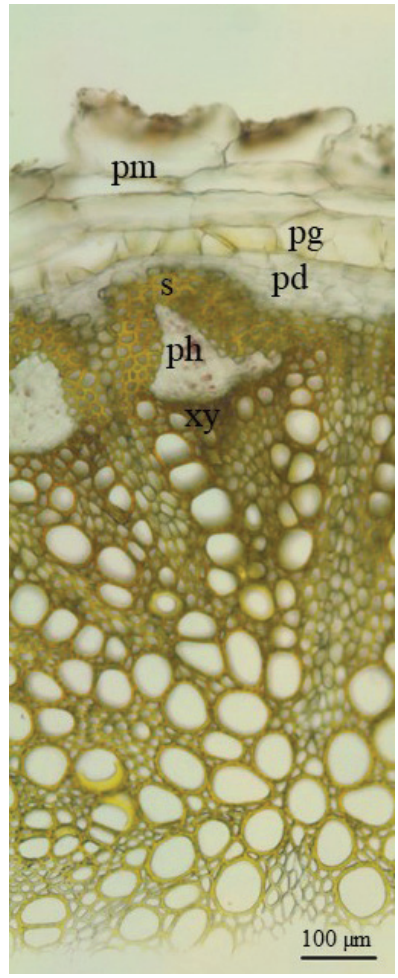

Figure 1. Transverse section of root in *C. orientalis*.
pd: phelloderm, pg: phellogen, ph: phloem, pm: phellem, s: sclerenchyma, xy: xylem

Stem anatomy

The epidermis is covered with a very thick cuticle layer in the transverse section of the stem. Its cells are single-layered, rounded, the adaxial and abaxial wall is thick, side walls are thin, the adaxial walls are curved outward, and the intracellular space is large. Stomata is observed. The cortex parenchyma cells, except cells in the oval shaped last layer, are longitudinally elongated, thin-walled, dense starchy, and consist of 2-4 rows of cells. Collateral type vascular bundles are arranged in a circle. The xylem are indistinctly concave where adjoining the phloem. Phloem are bounded externally by the pericyclic sclerenchymatous cap. The rays as 10-12 arms extend between vascular bundles, consist of 2-5 rows of cells laterally and contain very dense starch. Pith cells are thick-walled, sometimes with pitted walls, rounded, dense starchy, lignified cells, and intracellular spaces are large (Figure 2a, b, c, d). The above parts of the stem differ from the other parts in terms of hairs. The above parts of the stem covered by densely glandular (with a long stalk) and non-glandular (unicellular, silicified) types hairs, and other parts are bare (Figure 3a, b).

Leaf anatomy

The transverse section of leaf was observed as being broadly v-shaped and monofacial. The epidermis is composed of single-layer cells surrounded by a very thin cuticle layer. The shape of cells is ovoid and the outer walls are thick. The upper epidermis cells are larger than lower epidermis cells. There are non-glandular hairs (unicellular, silicified) and stomata on both the upper and lower epidermis. The mesophyll consists of tightly arranged parenchymatous cells. The palisade parenchyma consists of single-layer, long-cylindrical, thin-walled cells. The cells of the spongy parenchyma between the palisade layers are isodiametric, thin-walled, 2-6 rows and fills between the vascular bundles. Both palisade and spongy parenchyma cells

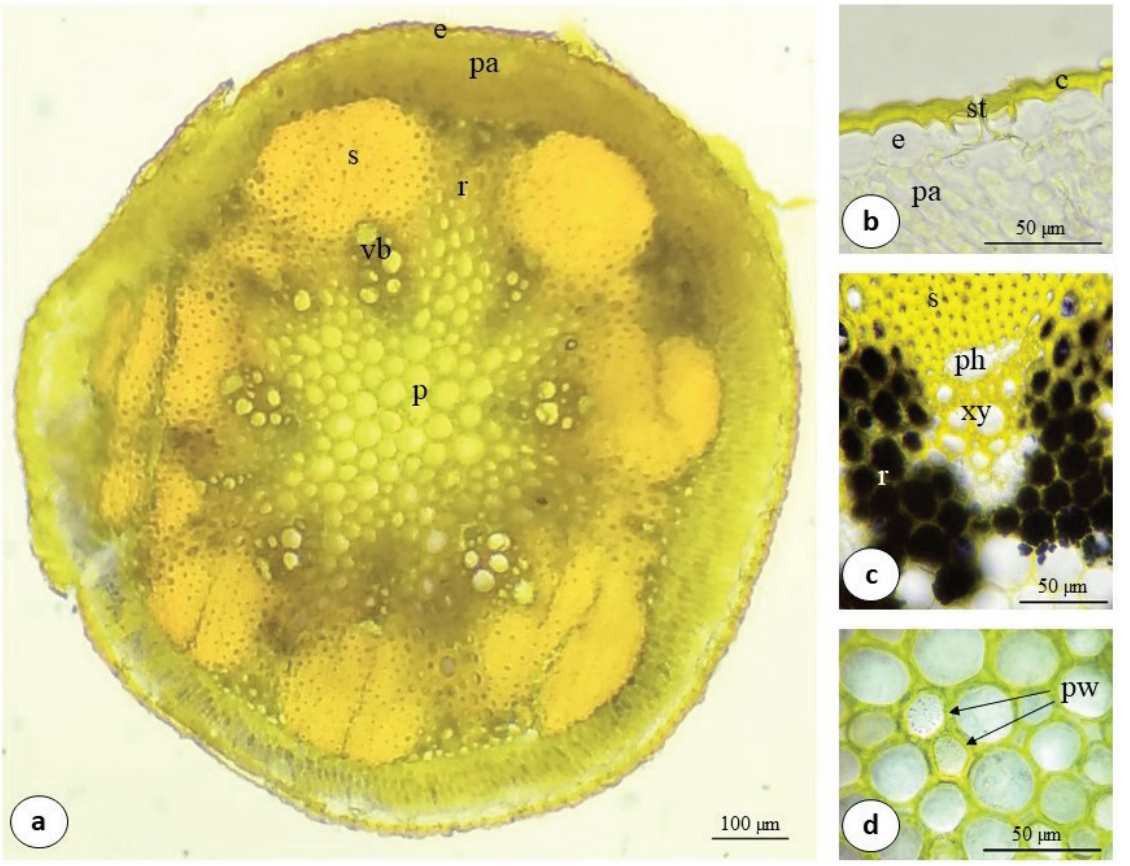

Figure 2. Transverse section of stem in *C. orientalis*.
c: cuticle, e: epidermis, p: pith, pa: cortex parenchyma, ph: phloem, pw: pitted walls of pith cells, r: ray, s: sclerenchyma, st: stomata, vb: vascular bundle, xy: xylem

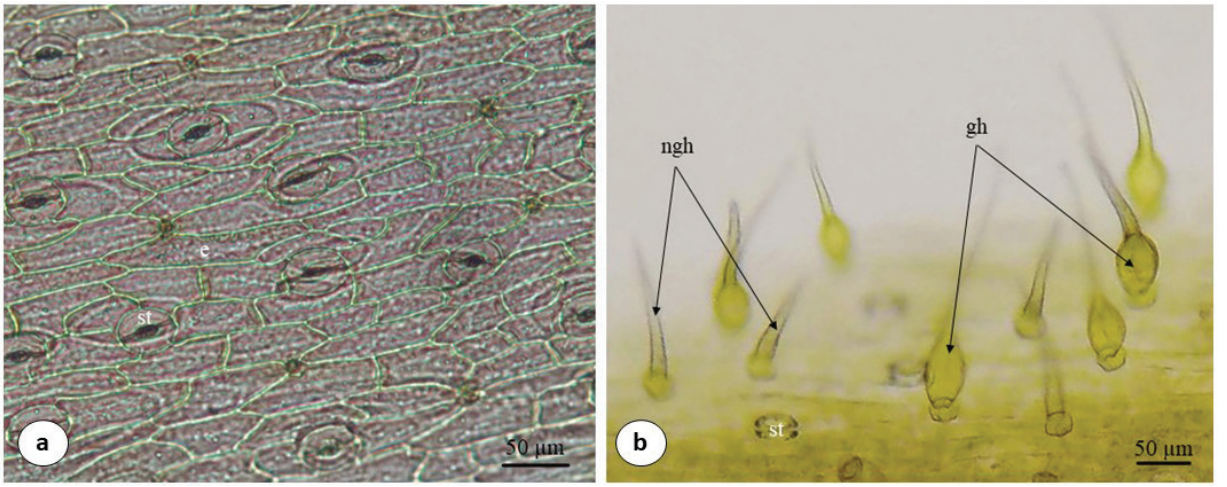

Figure 3. Surface sections of stem in *C. orientalis*.
e: epidermis, gh: glandular hair, ngh: non-glandular hair, st: stomata

contain abundant starch. The vascular bundles are surrounded by a compactly arranged bundle sheath (Figure 4a, b, c, d).

Both epidermal structures of the leaf were observed as similar in surface sections. The cells of epidermis with sinuous anticlinal walls contains ranunculaceous stomata and non-glandular hairs. The hairs are unicellular, silicified and slightly bowed (Figure 5a, b, c, d).

According to Metcalfe & Chalk (1965); the root of Ranunculaceae has a sclerenchymatous pith region in anatomical structure, a sclerenchymatous cap located on the adaxial side of vascular

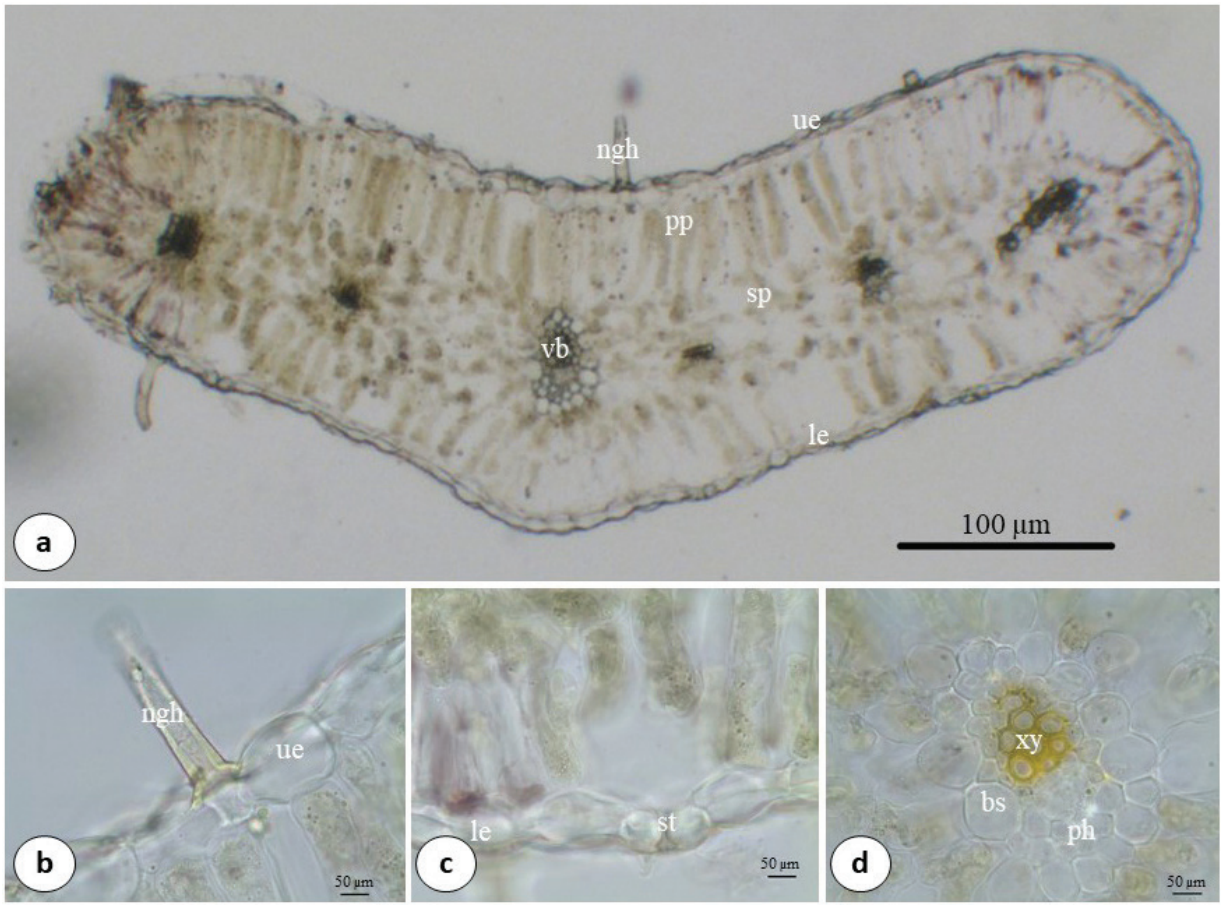

Figure 4. Transverse section of leaf in *C. orientalis*.
bs: bundle sheath, le: lower epidermis, ngh: non-glandular hair, pp: palisade parenchyma, ph: phloem, sp: spongy parenchyma, st: stomata, ue: upper epidermis, vb: vascular bundle, xy: xylem

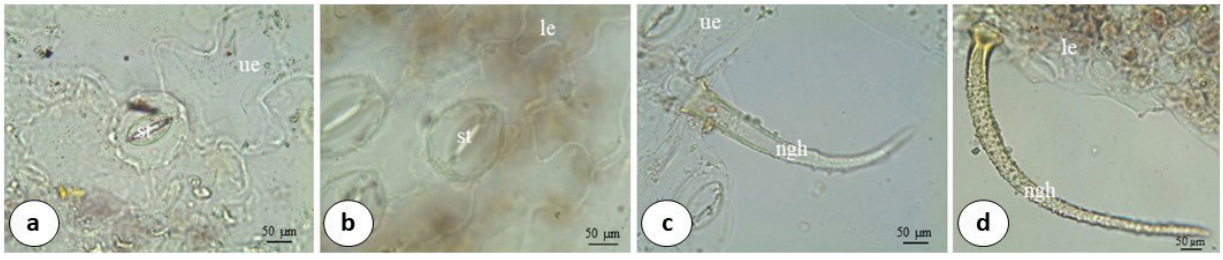

Figure 5. Surface sections of leaf in *C. orientalis*.
le: lower epidermis, ngh: non-glandular hair, st: stomata, ue: upper epidermis

bundles. The *C. orientalis* root, as characterized by the periderm layer, pericyclic sclerenchymatous cap and vessels embedded in the sclerenchymatous pith according to the results, is branched into 10-12 radial multiples polyarch xylem. Generally the Ranunculaceae family root is known as tetrarch (Maxwell, 1893; Yentür, 1995). However, the number of xylem arms also correlates with the viability and size of the root (Yentür, 1995). Periderm layers can be clearly distinguished from each other, as phellem, phellogen, and phelloderm. Additionally, cortex parenchyma cells are absent in anatomical features of the *C. orientalis* root.

The results show that the stem of *C. orientalis* is characterized by a monolayer epidermis with stomata and thick cuticle layer. The epidermis layer has glandular and non-glandular hairs, but only on above parts of the stem, other parts are bare. The cortex parenchyma and sclerenchymatous clusters are in a regular ring form. Vascular bundles in the stem are a collateral type, adaxial side of phloem enclosed by a sclerenchymatous cap. The concave xylem is found in abaxial side of the phloem. Lignified pith cells have large intercellular space and sometimes pitted walls were observed. According to Metcalfe & Chalk (1965), the stem of the Ranunculaceae family has a collateral-type vascular bundle with concave xylem and pericyclic sclerenchyma

ring, with additional non-glandular hairs on the stem. Pith cells are also lignified. What makes this report different is that glandular hairs were observed on above parts of the stem.

According to Metcalfe & Chalk (1965), the Ranunculaceae family has the characteristics of a monofacial or centric leaf. The epidermis has ranunculaceous stomata with glandular and non-glandular hairs. In addition, the lower epidermal cell anticlinal walls are sinuous. Except for the presence of glandular hair, the data of Metcalfe & Chalk (1965) agrees with the findings in this report. In addition, the report results showed that the upper epidermis of monofacial leaf was also sinuous. The non-glandular hairs of the leaf are unicellular, silicified and slightly bowed.

In conclusion, the root, stem and leaf anatomy of *C. orientalis* was studied for the first time. The anatomical features described in detail with reference reports could be helpful in future taxonomic studies.

Peer-review: Externally peer-reviewed.

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

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

Financial Disclosure: Authors declared no financial support.

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Istanbul J Pharm 51 (3): 403-410
DOI: 10.26650/IstanbulJPharm.2021.909570
Original Article

Morpho-anatomical and palynological studies on *Glaucosciadium cordifolium* (Boiss.) B.L. Burtt & P.H. Davis (Apiaceae) from Turkey

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Cite this article as: Saltan, N., Kaya, A., Dinc, M., & Doğu, S. (2021). Morpho-anatomical and palynological studies on *Glaucosciadium cordifolium* (Boiss.) B.L. Burtt & P.H. Davis (Apiaceae) from Turkey. *İstanbul Journal of Pharmacy, 51*(3), 403-410.

ABSTRACT

Background and Aims: The aim of the present study was to determine the morphological, micromorphological, anatomical and palynological characteristics of *Glaucosciadium cordifolium* (Boiss.) B.L. Burtt & P.H. Davis
Methods: Measurements were performed on approximately 30 different samples for morphological study. The fruit micromorphologies and pollen properties were examined using scanning electron microscopy. For anatomical studies, all sections prepared from stem, leaf and fruit were taken by hand.
Results: Cross-sections of stem, leaf and fruit are examined and a detailed anatomical description is presented and supported by photographs. It is a glaucous perennial herb with a height of 34-180 cm which has an acrid smell when bruised. The fruit is a dry schizocarp, glabrous and oblong-elliptic. The mericarp is elliptic in the transverse section which is winged in the lateral. The fruit surface ornamentation of the genus is occasionally wavy and striped and the upper surface simple, short and scaly. The stomata is observed.
Conclusion: In this study, morphological, anatomical and palynological properties of the *Glaucosciadium cordifolium* are described and illustrated for the first time. In addition, the palynological results confirmed the stenopalynous characteristic of the family Apiaceae, and revealed that the pollen grains of the genus are perprolate in shape.

Keywords: Anatomy, Apiaceae, *Glaucosciadium cordifolium*, morphology, palynology

INTRODUCTION

Apiaceae, a family known for its characteristic flower structure, includes 464 genera and 3780 species (Calviño, Teruel & Downie, 2016). Moreover, this family, which is capable of being the eighth largest family,consists of about 455 species occurring in 33% of Turkey's endemic (Davis, Mill & Tan, 1988; Duman, Guner, Ozhatay, Ekim & Baser, 2000).

Anatomy and morphology are taxonomically very important in Apiaceae and nearly all traditional classification systems of the family have relied on fruit characteristics. Apiaceae species have secretory cavities (vittae) in roots, petioles, stems, leaves and fruits, which carry schizogenic fatty ducts containing resin, oil or mucilage (Duman et al., 2000; Metcalfe & Chalk, 1950). Although anatomical characters are used to distinguish between closely related species and genera in the Apiaceae family, they are not always as useful as morphological characters (Akalın Uruşak & Kızılarslan, 2013).

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Submitted: 04.04.2021
Revision Requested: 24.07.2021
Last Revision Received: 01.08.2021
Accepted: 23.08.2021
Published Online: 09.12.2021

It is also a solution for the taxonomic problems that can be encountered in species determination to reveal and evaluate all the characters of the plant together.

According to the Flora of Turkey, the genus *Glaucosciadium* B.L.Burtt & P.H.Davis is represented by one taxon in Turkey and two taxa in the world (Hedge & Lamond, 1982). *G. cordifolium* was published by Burtt and Davis in Kew Bulletin in 1949. *G. cordifolium*, which grows on the stony river banks, slopes and chalk slopes of Southern and Central Anatolia, is a monotypic species and is known as "Çağşır otu" and "Sakar otu" in the regions where it grows. The plant, which has an acrid smell when crushed, is used as an aphrodisiac in Turkish folk medicine, mixed with honey (Güner, 2012; Özhatay & Koçak, 2011; Hedge & Lamond, 1982). The aim of this paper is to give a detailed description of the morphological and anatomical features and pollen characteristics of *G. cordifolium*. Our findings will contribute to the systematics of the Apiaceae family. Although there are a limited number of studies on the chemical content of *G. cordifolium*, the fact that there are no studies on the morphology, anatomy and pollen properties makes the study important.

MATERIALS AND METHODS

Plant material

G. cordifolium was collected from the provinces of Kütahya (B2: Kütahya, Uşak-Gediz road, 3. km after the Uşak junction, 658 m., ESSE 15001) and Karaman (C4: Karaman, Ermenek, Ermenek-Balkusan road 4. km, 1650 m., ESSE 15500) in Turkey during flowering time in July 2014. Herbarium specimens, prepared by pressing and drying of the plant, were stored in the Herbarium of the Faculty of Pharmacy of Anadolu University, in Eskisehir, Turkey (ESSE).

Morphology

All measurements regarding the morphological characters of the plant were made on living samples. Measurements were performed on approximately 30 different samples, and land notes were used for some morphological characteristics. General view, flower-fruit in compound umbel, single flower-fruit and petal of specimen were drawn. The flower and fruit parts of the plant were drawn using the drawing tube of the Olympus SZX12 stereo microscope.

Anatomy

Some of the plant samples were preserved in 70% ethanol to be used in anatomical studies. All sections prepared from stem, leaf and fruit were taken by hand, after staining with Sartur reactive (Çelebioğlu & Baytop, 1949), the sections were taken into glycerin-gelatine and the microscope slides were made permanent with Canada Balsam. At least 30 preparations were examined for each structure and photographs of the plant parts were taken with a light microscope (Olympus BX51T). The width and length of the epidermal cells, as well as the number of stomata and epidermal cells per unit area were determined for both leaf surfaces and the stomatal index (SI) as a percentage was calculated (Meidner & Mansfield, 1968).

Palynology

The pollen of the plant, taken on double-sided adhesive tape,was mounted on SEM stubs, covered with gold and photographed with Zeiss Ultra Plus Fesem. In pollen terminology, characteristic structures were adopted from Faegri & Iversen (1975) and Punt,Hoen, Blackmore, Nilsson & Le Thomas (2007).

RESULTS AND DISCUSSION

Morphological properties

Glaucosciadium is a monotypic genus, which is distributed in South and Central Anatolia. Since its description (Burtt & Davis, 1949), a detailed study about its anatomy, morphology and pollen properties on it had never been conducted before.

Description

Creeping, perennial, glaucous, erect, 34-180 cm long and 3-10 mm diameter, glabrous plant. Stem branched and pronounced stripes, with an acrid smell when bruised. Basal leaves 1-2 pinnate, ovate-triangular, 9-53 × 4-26 cm. Basal leaflets, widely ovate, rarely reniform, 10-35 × 8-90 mm, apex mucronate, base cordate - truncate, reticulate veined, sessile or up to 75 mm handle. Upper leaves sheath-like, elliptic-oblong, 5-25 × 10 mm, apex acute-obtuse, divided 1-3 segments at the top, base ocrea. Bracts 3-6, linear-lanceolate to elliptic-oblong, 4-27 × 2-16 mm. Bracteoles absent. Umbells compound, wide spread, unequal 4-10 rayed, rays 5-45 mm

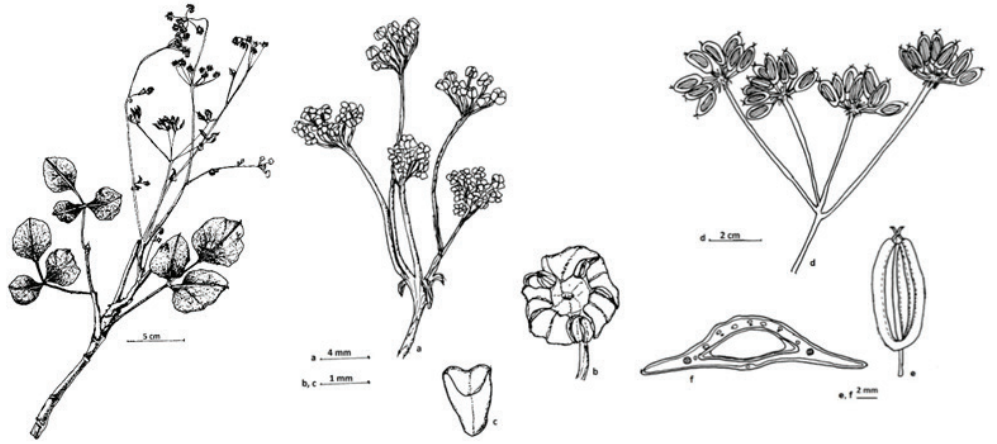

Figure 1. *G.cordifolium* ESSE 15001, Kütahya; a-compound umbel-flower, b-flower, c- petal, d-compound umbel- fruit, e-fruit; f- fruit cross section (Saltan, 2015).

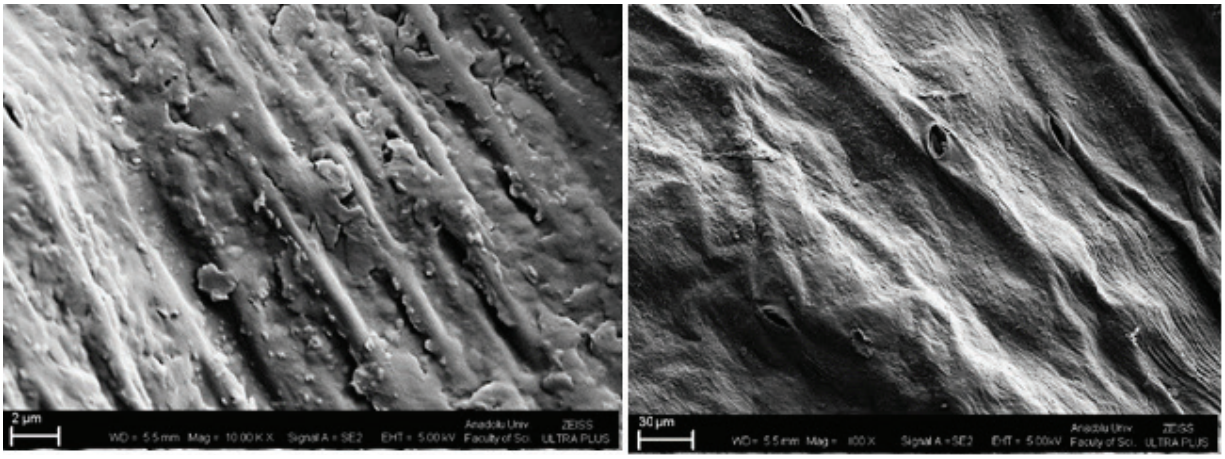

Figure 2. Scanning electron micrographs (SEM) of fruit surface of *G. cordifolium* (Saltan, 2015).

in flower, 17-100 mm in fruit. Flowers 7-23, orbicular, actinomorphic. Outer flowers of umbel hermaphrodite, 4-10, pedicels 4-5 mm. Sepals absent. Petals creamy white, 5, equal, free, 0.7-1.5 x 0.8-1 mm, significantly internal curved. Pedicels with glandular hairs. Stamens 5, free, cream, 0.4-0.5 × 0.2-0.4 mm, versatile sometimes basifixed. Filaments white and 0.5-1 mm. Ginekeum 0.8-1 × 1.2-2 mm, stylopodium 0.7 × 1 mm. Ovary inferior, of 2- united carpels. Fruit is a dry schizocarp, glabrous, oblong-elliptic, 9-19 × 3-6 mm. Ridges conspicuous 1-2 mm, 6-12 on dorsale, 2 on side (Figure 1). The fruit surface is occasionally wavy and striped, upper surface simple, short scaly. The stomata is observed (Figure 2).

Morphological features of *G. cordifolium* specimens collected from two different localities were compared with the description in Flora of Turkey, and some morphological features were found to be more variable than previously reported. When the plant samples collected from Kütahya and Karaman were evaluated morphologically, it was observed that they were highly similar. However, the leaves in the plant collected from Karaman were larger than those collected from Kütahya, and the pedicel and ray lengths were also higher. In addition, measurements related to many characters not in the Flora of Turkey were given in Table 1. While the stem length was reported to be up to 180 cm, it was indicated as 40-150 cm in Flora of Turkey. These differences are thought to be caused by geographical and climatic-edaphic factors (time of flowering, altitude, climate). When the findings were evaluated, it was determined that the dimensions of the plant parts were compatible with the Flora of Turkey data, in addition, the limits of variation of the species in the Flora of Turkey were expanded (Table 1). According to Burtt & Davis (1949), *Glaucosciadium*, which is distributed in the Old World, especially in Eurasia and Africa, resembles *Peucedanum*, a heterogeneous genus of 100-120 species, but the absence of commissural vittae shows that *Glaucosciadium* can certainly be isolated from *Peucedanum* (Pimenov & Leonov, 1993). The fruits of *G. cordifolium* are 9-14 × 5-6 mm and elliptic. Mericarp is elliptic in the transverse section which is winged in the lateral. Oil ducts were embedded in mesocarp. While 6-12 vittae were seen onthe dorsal side, there were no oil ducts on the commissural side. Fruit features almost agree with those reported in the Flora of Turkey (Hedge & Lamond, 1982). Fruit surface is occasionally wavy and striped, the upper surface is simple, short and scaly. The stomata is observed. Similarly, Saltan & Kaya (2020) reported that the fruit surface of *C. siifolium* Boiss. & Heldr is wavy and striped in places, simple, short and scaly.

In 2014, Zhou et al. examined the epidermal characters of 21 species of *Peucedanum* in the leaf using light microscopy and scanning electron microscopy, and found that stomata in all species were anisocytic, anomocytic or staurocytic in the abaxial epidermis, and in 12 species, there were stomata on both sides (Zhou, Wang, Gong & Liu, 2014). Zhang & He (2009) described six *Peucedanum* species growing in Southwest China and reported that *P. praeruptorum* had stomata only in the lower epidermis (Xuemei & Xingjin, 2009). On the other hand, it was reported that the leaves of *Ferula* (Karakaya, Bostanlık & Kılıç, 2018), *Prangos* (Ahmed, 2008) and *Seseli* (Güner & Duman, 2013) were monofacial as *G. cordifolium*.

The observation of different types of stomata (anomocytic, anisocytic and parasitic) on both leaf surfaces and the fact that the leaf is monofacial and mesomorphic show that our findings are similar to previous studies with close genera.

Anatomical properties

Stem anatomy

Transverse sections taken from the middle part of the stem were observed as follows. Stem were nearly round and ripped. The epidermis is composed of a single layer of oval, square, oblong to rectangular. A regular arrangement of cells in the epidermis is observed. Upper surface is covered with a thin cuticle. The cortex tissue consists of 5-8 layered chlorenchyma tissue (= photosynthesizing tissue) which comprises dense parenchyma cells containing chloroplasts and an ergastic substance located below the epidermis. Several rows of lightly crushed parenchyma cells and 3-4 layered parenchyma cells which contain an ergastic substance were located under this layer. Sclerenchyma tissue is well-developed below the parenchyma and located in groups with 10-13 layers. The collenchyma tissue, which consists of 12-15 layered ovoid or triangular

Table 1. Comparison of the morphological characters of *G. cordifolium.*

Characters	Kütahya province	Karaman province	Flora of Turkey (Hedge & Lamond, 1982)
Stem	34-180 cm	35-175 cm	40-150 cm
Basal leaves	Ovate-triangular 9-53 × 4-15 cm petiole 35-80 mm	Ovate-triangular 10-30 × 6-26cm petiole 50-80 mm	Ovate-triangular 10-40 × 7-25 cm
Leaflets	10-65 × 8-70 mm, sessile or up to 65 mm ovate sometimes reniform	35-63 × 30-90 mm sessile or up to 75 mm ovate sometimes orbiculate	1.8-8 × 1.9-9 cm ovate
Upper leaves	5-25 × 9 mm Sheat-like, sometimes elliptic-oblong	7-25 × 10 mm Sheat-like, sometimes elliptic-oblong	-
Bracts	4-25 × 2-10 mm linear-lanceolate	5-27 × 2-16 mm Lanceolate	-
Bracteoles	3, linear 1-5 × 0.5-3 mm	3-5, linear 1-5 × 0.5-3.5 mm	3-5
Ray	4-10 5-35 mm (in flowers) 17-100 mm (in fruits)	4-10 5-45 mm (in flowers) 19-110 mm (in fruits)	5-13 10-75 mm (in flowers)
Flowers	7-23 per umbellule	9-22 per umbellule	15- 25 per umbellule
Pedicel	0.5-5 mm (in flowers) 1-6 mm (in fruits)	1.5-7 mm (in flowers) 2-8 mm (in fruits)	-
Petal	White, 1-1.5 × 0.8-1 mm mm	White, 0,7-1 × 0,9-1 mm	-
Stamen **Anter**	5, free, versatile, bazifiks 0.3 × 0.5 mm, brown 0.5-1 mm, white	5 free, versatile, bazifiks 0.2 × 0.5 mm, brown 1.5-2 mm, white	-
Ginekeum	0.8-1 × 1.2-2 mm	0.8-1 × 1.5-2 mm	
Fruit	9-14 × 5-6 mm oblong elliptic, brown	9-19 × 3-6 mm oblong elliptic, brown	10-12 x 5-6.5 mm
Mericarps	subconvex 5 ridges 8-9 vittae in dorsal, 2-3 vittae in lateral commissures 8 vittae	subconvex 5 ridges valleculae 3-4 vittae commissures 6-8 vittae	oblong–elliptic 5 ridges
Stylopodium	0.4 × 0.9 mm, conical	0.5 × 0.9 mm, conical	-

cells, is located between epidermis, secretory canals and vascular bundles. The secretory canals, which surround 8-12 secretory cells, are embedded below the parenchyma cells. Vascular bundles are numerous (17-24) and arranged in a ring. Phloem and xylem are partly separated from one another by parenchyma and sclerenchyma tissue. Phloem consists of flattened, irregular cells, 8-10 layers. The pith located in the center of the stem is completely filled with large polygonal parenchymatic cells (Figure 3).

Leaf anatomy

Anatomical features have been revealed by examining the transverse and superficial sections of the leaf. In transverse sections, upper and lower epidermis comprise uniseriate rectangular to quadrangular cells. The upper walls are generally thicker than the lower and side walls, with both epidermal surfaces covered by a cuticle. Mid-rib was swollen and included a larger vascular bundle compared to the other parts of the mesophyll. Different types of stomata (anomocytic, anisocytic and parasitic) were observed on both surfaces of the leaf, which were located at the same level as the epidermal cells (mesomorphic) (Figure 5). While the leaf stomata index was 15.38 on the upper side, the lower side of the leaf was found to be 15.06. The parameters of stomata and epidermis were given in Table 2. According to Table 2, percent lower leaf epidermis and stomata number is similar for both faces. Leaf is monofacial. Mesophyll comprises 2-seriate palisade parenchyma cells which contain chloroplasts. 2-4 seriate spongy tissue is located between palisade tissue. 6-8 layers of collenchyma are located below lower epidermis. Secretory canals were surrounded by 8-12 layers of secretory cells. Vascular bundles were located in the parenchymatic tissue. Sclerenchyma is located between phloem and xylem (Figure 4).

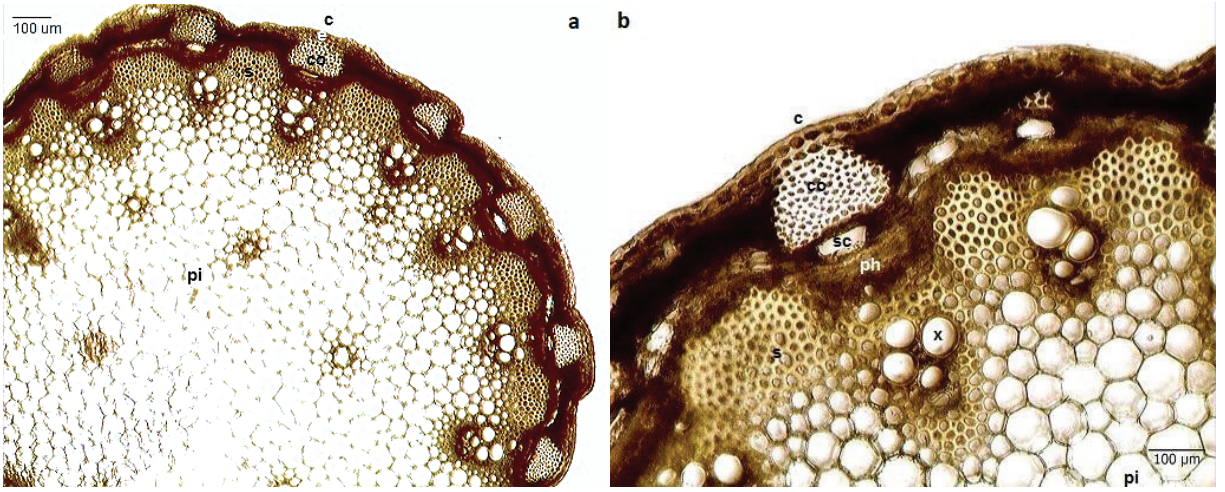

Figure 3. Cross-sections of the stem of *G. cordifolium* (a-Kütahya, b-Karaman): c- cuticle, e- epidermis, co- collenchyma, sc- secretory canal, s- sclerenchyma, ph- phloem, x-xylem, pi- pith.

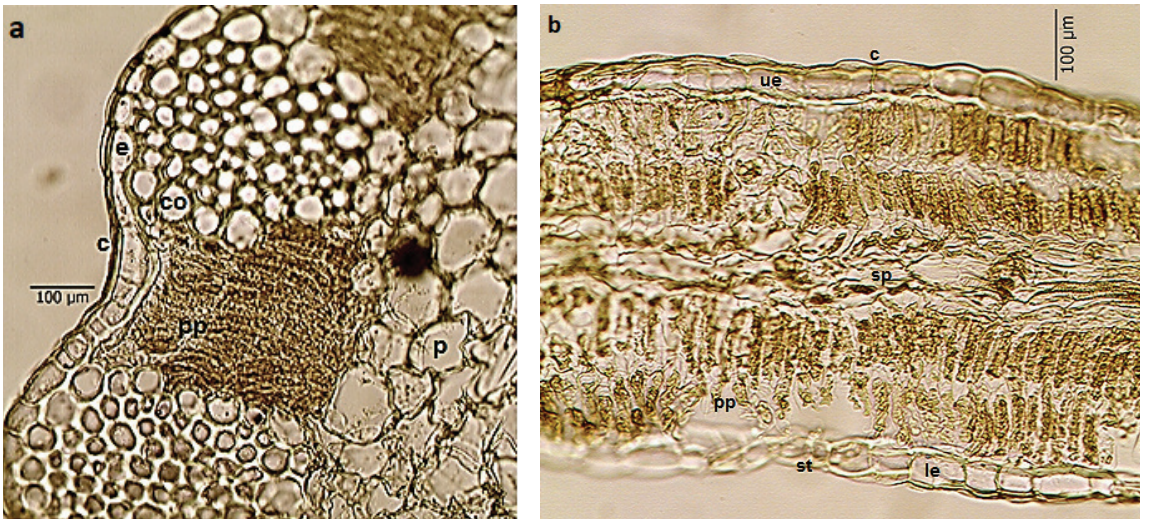

Figure 4. Cross-sections of the leaf of *G. cordifolium* (a- Kütahya, b- Karaman): c- cuticle, e- epidermis, co- collenchyma, p- parenchyma, pp- palisade parenchyma, sp- spongy parenchyma, le-lower epidermis, st- stomata, vb- vascular bundle, ue- upper epidermis.

Table 2. Comparison of epidermis and stomata in upper and lower surfaces of *G. cordifolium* leaves.

	Adaxial surface	Abaxial surface
Number of stomata (mm²)	12 (±2)	11 (±2)
Number of epidermis cells (mm²)	66 (±5)	62 (±5)
Stomata index (SI)	15.38	15.06
Stomata width (µm)	12-20	12-16
Stomata length (µm)	15-24	13-20
Width of epidermis cells (µm)	10-35	12-34
Length of epidermis cells (µm	19-49	14-55

Fruit anatomy

In the cross sections taken from the middle part of the fruit, it was seen that the fruit was a schizocarp with two mericarps. In addition, the mericarp is elliptic in the transverse section and is winged in the lateral. Exocarp (the outer epidermis) is composed of a single layer of square and rectangularly arranged cells.The upper surface is covered with a cuticle. Mesocarp consists of 4-6 layered, oblong-ovoid, polygonal parenchymatic cells with thin walls. Oil ducts (vittae) were embedded in the mesocarp. 6-12 vitae were seen on the dorsal side and there were no oil ducts on the commissural side. There was a vascular bundle in the wings. Collenchyma was located near the secretory canals and is small in clusters. The endocarp (the inner epidermis) consists of a single layer of narrow, long cells with thin walls. The testa was located under endocarp. The endosperm was well developed. The embryo was sunk into the endosperm (Figure 6).

There are less anatomical studies on vegetative organs compared to those on generative organs in the family Apiaceae. In this study, it was observed that the leaves of *G. cordifolium* were equifacial, while the leaves of *Chaerophyllum astrantiae* Boiss. & Bal. and *C. aureum* L. were reported to be bifacial by

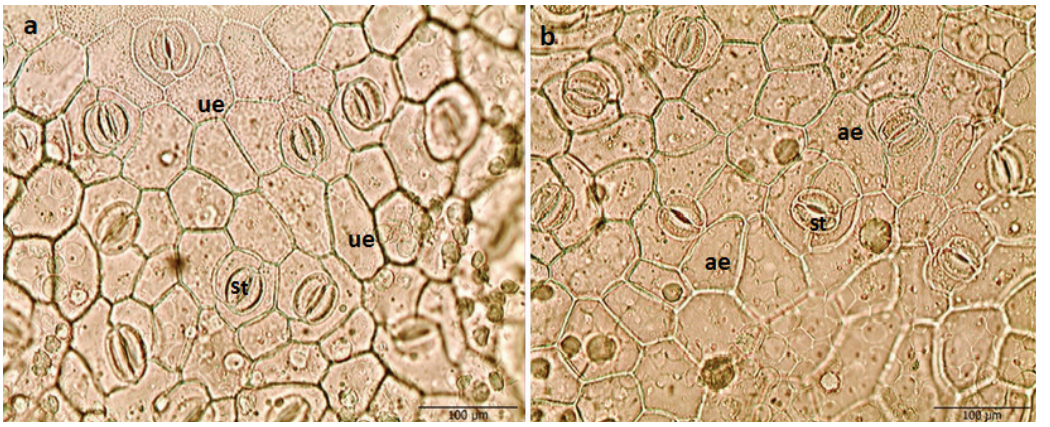

Figure 5. Surface-sections of the leaf of *G. cordifolium*: le-lower epidermis, st- stomata, ue- upper epidermis (a: upper surface, Kütahya, b: lower surface, Karaman).

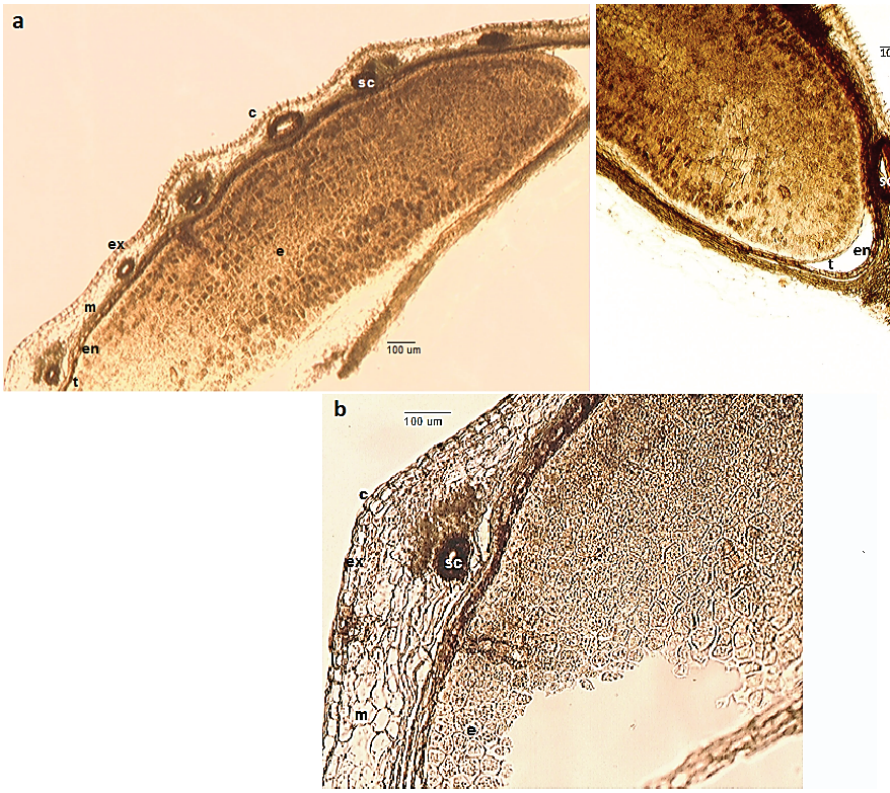

Figure 6. Cross-sections of the fruit of *G. cordifolium* (a-Kütahya, b-Karaman): c- cuticle, ex- exocarp, m- mesocarp, en- endocarp, sc- secretory canal, t- testa, e- endosperm.

Yılmaz & Tekin (2013). In a different study, it has been shown that the leaf of *C. siifolium*, which is another monotypic species in the Apiaceae family, is bifacial and that the epidermis cells only have wavy walls on the lower part of the leaf (Saltan & Kaya 2020). The results show that while mesophyll anatomy is not useful for interspecific classification, it has taxonomic importance in generic classification in the family Apiaceae.

While it has been reported that the collateral type vascular bundles are 13-17 in *C. siifolium*, and the pith consists of 7-10 cell rows, in *G. cordifolium* there are 17-24 vascular bundles and the piths are composed of 4-8 cell rows. In addition, it was stated that the parenchyma cells were fragmented and the pith was empty in *C. siifolium* (Saltan & Kaya 2020). However, in *G. cordifolium* the pith region is well developed and there are secretory channels in places.

There are 4 dorsal vallecular and 2 ventral commissural ducts in the mericarps of *Peucedanum* and *Chaerophyllum* species (Parolly & Nordt, 2005; Yılmaz & Tekin, 2013), 2-10 dorsal vallecular ducts and 2-6 commissural vittae in those of *Pimpinella* species in Iran (Khajepiri, Ghahremaninejad & Mozaffarian, 2010), but only 4 dorsal vitae (oil ducts) and no commissural vittae in the mericarps of *G. cordifolium*. The occurrence of sclerenchymatic

tissue in the mesocarp shows differences among the species in the family Apiaceae. In contrast to some *Peucedanum*, *Chaerophyllum* and *Angelica* species (Parolly & Nordt, 2005; Yankova, 2004; Yilmaz & Tekin, 2013) sclerenchyma is completely lacking in mericarp in the *Pimpinella* species in Iran (Khajepiri et al., 2010) as seen in *G. cordifolium*. Vascular bundles occur in the commissural or dorsal face of mesocarp in Apiaceae. While there are commissural vascular bundles in fruits of *Pimpinella* species (Khajepiri et al., 2010), commissural face of mesocarp lacks vascular bundles in *Peucedanum longibracteolatum* (Parolly & Nordt, 2005) as seen in *G. cordifolium*. In addition, both *Peucedanum longibracteolatum* and *G. cordifolium* include rudimentary oil ducts associated with the vascular bundles in their mericarps.

Palynological properties

The pollen grains of *G. cordifolium* were radially symmetrical, isopolar, and tricolporate. Their shape is euprolate-perprolate. In SEM, dimensions ranges were: polar axis 20.8-31.1 µm, equatorial axis 11.5-15.1 µm, colpus length 17.6-25.4 µm, colpus width 0.4-0.8 µm, pore length 0.8-1.0 µm and pore width 3.5-4.0 µm. The ratio of P/E (Polar axis/Equatorial axis) is 1.80-2.05 µm. The exine sculpturing is rugulate (Figure 7).

Identifying the pollen exine is useful for detecting relationships between species. Apiaceae is a stenopalynous family. The pollen grains and fruit surface belonging to species were firstly photographed via scanning electron microscope (SEM). Except for *G. cordifolium*, the pollen properties of the taxa studied were here described for the first time. Pollen morphology of the examined specimen exhibits some variation in size, shape and sculpture. Saltan & Kaya (2020) determined that the shape of *C. siifolium* pollen was perprolate and the ornamentation was rugulate-striat in the intermediate region and striate in the poles. Perveen & Qaiser (2006), who defined 3 types of pollen in the Apiaceae (Umbelliferae) family according to the exine pattern, determined that the pollen grains were generally tricolporate, the shape of the grains expanded to the prolate-perprolate and the P/E ratio varied between 1.2 and 2.6. P/E ratio is similar toour results (Perveen & Qaiser, 2006). Cerceau-Larrival (1962) identified the pollen of Umbelliferae with 5 types according to the P/E ratio. These types are as follows: subrhomboidal (type 1, P/E: 1-1.5), subcircular (type 2, P/E: 1-1.5), oval (type 3, P/E: 1.5-2), subrectangular (type 4, P/E: 2), and equatorially constricted (type 5, P/E: over 2). When our data is compared with previous studies with close genera, the pollen of *Glaucosciadium* is similar to type 3 with respect to the P/E ratio.

In conclusion, the morphological features of *G. cordifolium* were compared with those in two different localities and Flora of Turkey. Similar morphological features were observed in plants collected from both localities. When the characteristics of the plants collected from different localities were compared, the effect of environmental differences and climatic conditions on the botanical characteristics of the plant were revealed. In addition to the morphological description given in the book Flora of Turkey, the measurements of the new diagnostic char-

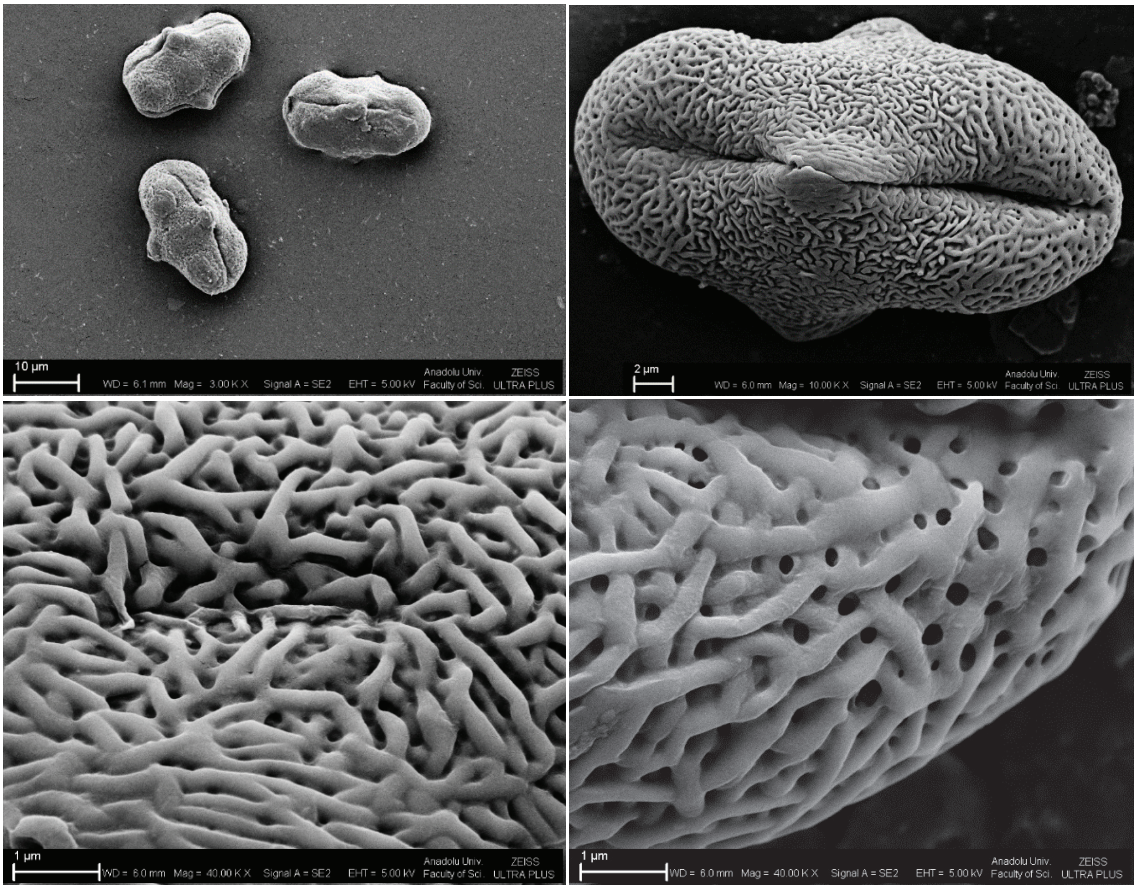

Figure 7. *G. cordifolium*, sculpturing of pollen grain in SEM (Saltan, 2015).

acters of *G. cordifolium* were revealed in this study. Moreover, the description of the studied samples were enlarged with this research. More clearly, the following characters were found to be important for distinguishing *G. cordifolium*: stem, leaf, bract and flower dimensions; the state of secretory canals, number of vascular bundles in the stem; leaf and stoma types and stomata index rates in the leaves; shape, surface ornemantation and number of vittae in the fruit; shape, size and exine sculpture in the pollen. We believe that the time of flowering, altitude, geographical and climatic-edaphic factors may be important. Various factors, both endogenous and exogenous, can affect the morphological characteristics of *G. cordifolium*. Also, the results of this study would support classification based on morphology.

CONCLUSION

Although studies with other Apiaceae species are frequently encountered in the literature, there is very little information about *G. cordifolium*. To our knowledge, this is the first comparative report on the morpho-anatomical and palynological features of *G. cordifolium*.

In this study, in addition to internal and external morphological features of *G. cordifolium*, detailed information is given about the pollen morphology and anatomical features of stem, leaf and fruit. Moreover, the descriptions of the species are expanded and the drawings of the morphological characters of the species, as well as photographs of their anatomical parts and pollen structures are presented for the first time in this study.

We believe that the obtained morphological findings will improve the morphology information of *G. cordifolium* for systematic purposes and the other findings can form a basis for future studies.

Peer-review: Externally peer-reviewed.

Ethics Committee Approval: There are no ethical issues after the publication of this manuscript.

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

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

Financial Disclosure: This study was supported by Scientific Research Projects of Anadolu University with the grant number 1406S314.

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Istanbul J Pharm 51 (3): 411-418
DOI: 10.26650/IstanbulJPharm.2021.854023
Review Article

Prevention and treatment of thrombocytopenia in dengue patients: A narrative review

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Cite this article as: Siddique, N. (2021). Prevention and treatment of thrombocytopenia in dengue patients: A narrative review. *İstanbul Journal of Pharmacy, 51*(3), 411-418.

ABSTRACT

Background and Aims: Dengue fever is a major health concern, especially in developed countries, because it often leads to undifferentiated febrile illness such as dengue haemorrhagic fever and dengue shock syndrome or expanded dengue syndrome, which are often reported with thrombocytopenia (TCP) and turn out to be hazardous to life. The WHO reported 23 million dengue cases and 82,000 deaths between 1955-2012. 2019 was a hallmark for the dengue epidemic from Latin America to South Asia. Due to unavailability of a dengue vaccine and antiviral therapies, additional medical attention and effort are required to treat thrombocytopenia (TCP). These approaches include thrombopoietin receptor agonists such as Oprelvekin, Eltrombopag, Romiplostim and corticosteroids, and platelet transfusion and intravenous immunoglobulin transfusions. The outcomes might not be achievable unless the proper assessment is carried out to evaluate these approaches to treat TCP in dengue patients. This narrative review aims to summarize the findings regarding the treatment of TCP in patients affected by the dengue virus.

Methods: A total of 55 published articles, including clinical trials, descriptive studies and case studies were reviewed regarding treatment of TCP in the context of dengue infection. The electronic databases PubMed, Cochrane Library, Google Scholar and Science Direct were searched for articles published from January 1990 to June 2020.

Conclusions: We concluded that dengue fever is becoming a global health concern. Premedical attention like the use of corticosteroids, Thrombopoietin receptor agonist (TPO-R) agonists and transfusions like (PT & IVIG) are not fully assessed by double-blind randomized clinical trials and cost-effectiveness analysis. However, as compared to other treatment approaches, the use of TPO-R agonists seems to be more suitable in TCP management due to its easy access and compliance.

Keywords: Thrombocytopenia in dengue, Dengue fever, TPO-R agonist

INTRODUCTION

Dengue is a major human insect-borne viral disease and its pathogen Dengue Virus (DV) is a single strand ribonucleic virus belonging to the family Flaviviridae (Monath, 2007). Approximately 23 million dengue cases and 82,000 deaths were reported by the World Health Organization (WHO) from 1955 to 2012 (Ruberto, Marques, Burke, & Van Panhuis, 2015). The year 2019 was marked with increased epidemics of dengue cases from Latin America to Southeast Asia. According to the Pan American Health Organization (PAHO), 236,372 cases were reported and 80,000 tests confirmed cases, while 68 deaths were solely found in America. It is estimated that cases of dengue in 2019 increased by up to 40% compared to the cases in 2018 (Hosangadi, 2019). In December 2019, WHO Pakistan and the Ministry of National Health Services, Regulations and Coordination (MNHSR&C) announced that there were 998 new cases in Pakistan. In the same epidemiological week (week 51), 100 cases were confirmed in Sudan.

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Submitted: 05.01.2021
Revision Requested: 29.04.2021
Last Revision Received: 02.05.2021
Accepted: 05.08.2021
Published Online: 10.12.2021

Clinical presentations of dengue infection vary from symptomless to serious diseases and can be one of the causes of death if left untreated. The symptomatic cases of dengue infection often show undifferentiated febrile illness; i) Dengue Fever (DF), ii) Dengue Haemorrhagic Fever (DHF), iii) Dengue Shock Syndrome (DSS) or Expanded Dengue Syndrome (EDS) (Kalayanarooj, 2011). DF stated as a non-specific febrile state, which is generally denoted as the transmission of the virus from an infected person through mosquito bite to another person, results in a mild to severe fever, general body pain, weakness, arthralgia, myalgia and rashes on the skin**.** On the other hand, DHF is observed among 1% - 5% of cases and leads to some critically serious issues such as plasma leakage, haemorrhage, ascites and reduction in leukocytes and platelets (Roy, 2019). Thrombocytopenia (TCP) is a medical condition in which blood has a lower than normal number of blood cell fragments called platelets or thrombocytes, in other words, less than 150 $\times 10^9$ /µl.The normal number of platelets per microliter of blood in adults is between the ranges of 150,000 to 450,000 platelets/µl. TCP explicitly rises for different reasons. These include types of cancers, hazardous chemicals and combinations of different regimens of drugs like chemotherapies, non-steroidal anti-inflammatory drugs, involvement of genetic inheritance and viral infections (Izak & Bussel, 2014; NHLBI, 2020). Specifically in dengue infected patients, momentary as well as transient disorder occurs during DF, which is likely to be caused by the consistently high viral load and contributes to malfunction of the endothelial vascular system. Disruption of the coagulation mechanism may lead to plasma discharge, shock and haemorrhages (WHO, 2009). Reduction in platelet production and subsequent clearance of platelets from peripheral blood in DV infection is a major contributing factor of thrombocytopenia, but platelet destruction is more prominent in the case of DV infection. The exact mechanism of platelet destruction is unknown, however the binding of antibody-opsonized DV with CD32 (FcyRll) on the thrombocytes marks the thrombocyte for destruction by phagocytosis from macrophages (Pesaro, D'Amico, & Aranha, 2007; Sridharan et al., 2013). TCP and haemorrhages are common faces of DF and DHF because TCP mostly disappears by itself on the 10th day of the onset of the dengue infection; very rare cases have been confronted to have a delayed TCP after the 10th day. However, all these delayed cases of TCP behave exactly like Immune TCP Purpura (ITP) (Verma, Hamide, Wadhwa, & Sivamani, 2013). ITP is a common blood disorder, which is denoted by muco cutaneous haemorrhage and decreased thrombocytes. This is an auto-immune disorder in which pathogenic immunoglobulin has an affinity to bind to the host thrombocytes or DV attaches itself to platelets, causing their rapid destruction. ITP is further categorized into two etiological dependent categories which are primary and secondary or timely based; it depends on duration after diagnosis, such as acute or chronic (Leong & Srinivas, 1993; Recht, 2009).

METHODS

This theoretical narrative review is merely grounded on hypotheses and research papers showing the positive or negative results with corticosteroids, TPO-R agonist platelet transfusion and IVIG transfusion in patients affected by DF, DHF and DSS after going through a web-based search on different search engines. The electronic databases PubMed, Cochrane Library, Google Scholar and Science Direct were searched for articles published from January 1990 to June 2020. A total of 55 published articles, including clinical trials, cross-sectional studies, case reports and meta-analysis reviews, were retrieved and reviewed regarding the treatment approaches in Dengue-associated TCP, including simple dengue fever, dengue haemorrhagic fever and dengue shock syndrome or expanded dengue syndrome. Out of 55 published research studies, 26 studies were reviewed according to the type of treatment to extrude outcomes that favour the reversal of TCP or platelets increase favours the treatment or not.

Approaches to treat thrombocytopenia

Prevention of TCP according to the World Health Organization

According to the WHO, treatment and management protocols of dengue patients are based on "Group A, B & C". In category A, dengue patients are mostly reported to have a high grade fever and are given only Paracetamol every six hours in 24 hours (along with oral rehydration salts) to reduce fever. This is indicated and other antipyretic drugs are avoided because they are suspected to induce more TCP. Group B consists of patients who reach the critical phase and are hospitalized followed by IV solutions (normal saline, ringer lactate) to avoid plasma loss. Group C patients are often serious, mostly suffering from dengue shock and other dengue haemorrhagic complications, which lead to plasma leakage, respiratory distress, severe haemorrhages and other organs' impairment. However, these patients are treated with isotonic crystalloid solution, blood transfusion (in case of severe bleeding) and platelet transfusion (for severe TCP) including other important steps detailed in Table 1. (WHO et al., 2009)

Use of Thrombopoietin Receptor Agonist (TPO-R) in prevention of TCP

i. Use of Recombinant Human Interleukin 11 (rhIL-11; Oprelvekin) in TCP

Recombinant Human Interleukin 11(rhIL-11), also known as Oprelvekin and authorized by the US FDA in 1997, was indicated for severe TCP. The rhIL-11 is a cytokine that acts together with different hematopoietic and non-hematopoietic cells. It is developed in E-coli by Recombinant DNA technology and this varies by the absence of the "amino-terminal praline residue" from endogenous interleukin 11 (Dorner, Goldman, & Keith, 1997; Health, 2017). RhIL-11 is a thrombopoietic growth factor; it promotes the proliferation of megakaryocyte progenitor cells and increases the production of thrombocytes (Medscape, 2020). Evaluation of rhIL-11 from two clinical trials conducted in Pakistan found over 40 patients with less than 30K/µL platelets. RhIL-11was found to have improved platelet count at convalescence. Furthermore, platelet counts increased by 20K/µL over baseline at 48 hours, without any bleeding complications among the placebo group (Nisar, Shaban, & Shah, 2018; Suliman, Qayum, & Saeed, 2014). Rodriguez et al, reported a dengue patient followed by anaemia, leukopenia, TCP. According to his studies, treatment started with steroidal interventions of

Table 1. World Health Organization-approved therapeutic approaches (Organization et al., 2009).

Category	Description	Treatment	Dosing	Purpose	Caution
Group (A)	Developing warning signs	Oral rehydration salt (ORS)	Adequate fluid intake	Substitute for fever and vomiting losses	Sugar-glucose fluids can exacerbate the effect of hyperglycaemia.
		Paracetamol	-	To relieve high fever	Avoid other NSAIDs.
Group (B)	A patient who developed warning signs	0.9% Normal saline, Ringer's lactate, or Hartmann's solution	Start with 5 - 7 ml/kg/hour for 1 - 2 hours; reduce according to clinical response	To replace losses from plasma leakage	Monitor warning signs such as haematocrit, white blood cell count, platelet counts.
Group (C)	1-severe plasma leakage leading to DS 2-fluid retention and respiratory distress 3-severe bleeding 4- severe organ impairment	1-Isotonic crystalloid solution 2-Blood Transfusion (for severe bleeding)	-	Plasma loss replacement	For obese patients, IBM should be used for calculating fluid infusion rates.
	Treatment of shock	Crystalloid solutions	5- 10 mL/kg/hr over one hour	Plasma loss replacement	Followed by reassessing the patient's vital signs
	Treatment of haemorrhagic complications	1-Platelet transfusions	-	for severe TCP	
		2- Blood transfusion	5–10ml/kg fresh-packed red cells or 10 - 20 mL/kg	Severe bleeding	1-Blood transfusion must be given with care because of the risk of fluid overload 2- Consider repeating the blood transfusion if there is further blood loss

IV hydrocortisone along with subcutaneous filgrastim 300mcg. Neutropenia was resolved after the 4th day of onset but TCP rose transiently and dropped unexpectedly even with daily transfusions of rh-IL-11 (Rodriguez-Mejorada, Rosel-Gomez, Rosado-Castro, & Ruiz-Argüelles, 2010) see Table 2.

ii. Use of Eltrombopag in prevention TCP

Eltrombopag olamine is a small molecule thrombopoietin receptor agonist (TPO-R) for oral administration. It interacts with the transmembrane domain of the TPO receptor (also known as cMpl), thus it increases thrombocyte production by signal-

Table 2. Recombinant Human Interleukin 11 (rhIL-11) in DF.

Sn	Study	Study Type	Age group	Disease	Dose	Platelet increase favours the rhIL-11	Outcome measures
01	Muhammad et al 2011	Randomized double-blind placebo control	<50Y N=20 Control group	Dengue Fever	1.5mg/SC	Yes	Significant Rise in Platelets
02	Jazirah Rehman 2011	Randomized double-blind placebo control	<50Y 40	Dengue Fever	1.5mg/SC	Yes	Significant Rise in Platelets **(Nisar et al., 2018; Suliman et al., 2014)**

ling cascades that induce proliferation and differentiation from bone marrow progenitor cells. However, it is being considered as a second treatment option in ITP patients (FDA, Aug 26, 2014; Vishnu & Aboulafia, 2016). Literature review from the last decade does not show much valuable data that supports the treatment of TCP in dengue patients with eltrombopag. Somehow, an ongoing phase II clinical trial of 100 dengue patients followed by DF, DHF and DSS in Dhaka India is pipelined to "evaluate the safety efficacy of eltrombopag in TCP" sponsored by (Incepta Pharmaceuticals Ltd) (Sajib Chakraborty, 2020)

iii. Use of Romiplostim in prevention of TCP

Romiplostim also belongs to the TPO- R agonist class of drugs. Its subcutaneous administration binds the romiplostim to TPO-R, which turns to receptor activation and results in increasing platelet production. Due to the absence of peptide sequence of endogenous TPO, cross-reactive antibodies were never reported. It is being broadly used to treat immune TCP as compared to other oral forms (Soff et al., 2019). The US FDA has approved its indication for adult patients and children above one year with ITP who suffered with an insufficient response to corticosteroids, immunoglobulins, or splenectomy (US. FDA, 2008). In vitro, it binds to c-Mpl receptors and Mpl-transfected cells (BaF3-Mpl cells). Like other TPO-R agonists, it is presumably internalized upon binding to the c-Mpl receptor, triggering activation of JAK-STAT and related pathways. When co-cultured with murine bone marrow cells, it shows the growth of colony forming unit (CFU) megakaryocytes and promotes the proliferation and maturation of megakaryocytes. Furthermore, it exhibited reflective platelet production in rats, mice and primates (Perez-Ruixo, Green, Doshi, Wang, & Mould, 2012). Rodriguez-Mejorada et al, reported a case series of a 56-year-old female dengue-positive patient followed by anaemia, leukopenia, TCP. Prior intention of filgrastim (300 mcg/day) and hydrocortisone (300 mg/day) received good results with sudden dropdown of platelets. However, secondary intention with romiplostim (4mcg/ kg/wk.) reduced TCP in the next 48 hours with continuous elevation without any side effects (Rodriguez-Mejorada et al., 2010).

IMMUNOLOGICAL APPROACHES IN PREVENTION OF TCP

***i. Intravenous Immunoglobulin* (IVIG)**

Intravenous Immunoglobulin (IVIG) is the blood product of about 1500 blood donors per batch made from the serum. It is the choice of treatment for antibody-deficient patients. IVIG is usually given thrice weekly (200mg/kg to 400mg/kg) to patients. A high dose of IVIG (hdIVIG) is frequently prescribed at 2g/kg/month, which is indicated as an immunomodulatory mediator in the context of immune and inflammatory conditions. Initially, hdIVIG was prescribed only in children for immune thrombocytopenic purpura (ITP). It was first established as an immunomodulatory mediator to manage ITP in adults as well (Jolles, Sewell, Misbah, & immunology, 2005). Kumar et al, reported a case of 30-year-old woman with ITP followed by a dengue-positive report. Treatment of IV platelets infusion could not indicate much of a desired outcome. Respectively, multiple transfusions of platelet units from single and multiple donors were provided, with no significant result. But, transfusion of IVIG (2 g/kg) over two days resulted an immediate rise in the platelet count, up to $35x10^9$/L, $78x10^9$/L and $130x10^9$/L 35 with IVIG at 12hrs, 36hrs and 72hrs (Kumar, Gupta, Soneja, & Biswas, 2018). Subbiah et al, also reported a dengue patient case, mentioned as (14×109/L) platelets. Five continuous days of IVIG infusion by 0.5 g/kg administration significantly helped the patient recover from dengue capillary leak syndrome (Subbiah, Mahajan, Yadav, & Agarwal, 2018). Respectively, Kumar et al, stated in a case report of a patient with a persistent decrease of platelets from 15×109/L to further low levels. Platelet transfusion and high doses of steroids were not successful. However, a transfusion of IVIG on day 9 followed by 1g/kg showed a profound response: day 10 – 40,000, day 11 – 90,000 and

Table 3. Use of IVIG in Dengue Fever-induced TCP.

Sn/Ref	Study Type	Country	Age/ Gender	Medical Condition	Dose	Platelet increase favours the rhIL-11	Outcome measures
(Kumar et al., 2018)	Case report	India	30 F	Dengue Fever ITP	IVIG at a dose of 2 g/ kg body weight over 2 days.	Yes	No significant difference in the platelet counts at the time of discharge. Similar early increase in platelet count with the use of anti-D.
(Subbiah et al., 2018)	Case report	India	45 M	stable graft function Dengue Positive TCP	0.5 g/kg of IVIG was administered daily for 5 days	Yes	The patient improved significantly with IVIG administration.
(Kumar et al., 2016)	Case report	India	21 F	DHF	1g/kg	Yes	Better response to IVIG and failure to respond to steroid and IV platelet infusion.

on day 12 – 125,000 per cu mm (Kumar, Prabhat, et al, 2016). Moreover, only one RCT in a 2007 study of 31 dengue patients by Dimaano et al reported that IV-IG was found to show better outcomes in correcting TCP in dengue patients as compared to the placebo group (Dimaano et al., 2007) (Table 3).

ii. Anti-D Immune Globulin

Anti-D immune globulin is an IgG fraction of plasma prepared from "D-Positive Red Cell Immunized" individuals and likely rich in polyclonal Anti-D specificities. This is used to avoid alloimmunization in Rh^{-ive} mothers giving birth to Rh^{+ive} infants. Anti-D immune globulin blocks Fcγ receptors and reduces immune destruction in patients followed by ITP. Hence, it recovers TCP in dengue patients (Brinc & Lazarus, 2009; Lazarus, Freedman, & Semple, 1998). Pannu et al reported in an open-label, randomized interventional study in 2007 on over 30 Rh+ive patients with serology-proven dengue positivity with severe TCP (platelet count ≤20,000/mm^3). The two groups were compared concerning their response in the form of a rise in platelet count. The primary outcome was a rise in platelet count ≥50,000/mm3 above the baseline after a period of 48 hours of administering an anti-D injection in the intervention group. The rise in platelets was more rapid in the intervention group and was significant at 24hrs, 36hrs, and 48hrs (p=0.0001, <0.0001, and <0.0001, respectively (Pannu et al., 2017). Kharya et al reported a 16-year-old patient with DHF and serious refractive TCP undergoing 6800/mm^3 of platelets value. It is observed that the average count of platelets after 48hrs followed by administration of IG Anti-D in patients showed a positive response by 37,800/mm^3 platelets (Kharya, Yadav, Katewa, Sachdeva, & Oncology, 2011). Anti-D immune globulin confirmed in RCT reported by Rajapakse et al, stated that dengue patients successfully recovered with anti-D immune globulin (Rajapakse, de Silva, Weeratunga, Rodrigo, & Fernando, 2017).

OTHER APPROACHES IN PREVENTION OF TCP

i. Use of Corticosteroids in prevention TCP

According to this review, inadequate evidence supported the effectiveness of corticosteroids (CS) in deranged immune systems due to dengue. Besides, use of CS is not specified in WHO guidelines for dengue management and treatment protocols. However, physicians objectively use corticosteroids to explain their use in an immunological context (Kularatne, 2005; Medin & Rothman, 2006). Clinical studies have shown conflicting outcomes and unconvincing results with corticosteroids treatments. Only a few positive responses came out by min et al, in a clinical trial in which children were treated with hydrocortisone hemi succinate followed by dose tapering for three days, but results seem to be significant in the children solely greater than eight years of age (Min, Aye, Shwe, & Swe, 1975). Verma et al reported a case of a 65 years old patient who was diagnosed with dengue and immune-mediated TCP. The patient was started with oral prednisolone 1mg/kg after the trial of four units of platelet transfusion, hence, CS responded to achieve the desired effects (Verma et al., 2013). Other RCTs and case reports mentioned in (Table 4) did not show an efficient haemodynamic improvement in favour of corticosteroids (hydrocortisone hemisuccinate, methyl prednisolone, prednisolone and dexamethasone) in DS and DHF (Boo, Lim, P'ng, Liam, & Huan, 2019; Kohli, Saharan, Lodha, & Kabra, 2008; Kularatne et al., 2009; Pongpanich, Bhanchet, Phanichyakarn, & Valyasevi, 1973; Sumarmo, Talogo, Asrin, Isnuhandojo, & Sahudi, 1982; Tassniyom, Vasanawathana, Chirawatkul, & Rojanasuphot, 1993; Widya & Martoatmodjo, 1975).

Table 4. Corticosteroid outcomes In Dengue associated TCP.

SN	Study	Study Type	Year	Drug	Dose	Age Group / No of Patients	disease	Favours Steroid	Outcome measures
1	(Kohli et al., 2008)	Case Report	2008	N/A	N/A IV	11 Yrs 01	DSS, DHF	No	Patient died
2	(Kularatne et al., 2009)	Placebo controlled study	2009	Dexamethasone	2mg / IV 8h for 24h	>12 Yrs 100	Acute DF	No	No significant response
3	(Tam et al., 2012)	Randomized placebo control	2012	Prednisolone	Lower dose 0.5 mg / kg Higher dose 2 mg / kg	05-20 Yrs	Dengue with hypovolemic shock	No	No reduction represented to counter the Dengue shock
4	Shailendra et al,2012) (Verma et al., 2013)	Case Report	2013	Prednisolone	1mg/kg/PO	65 Yrs 01	DF	Yes	Promptly responded to Prednisolone
5	(Boo et al., 2019)	Case Report	2019	Methyl prednisolone	1mg/kg/PO	13 Yrs 01	ITP post Dengue infection	NO	Patient died after three months

ii. Platelet transfusion

Platelet Transfusion (PT) is commonly used during or after the use of chemotherapy, for wounded/injured patients and for those who are undergoing surgery or an invasive investigation. Hypo-proliferative TCP requires PT; it is acquired in prophylactic strategies such as acute leukaemia, stem cell therapies and aplasias following intensive myeloid-suppressive antineoplastic therapies and dengue associated acute TCP (Humbrecht, Kientz, & Gachet, 2018; Roy, 2019). Lye et al reported that in 2009 at Tan Tock Seng Hospital Singapore, 256 dengue-infected patients had TCP followed by average 20,000/mm3 platelets. Without prior bleeding, of the 188 patients who were transfused with platelets. Although subsequent bleeding, platelet increment, and recovery were almost found similar between two groups of patients, those who received the transfusion compared to those who did not, had similar outcomes. Prophylactic PT was ineffectual in stopping bleeding in the patients (Lye, Lee, Sun, & Leo, 2009). In a study reported by Pothapregada et al, in 2012, involving children aged 0 - 12 years old, confirmed with DF. Out of a total of 17 children 17 were transfused with platelets units. Among the children, 12 had an average PC of over 20,000/mm^3, while the other children ranged between 20000/mm^3 and 50000/mm^3. Children who received platelet transfusions recovered completely (Pothapregada, Kamalakannan, & Thulasingam, 2015). Respectively, previous study reported prophylactic platelet transfusion leads to no significant difference between two groups (Archuleta et al., 2019; Assir & Ahmad, 2017; Lee et al., 2016). According to Soumya et al, there is a large lack of evidence-based platelet transfusion guidelines when patients develop bleeding symptoms in dengue infection. Inappropriate usage of platelets for stable patients with low platelet count causes decreased availability of platelets. There is a need for decreasing unwanted transfusions, which can be achieved by strict adherence to the British Committee for Standardization in Hematology (BCSH) Guidelines (Soumya, Das, & Kalyani, 2019) (Table 5).

FUTURE DIRECTION

Scientists should be focused on elaborating the role of all the approaches and treatment options in dengue associated with TCP for improving the production and maintenance of platelets to reduce morbidity and mortality. TPO-R agonists and growth factors such as RhIL-11 (oprelvekin) are the easiest approaches if these options are appropriately evaluated for their safety and effectiveness via RCTs in a large group of the population along with long-term exposure studies. The safety and efficacy profile of TPO-R agonist and RhIL-11 in dengue patients should be determined in order to choose the best dosage schedule in consideration of the potential side effects and adverse effects and other toxicities like mutagenicity, carcinogenicity, tumorigenicity and withdrawal effects on TCP.

CONCLUSION

Dengue is an insect-borne viral disease that requires medical attention because it may lead to TCP and life-threatening conditions. CS is the most common choice of drugs used to treat TCP, however, the WHO has not recommended it in its guidelines. CS is well known to have an immunosuppressant behavioural response in patients, especially in the case of DV. The antibody-virus complex is more prone to target the cell and leads to more destruction of thrombocytes. For future outcomes, researchers should thoroughly assess the use of CS in a randomised controlled trial. Although RhIL-11 is useful for treating chemotherapy-induced TCP, Rh IL-11 (Oprelvekin) is excessively used along with chemotherapy in major hospitals in Pakistan and other developed countries to counter chemotherapeutic-induced TCP. However, only two small studies reported in Pakistan support the use of RhIL-11 in the treatment of TCP due to dengue infection**.** Further, detail in this context requires proper assessment for safety, efficacy and risk factors. Similarly, very limited data is available about Romiplostim and eltrombopag for the reversal of TCP. This limited finding indicates the imperative consideration to choose these treatment modalities for improvement of deadly TCP in dengue. However, unbiased double-blind randomized clinical trials must be carried out to assess the efficacy, safety, side effects and cost-effectiveness.

In addition to TPO-R agonists, Anti-D immune globulin, Intravenous Immunoglobulin G (iv-ig) and platelet transfusion can

Table 5. Use of prophylactic transfusion of Platelets to cover TCP.

Sn / Ref	Year	Study Type	Country	Age / Nos	Medical Condition	Mean PT Units	Favours PT	Outcome measures
(Lye et al., 2009)	2009	Retrospective cohort	Singapore	(34) 256	Dengue infection TCP	4 Units	No	Not recovered with PT
(Pothapregada et al., 2015)	2015	Observational Study	India	(0-12) 261	Dengue infection DSS	N/A	Yes	Recovered completely with PT
(Lee et al., 2016)	2016	Non-Randomized observational study	Singapore	22-65 788	Dengue infection Bleeding	N/A	No	Not recovered with PT
(Assir & Ahmad, 2017)	2017	Open-Label Randomized Clinical Trial	Pakistan	N/A 372	Dengue infection without bleeding	4 Units	No	Recovered completely with PT

be considered to reduce TCP. However, these choices were found to have very limited literature support in case of dengue. Besides, IVIG is supported by few case reports that are conducted and reported in India only, while many studies suggest platelet transfusion favours the reversal of TCP but carries many other complications because PTs are obtained from multiple donors and are more prone to immunological responses in patients. A detailed insight into their role in the treatment of DHF TCP patients is urgently required. Keeping in view these complications with PT, IVIG and Anti-D immune globuline transfusion and others, TPO-R like eltrombopag and romiplostim and growth factor like Rh IL-11 (oprelvekin) are good and easy approaches which should be tried in the management of TCP in dengue patients.

Peer-review: Externally peer-reviewed.

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

Financial Disclosure: Authors declared no financial support.

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