İstanbul Journal of Pharmacy

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

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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).

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

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

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

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

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

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

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

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- Does the abstract clearly and accurately describe the content of the manuscript?
- Is the problem significant and concisely stated?
- Are the methods described comprehensively?
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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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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.

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

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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.

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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
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When a drug, product, hardware, or software program is mentioned within the main text, product information, including the name of the product, the producer of the product, and city and the country of the company (including the state if in USA), should be provided in parentheses in the following format: "Discovery St PET/CT scanner (General Electric, Milwaukee, WI, USA)"

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

REFERENCES

Reference Style and Format

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

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

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

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

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

Samples:

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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;

(Çavdar et al., 2003)

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

Basic Reference Types

Book

a) Turkish Book

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

b) Book Translated into Turkish

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

c) Edited Book

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

d) Turkish Book with Multiple Authors

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

e) Book in English

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

f) Chapter in an Edited Book

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

g) Chapter in an Edited Book in Turkish

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

h) Book with the same organization as author and publisher

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

Article

a) Turkish Article

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

b) English Article

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

c) Journal Article with DOI and More Than Seven Authors

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

d) Journal Article from Web, without DOI

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

e) Journal Article wih DOI

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

f) Advance Online Publication

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

g) Article in a Magazine

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

Doctoral Dissertation, Master's Thesis, Presentation, Proceeding

a) Dissertation/Thesis from a Commercial Database

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

b) Dissertation/Thesis from an Institutional Database

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

c) Dissertation/Thesis from Web

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

d) Dissertation/Thesis abstracted in Dissertations Abstracts International

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

e) Symposium Contribution

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

f) Conference Paper Abstract Retrieved Online

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

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

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

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

h) Proceeding in Book Form

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

i) Paper Presentation

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

Other Sources

a) Newspaper Article

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

b) Newspaper Article with no Author

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

c) Web Page/Blog Post

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

d) Online Encyclopedia/Dictionary

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

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

e) Podcast

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

f) Single Episode in a Television Series

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

g) Music

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

REVISIONS

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

Accepted manuscripts are copy-edited for grammar, punctuation, and format. Once the publication process of a manuscript is completed, it is published online on the journal's webpage as an ahead-of-print publication before it is included in its scheduled issue. A PDF proof of the accepted manuscript is sent to the corresponding author and their publication approval is requested within 2 days of their receipt of the proof.

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Original Article

Design, synthesis and biological evaluation of novel sulfonamide hydrazones as α-glucosidase and α-amylase inhibitors

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ABSTRACT

Background and Aims: Diabetes mellitus is among the major hazards to global public health due to increasing incidence worldwide, and new therapeutic agents are urgently needed for the control of the disease. In this study, a novel series of sulfonamide hydrazones (**3a-i**) were synthesized and evaluated, *in vitro*, for α-amylase and α-glucosidase inhibitor activities.
Methods: Target compounds were prepared according to a high-yielded synthetic route. The *in vitro* antidiabetic activity of the compounds was analyzed by evaluating the inhibitory abilities on α-glucosidase and α-amylase enzymes. Acarbose was chosen as a reference in this study.
Results: Compounds **3d**, **3e**, **3g** and **3h** exhibited better α-glucosidase inhibitory activity compared to reference antidiabetic drug acarbose. Compound **3g** was the most active analogue, possessing an IC_{50} value of 65.27 μg/mL. **3d**, **3e**, **3g** and **3h** showed similar α-amylase inhibitory activity compared to acarbose when tested at high concentrations. However, their IC_{50} values were much higher compared to that of reference acarbose.
Conclusion: The most active analogue **3g** was found to be two times more active than acarbose. The addition of a bulky group to the 4-position of the cyclohexane ring seemed to have a positive effect on antidiabetic activity. The new hydrazone derivatives reported in this study are potentially promising candidates for developing new antidiabetic agents.

Keywords: Antidiabetic activity, Sulfonamide, Hydrazone, α-amylase, α-glucosidase

INTRODUCTION

Diabetes mellitus is a metabolic disease identified by chronic hyperglycemia that causes defects in insulin secretion, insulin action or both. Diabetes is one of the major threats to global public health due to increasing incidence worldwide (Toniolo et al., 2019). Nearly 422 million people worldwide currently have diabetes, especially in low-and middle-income countries, and about 1.6 million deaths are linked to diabetes each year (WHO, 2021). Of the three major types of diabetes, type 2 diabetes is much more common (accounting for nearly 90% of all cases) than either type 1 or gestational diabetes. Type 2 diabetes, formerly called non-insulin-dependent, or adult-onset, is caused by insufficient use of insulin by the body (DeFronzo et al., 2015). One of the therapies for type 2 diabetes is the inhibition of the key enzymes that digest carbohydrates, such as α-amylase and α-glucosidase. α-Glucosidase inhibitors (AGIs) are a class of oral antidiabetic drugs that are widely used in the treatment of type 2 diabetes. Acarbose is the most commonly used AGI,

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and also the most widely studied one. Voglibose and miglitol are the other AGIs available commercially. AGIs are saccharides that competitively inhibit the α-glucosidase enzyme that converts complex non-absorbable carbohydrates into simple absorbable carbohydrates and result in a reducing effect on postprandial blood glucose and insulin levels (Hossain, Das, Ghosh, & Sil, 2020; Padhi, Nayak, & Behera, 2020). AGIs also inhibit the pancreatic α-amylase enzyme that hydrolyzes the starches into oligosaccharides, thus having a dual effect on complex carbohydrates (Proença, Ribeiro, Freitas, & Fernandes, 2020). However, their use has been limited due to severe gastrointestinal side effects and limited effect on fasting glucose levels. Thus, it is crucial to discover new AGIs as preclinical drug candidates that have fewer adverse reactions. A great number of new compounds with *in vitro* or *in vivo* α-glucosidase inhibitory activity has been reported in recent literature. Most of them are sugar-mimic compounds that have been designed on the basis of the structure of glucose like commercially available AGI drugs. There are also many compounds without a sugar-mimic framework that show favorable α-glucosidase inhibitory activity (Liu & Ma, 2017).

The hydrazone functional group is an important pharmacophore in medicinal chemistry, due to its chemical and biological properties, as well as its structural versatility. Many acyl/aryl/aroyl hydrazones with a diversity of heterocyclic spacers have been studied for their broad spectrum of biological activities, including anticancer (Nasr, Bondock, & Youns, 2014), antituberculosis (Koçyiğit-Kaymakçıoğlu et al., 2006), antimicrobial (Metwally, Abdel-Aziz, Lashine, Husseiny, & Badawy, 2006) and anti-inflammatory (Moldovan et al., 2011) properties. Two different series of aroyl hydrazone derivatives, 2-indolylcarbohydrazones (Taha et al., 2015) and benzimidazole hydrazones (Zawawi et al., 2016) have been reported to exert notable *in vitro* inhibitory activity against α-glucosidase enzyme. In a newly published study by Wang et al. (Wang et al., 2017), a chromone hydrazone derivative with 4-sulfonamide substitution at the phenyl part of the hydrazide was described as a promising inhibitory agent against α-glucosidase.

This report is based on the synthesis and structural characterization of new cyclohexanone benzoylhydrazone derivatives carrrying a sulfonamide moiety on the benzene ring. The newly synthesized compounds were screened for inhibition of *in vitro* α-glucosidase and α-amylase activities.

MATERIAL AND METHODS

Chemistry

The chemicals were provided by Sigma-Aldrich. Melting points (m.p.) were uncorrected and determined with a Buchi B-540 melting point apparatus. Spectroscopic data were recorded as follows: Shimadzu IRAffinity-1 FTIR spectrophotometer for IR, Varian Mercury-400 MHz for ^{1}H NMR (DMSO-d_6) spectra, Varian^{UNITY} INOVA-125 MHz for ^{13}C-NMR (APT) (DMSO-d_6) spectra. Elemental analyses were run on a Thermo Finnigan Flash EA 1112 Series elemental analyzer (cy: cyclohexane, phenyl: ph).

2-Methoxy-4-sulfamoylbenzhydrazide (2)

$NH_2NH_2.H_2O$ (98%, 0.008 mol) was added to a solution of **1** (1.22 g, 0.005 mol) in ethanol (25 mL). The reaction mixtures were heated under reflux for 8 hours. The resulting mixture was cooled and allowed to stand overnight. The precipitate was filtered, washed with water and used without further purification.

General procedure for the synthesis of 4-(2-(3,4-(non) substituted cyclohexylidene)hydrazinecarbonyl)-3-methoxybenzene-1-sulfonamide (3a-i)

A mixture of compound **2** (1.22 g, 0.005 mol) with an appropriate cyclohexanone (0.006 mol) in absolute ethanol (10 mL) was refluxed for 5-7 hours. The reaction was followed up by TLC. After cooling, the reaction mixture was filtered. The purification was done with washing or recrystallization from ethanol.

4-(2-cyclohexylidenehydrazinecarbonyl)-3-methoxybenzene-1-sulfonamide (3a)

White powder (85%); m.p: 231-234 °C; IR (KBr): υ_{max} 3315, 3275, 3182 (N-H), 1643 (C=O), 1633, 1595, 1508, 1483 (C=N, C=C), 1325, 1161 (S=O). ^{1}H NMR (DMSO-d_6): δ 10.74, 10.61 (1H, 2s, NH), 8.11 (1H, d, *J*=2.5 Hz, H2), 7.87 (1H, dd, *J* = 8.7, 2.5 Hz, H6), 7.32 (2H, s, SO_2NH_2), 7.30 (1H, d, *J*= 8.7 Hz, H5), 3.94, 3.78 (3H, 2s, OCH_3), 2.23-2.40 (4H, m, CH_2-cy), 1.75-1.46 (6H, m, CH_2-cy). ^{13}C-NMR (DMSO-d_6): δ 163.53, 160.85 (C=N, C=O), 159.44 (C_3), 136.88 (C1), 130.19, 128.66 (C5,6), 123.62 (C4), 112.89 (C2), 57.25 (OCH_3), 35.38, 27.84, 27.19, 26.03, 25.50 (cy-C). Anal. calcd. for $C_{14}H_{19}N_3O_4S$ (325.38) C: 51.68, H: 5.89, N: 12.91. Found C: 51.82, H: 6.10, N: 12.89.

3-methoxy-4-[2-(3-methylcyclohexylidene)hydrazinecarbonyl]benzene-1-sulfonamide (3b)

White needles (81%); m.p: 237-240 °C; IR (KBr): υ_{max} 3346, 3323, 3176 (N-H), 1668 (C=O), 1633, 1593, 1537, 1479 (C=N, C=C), 1332, 1170 (S=O). ^{1}H NMR (DMSO-d_6): δ 10.74, 10.62 (1H, 2s, NH), 8.11 (1H, 2d, *J* = 2.5 Hz, H2), 7.87 (1H, 2dd, *J* = 8.7, 2.5 Hz, H6), 7.31-7.30 (3H, m, H5 and SO_2NH_2), 3.94, 3.77 (3H, 2s, OCH_3), 2.69 (1H, t, *J*=13 Hz, CH/CH_2-cy), 2.43-2.28, 2.21-2.09 (1H, m, CH/CH_2-cy), 2.00-1.78 (2H, m, CH/CH_2-cy), 1.76-1.53 (3H, m, CH/CH_2-cy), 1.52-1.30 (1H, m, CH/CH_2-cy), 1.26-1.06 (1H, m, CH/CH_2-cy), 0.83, 0.95 (3H, 2d, *J* = 6.4 Hz, CH_3). ^{13}C-NMR (DMSO-d_6): 163.27, 160.87 (C=N, C=O), 159.41 (C_3), 136.89 (C1), 130.20, 128.68 (C5,6), 123.70 (C4), 112.89 (C2), 57.25 (OCH_3), 35.73, 34.89, 24.81 (cy-C), 33.70, 32.78 (cy-C3), 22.37 (CH_3). Anal. calcd. for $C_{15}H_{21}N_3O_4S$ (339.40) C: 53.08, H: 6.24, N: 12.38. Found C: 53.14, H: 6.55, N: 12.37.

3-methoxy-4-[2-(4-ethylcyclohexylidene)hydrazinecarbonyl]benzene-1-sulfonamide (3c)

White powder (84%); m.p: 238-240 °C; IR (KBr): υ_{max} 3340, 3184 (N-H), 1660 (C=O), 1598, 1483 (C=N, C=C), 1334, 1168 (S=O). ^{1}H NMR (DMSO-d_6): δ 10.73, 10.61 (1H, 2s, NH), 8.12 (1H, d, *J*=2.5 Hz, H2), 7.88 (1H, dd, *J* = 8.7, 2.5 Hz, H6), 7.32 (2H, s, SO_2NH_2), 7.31 (1H, d, *J*=8.7 Hz, H5), 3.94, 3.78 (3H, 2s, OCH_3), 2.95, 2.75 (1H, 2d, *J*= 14.0 Hz, CH/CH_2-cy), 2.45-2.34 (1H, m, CH/CH_2-cy), 2.23 (1H, td, *J*=13.7, 4.9 Hz, CH/CH_2-cy), 2.06-1.70 (4H, m, CH/CH_2-cy), 1.52-0.98 (4H, m, CH/CH_2-cy and 4-CH_2CH_3-cy), 0.87 (3H, t, *J*= 7.4 Hz, CH_3). ^{13}C-NMR (DMSO-d_6): 163.63, 160.82 (C=N, C=O), 158.62 (C3), 136.89 (C1), 130.21, 128.68 (C5,6), 123.58 (C4), 112.89 (C2), 57.25 (OCH_3), 37.96 (cy-C4), 34.53, 32.72, 26.93, 24.81 (cy-C), 31.61 (CH_2), 11.98 (CH_3). Anal. calcd. for $C_{16}H_{23}N_3O_4S$ (353.43) C: 54.37, H: 6.56, N: 11.89. Found C: 54.05, H: 6.83, N: 11.99.

3-methoxy-4-[2-(4-propylcyclohexylidene)hydrazinecarbonyl]benzene-1-sulfonamide (3d)

White powder (90%); m.p: 248-250 °C; IR (KBr): υ_{max} 3338, 3190 (N-H), 1658 (C=O), 1598, 1525, 1483 (C=N, C=C), 1334, 1166 (S=O). ^{1}H NMR (DMSO-d_6): δ 10.74, 10.61 (1H, 2s, NH), 8.12 (1H, d, *J*=2.5 Hz, H2), 7.88 (1H, dd, *J* = 8.8, 2.5 Hz, H6), 7.32 (2H, s, SO_2NH_2), 7.30 (1H, d, *J*= 8.8 Hz, H5), 3.94, 3.77 (3H, 2s, OCH_3), 2.94, 2.74 (1H, 2d, *J* = 14.0 Hz, CH/CH_2-cy), 2.45-2.35 (1H, m, CH/CH_2-cy), 2.23 (1H, td, *J*=13.3, 4.9 Hz, CH/CH_2-cy), 2.03-1.72 (3H, m, CH/CH_2-cy), 1.60-1.46 (1H, m, CH/CH_2-cy), 1.38-1.00 (6H, m, CH/CH_2-cy and 4-$CH_2CH_2CH_3$-cy), 0.86 (3H, t, *J*= 7.2 Hz, CH_3). ^{13}C-NMR (DMSO-d_6): 163.58, 160.82 (C=N, C=O), 159.44 (C3), 136.89 (C1), 130.20, 128.68 (C5,6), 123.56 (C4), 112.97 (C2), 57.24 (OCH_3), 38.26, 34.55, 33.09, 32.22 (cy-C), 36.03 (cy-C4), 26.97, 20.13 (CH_2), 11.98 (CH_3). Anal. calcd. for $C_{17}H_{25}N_3O_4S$ (367.46) C: 55.57, H: 6.86, N: 11.44. Found C: 55.78, H: 7.23, N: 11.48.

3-methoxy-4-[2-(4-phenylcyclohexylidene)hydrazinecarbonyl]benzene-1-sulfonamide (3e)

White needles (94%); m.p: 275-279 °C; IR (KBr): υ_{max} 3350, 3190 (N-H), 1666 (C=O), 1598, 1521, 1487 (C=N, C=C), 1334, 1168 (S=O). ^{1}H NMR (DMSO-d_6): δ 10.83, 10.69 (1H, 2s, NH), 8.12 (1H, d, *J*=2.5 Hz, H2), 7.88 (1H, dd, *J* = 8.8, 2.5 Hz, H6), 7.40-7.09 (8H, m, H5, ph-H and SO_2NH_2), 3.94, 3.81 (3H, 2s, OCH_3), 2.97-2.73 (2H, m, CH/CH_2-cy), 2.59-2.36 (2H, m, CH/CH_2-cy and DMSO-d_6), 2.23-1.86 (3H, m, CH/CH_2-cy), 1.77-1.47 (2H, m, CH/CH_2-cy). ^{13}C-NMR (DMSO-d_6): 162.57, 160.96 (C=N, C=O), 159.45 (C3), 146.22, 126.62 (ph-C), 136.88 (C1), 130.21, 128.86 (C5,6), 123.66 (C4), 112.89 (C2), 57.24 (OCH_3), 42.94 (cy-C4), 35.15, 34.27, 33.22, 27.53 (cy-C). Anal. calcd. for $C_{20}H_{23}N_3O_4S$. 1/4H_2O (406.07) C: 59.18, H: 6.16, N: 10.35. Found C: 59.07, H: 5.85, N: 10.46.

3-methoxy-4-{2-[4-(trifluoromethyl)cyclohexylidene] hydrazinecarbonyl}benzene-1-sulfonamide (3f)

White powder (78%); m.p: 210-214 °C; IR (KBr): υ_{max} 3340, 3205 (N-H), 1662 (C=O), 1600, 1529, 1485 (C=N, C=C), 1338, 1166 (S=O). ^{1}H NMR (DMSO-d_6): δ 10.85, 10.68 (1H, 2s, NH), 8.09 (1H, d, *J*=2.5 Hz, H2), 7.88 (1H, dd, *J* = 8.8, 2.5 Hz, H6), 7.33 (2H, s, SO_2NH_2), 7.31 (1H, d, *J*= 8.7 Hz, H5), 3.94, 3.78 (3H, 2s, OCH_3), 2.86 (1H, d, *J* = 14.3 Hz, CH/CH_2-cy), 2.72-2.58 (1H, m, CH/CH2-cy), 2.36 (1H, td, *J* = 14.2, 4.9 Hz, CH/CH_2-cy), 2.16-1.88 (4H, m, CH/CH_2-cy), 1.55-1.31 (2H, m, CH/CH_2-cy). ^{13}C-NMR (DMSO-d_6): 161.24, 161.02 (C=N, C=O), 159.41 (C3), 136.86 (C1), 130.23, 128.64 (C5,6), 128.28 (q, *J*=277 Hz, CF_3), 123.67 (C4), 112.88 (C2), 57.22 (OCH_3), 40.16 (d, *J*= 20 Hz, C4), 32.76, 25.52, 25.14, 23.99 (cy-C). Anal. calcd. for $C_{15}H_{18}F_3N_3O_4S$ (393.38) C: 45.80, H: 4.61, N: 10.68. Found C: 45.50, H: 4.30, N: 11.05.

3-methoxy-4-[2-(4-(2-methylbutan-2-yl)cyclohexylidene)hydrazinecarbonyl]benzene-1-sulfonamide (3g)

White powder (80%); m.p: 228-232 °C; IR (KBr): υ_{max} 3340, 3246, 3186 (N-H), 1651 (C=O), 1598, 1514, 1483 (C=N, C=C), 1336, 1165 (S=O). ^{1}H NMR (DMSO-d_6): δ 10.74, 10.61 (1H, 2s, NH), 8.13 (1H, d, *J*=2.5 Hz, H2), 7.88 (1H, dd, *J* = 8.8, 2.5 Hz, H6), 7.31 (2H, s, SO_2NH_2), 7.30 (H, d, *J*= 8.8 Hz, H5), 3.95, 3.77 (3H, 2s, OCH_3), 2.87-2.77 (1H, m, CH/CH_2-cy), 2.45-2.38 (1H, m, CH/CH_2-cy), 2.22 (1H, td, *J*= 13.4, 4.8 Hz, CH/CH_2-cy), 2.04-1.69 (3H, m, CH/CH_2-cy), 1.46-1.01 (5H, m, CH/CH_2-cy and 4-$C(CH_3)_2CH_2CH_3$-cy), 0.80-0.74 (9H, m, CH_3). ^{13}C-NMR (DMSO-d_6): 163.58, 160.75 (C=N, C=O), 159.45 (C3), 136.91 (C1), 130.24, 128.74 (C5,6), 123.58 (C4), 112.91 (C2), 57.28 (OCH_3), 44.09 (cy-C4), 35.05, 32.74, 27.35 (cy-C), 24.58 (CH), 8.52 (CH_3). Anal. calcd. for $C_{19}H_{29}N_3O_4S$ (395.51) C: 57.70, H: 7.39, N: 10.62. Found C: 57.54, H: 7.58, N: 10.67.

3-methoxy-4-[2-(4-cyano-4-phenylcyclohexylidene)hydrazinecarbonyl]benzene-1-sulfonamide (3h)

White powder (82%); m.p: 257-263 °C; IR (KBr): υ_{max} 3394, 3352, 3209 (N-H), 1643 (C=O), 1597, 1566, 1485 (C=N, C=C), 1338, 1166 (S=O). ^{1}H NMR (DMSO-d_6): δ 10.98, 10.78 (1H, 2s, NH), 8.11 (1H, d, *J*=2.5 Hz, H2), 7.89 (1H, dd, *J* = 8.8, 2.5 Hz, H6), 7.57 (2H, d, *J*= 7.9 Hz, ph-H2,6), 7.44 (2H, t, *J*= 7.6 Hz, ph-H3,5), 7.37 (1H, d, *J*= 7.9 Hz, ph-H4), 7.34 (2H, s, SO_2NH_2), 7.32 (1H, d, *J*=8.9 Hz, H5), 3.95, 3.81 (3H, 2s, OCH_3), 3.05-2.95 (1H, m, CH_2-cy), 2.69-2.57 (2H, m, CH_2-cy), 2.37-2.07 (5H, m, CH_2-cy). ^{13}C-NMR (DMSO-d_6): 161.19, 159.50 (C=N, C=O), 159.06 (C3), 140.07, 129.54, 126.16 (ph-C), 122.23 (CN), 136.88 (C1), 130.31, 127.08 (C5,6), 123.58 (C4), 112.91 (C2), 57.23 (OCH_3), 43.67 (cy-C4), 36.57, 36.32, 35.37, 32.42, 25.29 (cy-C). Anal. calcd. for $C_{21}H_{22}N_4O_4S$ (426.48) C: 59.14, H: 5.20, N: 13.14. Found C: 59.19, H: 5.39, N: 12.99.

3-methoxy-4-[2-(4-acetylaminocyclohexylidene)hydrazinecarbonyl]benzene-1-sulfonamide (3i)

White needles (76%); m.p: 239-244 °C; IR (KBr): υ_{max} 3317, 3211, 3190 (N-H), 1658 (C=O), 1595, 1529, 1479 (C=N, C=C), 1332, 1165 (S=O). ^{1}H NMR (DMSO-d_6): δ 10.79, 10.64 (1H, 2s, NH), 8.09 (1H, d, *J*=2.5 Hz, H2), 7.90-7.75 (2H, m, H6 and N**H**$COCH_3$), 7.33 (2H, s, SO_2NH_2), 7.30 (1H, d, *J*= 8.6 Hz, H5), 3.93, 3.79 (3H, 2s, OCH_3), 2.74-2.63 (1H, m, CH/CH_2-cy), 2.46-2.25 (2H, m, CH/CH_2-cy), 2.19-1.84 (4H, m, CH/CH_2-cy), 1.78 (3H, s, NHCOC**H**$_3$), 1.50-1.18 (2H, m, CH/CH_2-cy). ^{13}C-NMR (DMSO-d_6): 169.22 ($NHCOCH_3$), 162.29, 161.01 (C=N, C=O), 159.45 (C3), 136.84 (C1), 130.21, 128.59 (C5,6), 123.74 (C4), 112.87 (C2), 57.22 (OCH_3), 46.40 (cy-C4), 32.94, 32.30, 31.15, 25.40 (cy-C), 23.21 ($COCH_3$). Anal. calcd. for $C_{16}H_{22}N_4O_5S$. 1/2H_2O (391.43) C: 49.10, H: 6.13, N: 14.32. Found C: 49.62, H: 5.89, N: 14.50.

Biological activity

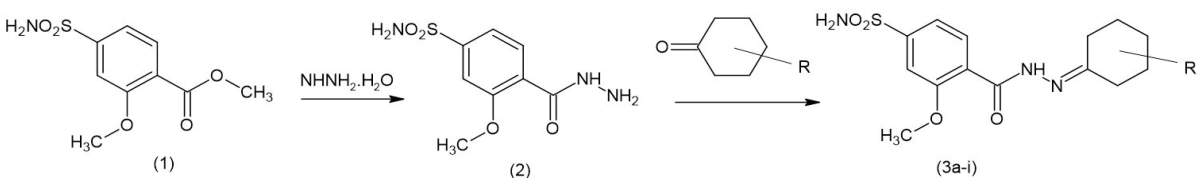
The antidiabetic activity

The antidiabetic activity of compounds was analyzed by measuring the inhibitory effects on α-glucosidase and α-amylase enzymes. α-Amylase, α-glucosidase, 3,5-dinitrosalicylic acid (DNS), acarbose, dimethyl sulfoxide (DMSO), *p*-nitrophenyl α-D-glucopyranoside (*p*NPG) and starch were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade. For the assays, the synthesized compounds and acarbose were dissolved in DMSO to form a 5 mg/mL stock solution. Different concentrations of the compounds and acarbose were prepared for use in the analyses by dilution of the stock solution with DMSO.

α-Glucosidase inhibitory activity

The α-glucosidase inhibitory activities of the compounds were determined by Bothon et al.'s method, with some modifications (Bothon et al., 2013). 10 µL of the compounds' solution, 90 µL of Na-phosphate buffer (pH 6.8) and 50 µL of α-glucosidase solution (1 U/mL) were mixed and incubated at 37°C for 10 min. After incubation, 50 µL of *p*NPG solution was added and the absorbance increase was measured at 405 nm. Acarbose

Scheme 1. Synthesis of compounds 3a-i.



Compound	R	Compound	R	Compound	R
3a	H	**3d**	4-C_3H_7	**3g**	4-$C(CH_3)_2CH_2CH_3$
3b	3-CH_3	**3e**	4-C_6H_5	**3h**	4-CN, 4-C_6H_5
3c	4-C_2H_5	**3f**	4-CF_3	**3i**	4-$NHCOCH_3$

was used as the standard. The α-glucosidase inhibitory activity (%) was calculated according to the following formula:

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

α-Amylase inhibitory activity

The α-amylase inhibitory activities of the compounds were determined by Ali et al.'s method, with some modifications (Ali, Houghton, & Soumyanath, 2006). 10 µL of the compounds' solution, 40 µL of Na-phosphate buffer (pH 6.8) and 50 µL of α-amylase solution (3 U/mL) were mixed and incubated for 10 min at 25°C. Starch solution (50 µL, 0.75%) was added to the mixture and kept at 25°C for 5 min. Then, 75 µL of DNS reagent was added to stop the reaction. The mixture was kept at 85°C for 15 min and diluted with distilled water after cooling. The absorbances were measured at 540 nm against the blank. Acarbose was used as the standard. The α-amylase inhibitory activity (%) was calculated according to the following formula:

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

RESULTS AND DISCUSSION

Chemistry

A convenient method for the synthesis of hydrazones is illustrated in Scheme 1. The structures of the newly synthesized compounds were elucidated by spectrometric methods (microanalysis, IR, ^{1}H-NMR and APT). The IR spectra of the new hydrazone compounds (**3a–i**) exhibited the N-H and C=O stretching vibrations in the 3394-3176 cm^{-1} and 1668-1643 cm^{-1}, respectively. The presence of the characteristic signals for aliphatic protons (Cihan-Üstündağ & Çapan, 2012; Kocabalkanlı et al., 2017; Cihan-Üstündağ, Mataracı-Kara, & Çapan, 2019) in the cyclohexylidene residue at about δ 0.98-2.97 ppm in the ^{1}H NMR spectrum of **3a–i** confirmed the formation of the hydrazone compounds. The splitting patterns of the H2, H5 and H6 hydrogens on the aromatic ring were in accordance with the 1,2,4-trisubstituted benzene system. The NH protons resonated at about δ 10.98-10.61 ppm and OCH_3 protons resonated at about δ 3.95-3.77 ppm as two separate singlets. The multiplicity in these signals pointed to the presence of two isomeric forms due to the restricted rotation about the C=N double bond and the partial double bond character of CONH (C-N) bond, in accordance with earlier reports (Ulusoy-Güzeldemirci, Şatana, & Küçükbasmacı 2015; Ulusoy-Güzeldemirci, Pehlivan, Halamoğlu, & Kocabalkanlı, 2016; Apaydın, 2018; Cihan-Üstündağ et al., 2019). Previous X-ray crystallographic studies on 4-methyl/4-ethylcyclohexanone derived indole-hydrazones revealed that these compounds existed as two crystallographically independent molecules due to the restricted rotation and these pair of molecules were found to be connected to each other, forming N—H ··· O dimers (Türktekin-Çelikesir, Akkurt, Cihan-Üstündağ, Çapan, & Büyükgüngör, 2013; Akkurt, Türktekin-Çelikesir, Cihan-Üstündağ, Çapan, & Büyükgüngör, 2013). The ^{1}H-NMR spectrum of compound **3b** displayed two sets of signals for most of the protons, including aromatic and 3-CH_3 protons, as well as NH and OCH_3 protons. It is assumed that the methyl substituent at 3-position interrupts the symmetry of the molecule and gives rise to the formation of *E* and *Z* isomers for compound **3b** (Montalvo-Gonzalez, Montalvo-Gonzalez, & Ariza-Castolo, 2008; Cihan-Üstündağ et al., 2019). Carbon assignments were evaluated by performing APT experiments. The new C=N carbon signals resonated with the C=O signals at δ 163.63-159.50 ppm region and further supported the formation of hydrazone derivatives. The detailed spectral data of compounds **3a–i** are given in the Materials and Methods section.

Biological activity

α-Glucosidase and α-amylase inhibitory activity

The novel benzoylhydrazones (**3a–i**) were tested for *in vitro* antidiabetic activity against α-glucosidase and α-amylase enzymes. The inhibitory activity test results were expressed as percentage inhibition and IC_{50} values. IC_{50} values indicate the concentration that inhibits enzyme activity by 50%. IC_{50} values were calculated from dose-response curves (Figure 1), using Microsoft Excel software. The antidiabetic drug acarbose was used as the standard α-glucosidase and α-amylase inhibitor in the tests.

Compounds **3d**, **3e**, **3g** and **3h** showed high α-glucosidase inhibitory activity compared to acarbose, especially at concentrations of 125 and 250 µg/mL. **3e** and **3g** showed similar α-glucosidase inhibitory activity compared to acarbose at a concentration of 62.5 µg/mL. The most active analogue **3g**, with a *tert*-pentyl group at the cyclohexane ring, exhibited the highest α-glucosidase inhibitory activity with an IC_{50} of 65.27 µg/mL, while the IC_{50} value of the reference acarbose was found to be 122.25 µg/mL (Table 1). **3d**, **3e**, **3g** and **3h** showed similar

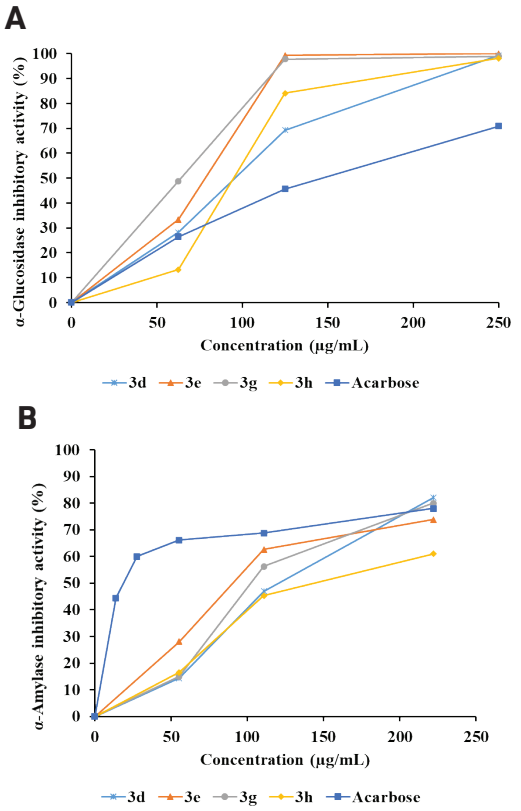


Figure 1. α-Glucosidase (**A**) and α-amylase (**B**) inhibitory activities (%) of compounds at different concentrations.

α-amylase inhibitory activity compared to acarbose at high concentrations, 222 and 111 μg/mL. However, they showed weak inhibitory activity at a concentration of 55.5 μg/mL and their IC_{50} values were much higher compared to that of reference acarbose (Table 2). None of the other tested hydrazone compounds had a meaningful efficacy on α-glucosidase and α-amylase enzyme activity at the highest concentrations tested. Introduction of a bulky substituent at position 4- of the cyclohexane system seemed to have a positive effect on antidiabetic activity.

CONCLUSION

This report is based on the synthesis and characterization of a new series of cyclohexanone benzoylhydrazones. The new hydrazones were evaluated for their *in vitro* α-glucosidase and α-amylase inhibitory activity. Of the nine compounds tested, four derivatives, **3d**, **3e**, **3g** and **3h,** were found to have an inhibitory effect on the enzymes tested. Compounds **3d**, **3e**, **3g** and **3h** exhibited better α-glucosidase inhibitory activity compared to reference antidiabetic drug acarbose. Compound **3g** was the most active agent tested (IC_{50}:65.27 μg/mL), being two-fold more active than acarbose. **3d**, **3e**, **3g** and **3h** showed similar α-amylase inhibitory activity compared to acarbose when tested at high concentrations. However, they were found to be weakly active at a concentration of 55.5 μg/mL and their IC_{50} values were much higher compared to that of reference acarbose. The existence of a bulky group at the 4-position of the cyclohexane system seemed to cause an increase in antidiabetic activity.

Table 1. α-Glucosidase inhibitory activity of compounds 3d, 3e, 3g and 3h.

	Inhibition (%)			IC_{50} (μg/mL)[a]
Compounds	**250 μg/mL**	**125 μg/mL**	**62.5 μg/mL**	
3d	99.37±0.77	69.26±0.99	28.27±2.43	102.28±2.44
3e	100.00±0.00	99.31±0.41	33.43±2.23	78.18±1.61
3g	98.94±0.84	97.82±0.16	48.87±3.04	65.27±1.46
3h	98.16±0.81	84.08±1.63	13.28±0.92	94.92±1.13
Acarbose	70.96±2.56	54.49±2.99	36.77±1.20	122.25±5.05

Table 2. α-Amylase inhibitory activity of compounds 3d, 3e, 3g and 3h.

	Inhibition (%)			IC_{50} (μg/mL)[a]
Compounds	**222 μg/mL**	**111 μg/mL**	**55.5 μg/mL**	
3d	82.04±1.30	46.96±1.10	14.31±1.42	135.30±1.76
3e	73.80±1.08	62.65±0.39	28.11±1.14	110.13±1.87
3g	80.02±2.68	56.24±2.22	14.98±1.89	128.55±3.17
3h	60.94±0.56	45.33±0.77	16.59±1.41	166.10±0.24
Acarbose	77.98±1.66	68.74±0.75	66.20±0.13	18.06±1.81

[a] IC_{50} values indicate the concentration that inhibits enzyme activity by 50%. IC_{50} values were calculated from dose-response curves (by plotting the percentage of inhibition against concentration) using Microsoft Excel software.
*Values represent the means of three replicates ± standard deviation.

Peer-review: Externally peer-reviewed.

Informed Consent: Written consent was obtained from the participants.

Author Contributions: Conception/Design of Study- Ç.B.A., G.H.Ç., T.Y.Ö., G.C.Ü.; Data Acquisition- Ç.B.A., G.H.Ç.; Data Analysis/Interpretation- Ç.B.A., G.H.Ç., T.Y.Ö., G.C.Ü.; Drafting Manuscript- Ç.B.A., G.H.Ç., T.Y.Ö., G.C.Ü.; Critical Revision of Manuscript- Ç.B.A., G.H.Ç., T.Y.Ö., G.C.Ü.; Final Approval and Accountability- Ç.B.A., G.H.Ç., T.Y.Ö., G.C.Ü.

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

REFERENCES

- Akkurt, M., Türktekin-Çelikesir, S., Cihan-Üstündağ, G., Çapan, G., & Büyükgüngör, O. (2013). *N*-(4-Ethylcyclohexylidene)-5-fluoro-3-phenyl-1*H*-indole-2-carbohydrazide. *Acta Crystallographica Section E*, *E69*, 1331. https://doi.org/10.1107/S1600536813020394
- Ali, H., Houghton, P. J., & Soumyanath, A. (2006). α-Amylase inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to Phyllanthus amarus. *Journal of Ethnopharmacology*, *107*(3), 449–455. https://doi.org/10.1016/j.jep.2006.04.004
- Apaydın, Ç.B. (2018). Fenoksiasetamid Yapısı Taşıyan Tiyazolidinonlar Üzerinde Çalışmalar. (Doctoral dissertation). Retrieved from https://tez.yok.gov.tr/UlusalTezMerkezi/tezDetay.jsp?id=MsUfCzS3veHBGNDkm8m_BA&no=91GPEzP_U0xS5UJCmhRx8A
- Bothon, F. T. D., Debiton, E., Avlessi, F., Forestier, C., Teulade, J. C., & Sohounhloue, D. K. C. (2013). In vitro biological effects of two anti-diabetic medicinal plants used in benin as folk medicine. *BMC Complementary and Alternative Medicine*, *13*(51), 1-8. https://doi.org/10.1186/1472-6882-13-51
- Cihan-Üstündağ, G. & Çapan, G. (2012). Synthesis and evaluation of functionalized indoles as antimycobacterial and anticancer agents. *Molecular Diversity*,*16*, 525-539. https://doi.org/10.1007/s11030-012-9385-y
- Cihan-Üstündağ, Mataracı-Kara, E., & Çapan, G. (2019). Synthesis, characterization, antibacterial and antifungal evaluation of novel cyclohexanone benzoylhydrazones. *Istanbul Journal of Pharmacy*, *49*(3), 142-147. https://doi.org/10.26650/IstanbulJPharm.2019.19022
- DeFronzo, R. A., Ferrannini, E., Groop, L., Henry, R. R., Herman, W. H., Holst, J. J. …Weiss, R. (2015). Type 2 diabetes mellitus. *Nature Reviews Disease Primers*, *1*, 1–23. https://doi.org/10.1038/nrdp.2015.19
- Hossain, U., Das, A. K., Ghosh, S., & Sil, P. C. (2020). An overview on the role of bioactive α-glucosidase inhibitors in ameliorating diabetic complications. *Food and Chemical Toxicology*, *145*, 111738. https://doi.org/10.1016/j.fct.2020.111738
- Kocabalkanlı, A.,Cihan-Üstündağ, G., Naesens, L., Mataracı-Kara, E., Nassozi, M., & Çapan, G. (2017). Diclofenac-Based hydrazones and spirothiazolidinones: Synthesis, Characterization, and antimicrobial properties. *Archieve der Pharmazie*, *350*, e1700010. https://doi.org/10.1002/ardp.201700010
- Koçyiğit-Kaymakçıoğlu, B., Elçin, O., Seda, U., Fatma, K., Nathaly, S., Sevim, R., & Dimoglo, A. (2006). Synthesis and characterization of novel hydrazide-hydrazones and the study of their structure-antituberculosis activity. European Journal of Medicinal Chemistry, *41*(11), 1253–1261. https://doi.org/10.1016/j.ejmech.2006.06.009
- Liu, Z., & Ma, S. (2017). Recent Advances in Synthetic α-Glucosidase Inhibitors. *ChemMedChem*, *12*(11), 819–829. https://doi.org/10.1002/cmdc.201700216
- Metwally, K. A., Abdel-Aziz, L. M., Lashine, E. S. M., Husseiny, M. I., & Badawy, R. H. (2006). Hydrazones of 2-aryl-quinoline-4-carboxylic acid hydrazides: Synthesis and preliminary evaluation as antimicrobial agents. *Bioorganic and Medicinal Chemistry*, *14*(24), 8675–8682. https://doi.org/10.1016/j.bmc.2006.08.022
- Moldovan, C. M., Oniga, O., Pârvu, A., Tiperciuc, B., Verite, P., Pîrnău, A. … Pop, R. (2011). Synthesis and anti-inflammatory evaluation of some new acyl-hydrazones bearing 2-aryl-thiazole. *European Journal of Medicinal Chemistry*, *46*(2), 526–534. https://doi.org/10.1016/j.ejmech.2010.11.032
- Montalvo-Gonzalez, R., Montalvo-Gonzalez, J.A., & Ariza-Castolo, A. (2008). Conformational and structural analysis of exocyclic olefins and ketimines by multinuclear magnetic resonance. *Magnetic Resonance in Chemistry*, *46*, 907-917. https://doi.org/10.1002/mrc.2259
- Nasr, T., Bondock, S., & Youns, M. (2014). Anticancer activity of new coumarin substituted hydrazide-hydrazone derivatives. *European Journal of Medicinal Chemistry*, *76*, 539–548. https://doi.org/10.1016/j.ejmech.2014.02.026
- Padhi, S., Nayak, A.K., & Behera, A. (2020). Type II diabetes mellitus: a review on recent drug based therapeutics. *Biomed Pharmacother*, *131*, 110708. doi:10.1016/j.biopha.2020.110708
- Proença, C., Ribeiro, D., Freitas, M., & Fernandes, E. (2021). Flavonoids as potential agents in the management of type 2 diabetes through the modulation of α-amylase and α-glucosidase activity: a review. *Critical Reviews in Food Science and Nutrition*, https://doi.org/10.1080/10408398.2020.1862755
- Taha, M., Ismail, N. H., Javaid, K., Imran, S., Anouar, E. H., Wadood, A. … Choudhary, M. I. (2015). Evaluation of 2-indolcarbohydrazones as potent α-glucosidase inhibitors, in silico studies and DFT based stereochemical predictions. *Bioorganic Chemistry*, *63*, 24–35. https://doi.org/10.1016/j.bioorg.2015.09.001
- Toniolo, A., Cassani, G., Puggioni, A., Rossi, A., Colombo, A., Onodera, T., & Ferrannini, E. (2019). The diabetes pandemic and associated infections: Suggestions for clinical microbiology. *Reviews in Medical Microbiology*, *30*(1), 1–17. https://doi.org/10.1097/MRM.0000000000000155
- Türktekin-Çelikesir, S., Akkurt, M., Cihan-Üstündağ, G., Çapan, G., & Büyükgüngör, O. (2013). 5-Fluoro-N0-(4-methylcyclohexylidene)-3-phenyl-1H-indole-2-carbohydrazide. *Acta Crystallographica Section E, E69,* 1211–1212. https://doi//10.1107/S1600536813018436
- Ulusoy Güzeldemirci, N., Şatana, D., & Küçükbasmacı, Ö. (2015). Synthesis and antimicrobial evaluation of some new hydrazone derivatives of 6-(4-nitrophenyl)imidazo[2,1-b]thiazole-3-aceticacid hydrazide. *Istanbul Journal of Pharmacy*, *45*(2), 127-138.
- Ulusoy Güzeldemirci, N., Pehlivan, E., Halamoğlu, Z., & Kocabalkanli, A. (2016). Synthesis and antiviral activity evaluation of some new cyclohexylidenehydrazide derivatives of 1,3-thiazole core. *Marmara Pharmaceutical Journal*, *20*, 207-215. https://doi.org/10.12991/mpj.20162019913
- Wang, G., Chen, M., Wang, J., Peng, Y., Li, L., Xie, Z. Z. … Li, W. (2017). Synthesis, biological evaluation and molecular docking studies of chromone hydrazone derivatives as α-glucosidase inhibitors. *Bioorganic and Medicinal Chemistry Letters*, *27*(13), 2957–2961. https://doi.org/10.1016/j.bmcl.2017.05.007
- World Health Organization (2021). WHO Health topics:Diabetes. Retrieved from https://www.who.int/health-topics/diabetes#tab=tab_1
- Zawawi, N. K. N. A., Taha, M., Ahmat, N., Wadood, A., Ismail, N. H., Rahim, F. … Abdullah, N. (2016). Benzimidazole derivatives as new α-glucosidase inhibitors and in silico studies. *Bioorganic Chemistry*, *64*, 29–36. https://doi.org/10.1016/j.bioorg.2015.11.006

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Original Article

Effect of metformin and metformin-sulfonylurea on lipid profile of type 2 *diabetes mellitus* patients: A cross-sectional study

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ABSTRACT

Background and Aims: In addition to lowering blood glucose levels, metformin also has a positive effect on the lipid profile by affecting gluconeogenesis and lipogenesis in the liver. Conversely, sulfonylurea is reported to possibly worsen the lipid profile and increase the risk of cardiovascular disease. Therefore, we would like to know whether there is a significant difference in the lipid profile of type 2 diabetes mellitus patients taking metformin as monotherapy and metformin-sulfonylurea as a combination since these two medicines are very commonly used in Indonesia.

Methods: A cross-sectional study was performed on 88 patients with type 2 diabetes mellitus who were restricted on metformin or metformin-sulfonylurea for equal to or more than 1 year. Subjects on metformin (n=37) and metformin-sulfonylurea (n=51) were asked to fast for at least 8 hours before blood sampling. We measured the lipid parameters from subjects' blood samples using a standardized enzymatic method.

Results: All basic characteristics of the study subjects in these two groups were matched. We found that total cholesterol, LDL-cholesterol, and triglyceride were lower and HDL-cholesterol was higher in the metformin group than the metformin-sulfonylurea group but not statistically significant ($p>0.05$). Multivariate analysis showed no significant differences for both therapies in any parameters before and after being adjusted by confounders. Only the increase in BMI contributed significantly to the increase in triglyceride.

Conclusion: This study presents no statistical differences in lipid profile after ≥1 year consumption of metformin and metformin-sulfonylurea combination.

Keywords: *Diabetes mellitus*, Metformin, Sulfonylurea, Lipid profile

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INTRODUCTION

Diabetes mellitus occurs due to the disruption of the endocrine system, making blood glucose levels abnormal and further causing complications in the human organ system (WHO, 2021). Patients with type 2 diabetes mellitus typically have obesity and insulin resistance, which could cause metabolic syndrome and impaired lipid metabolism (Jaiswal et al., 2014; Schofield, Liu, Rao-Balakrishna, Malik, & Soran, 2016). Hyperlipidemia in patients with type 2 diabetes mellitus can lead to many comorbidities, including cardiovascular diseases (Bangert, 2008; Chapman, et al., 2011). The American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) developed an overall approach for the glucose-lowering medication (antidiabetic) in type 2 diabetes mellitus (Davies et al., 2018). In this recommended algorithm, metformin is still the first-line oral antidiabetic drug. The combination of sulfonylurea and metformin is the second step in the management of patients with type 2 diabetes suggested by the ADA, EASD, and also the Indonesian Endocrinologist Association (PERKENI) (Adler, Shaw, Stokes, & Ruiz, 2009; PERKENI, 2015). Since many patients are put on both medications, an evaluation is not only needed for their capacity in lowering blood glucose levels but also their ability to prevent the progression of comorbidities (Davies et al., 2018). Metformin has been consumed by 60% of type 2 two diabetes patients worldwide due to lower long-term risk than other oral antidiabetic drugs (Berkowitz, et al., 2014). In addition to lowering blood glucose levels, metformin also affects the lipid profile of diabetes mellitus patients by affecting gluconeogenesis and lipogenesis in the liver (Brunton et al., 2005; Laisupasin, Thompat, Sukarayodhin, Sornprom, & Sudjaroen, 2013; Shaw et al., 2005). It is reported that metformin gives a positive effect to triglyceride, low-density lipoprotein cholesterol (LDL-c), and high-density lipoprotein cholesterol (HDL-c) levels after long usage (Busti, 2015). We also previously found that metformin was more effective than metformin-sulfonylurea in decreasing oxidative stress and urine albumin-to-creatinine ratio (UACR) (Sauriasari, Andriany, Sekar, & Azizahwati, 2017). In a meta-analysis reported by Rao, Kuhadiya, Reynolds, & Fonseca (2008), combination therapy of metformin and sulfonylurea significantly increased the risk of cardiovascular hospitalization or mortality, (fatal and nonfatal events) irrespective of the metformin monotherapy or sulfonylurea monotherapy. A recent cohort study also showed an increase in cardiovascular disease incidences in female patients with type 2 diabetes mellitus who use a combination of metformin and sulfonylurea for ten years (Li, Hu, Ley, Rajpathak, & Hu, 2014). Hypoglycemia, a frequent side effect due to sulfonylurea, is known as an important factor that affects cardiac performance (Middleton et al., 2017). Sulfonylureas have a small effect on lipids although they may statistically increase the level of free fatty acid (FFA) and triglyceride and decrease LDL-c and HDL-c (Chen et al., 2015). When compared to metformin, sulfonylureas could increase total cholesterol (TC) and LDL-c (Chen, et al., 2015). In this study, we would like to focus on a combination of metformin and sulfonylureas rather than sulfonylurea alone. We aimed to know whether there are significant differences between metformin-sulfonylurea compared to metformin alone on lipid profile since these two drugs are commonly prescribed in Indonesia. The study subjects were restricted to patients who use metformin or metformin-sulfonylurea for long term (≥ 1 year).

MATERIAL AND METHODS

This study was approved by The Ethics Committee, Faculty of Medicine, Universitas Indonesia - Dr. Cipto Mangunkusumo Hospital (Number:016/UN2.F1/ETIK/2018). Clinical examinations were undertaken using informed consent, and questionnaires were given to subjects before sampling takes place.

This cross-sectional study is part of the project aimed to compare metformin and metformin-sulfonylurea effectiveness by examining several clinical outcomes, including renal function (Sauriasari, Aristia, & Azizahwati, 2020) and lipid profile. We carried out the study by conducting a consecutive sampling on subjects with type 2 diabetes mellitus who were outpatients at Pasar Minggu Primary Health Care, Jakarta, Indonesia, from March to May 2018. The daily dose of metformin used by the patients was in the range of 500 mg 2-3 times daily and 850 mg 1-2 times daily. The sulfonylurea drug used by the patients in this study was is glimepiride 1-2 mg once daily. The exclusion criteria included patients over 25 years old who had been consistently taking metformin therapy or metformin-sulfonylurea therapy for at least one year before the sampling, based on the information provided in the medical record. All patients then were asked to fast for at least eight hours before the blood sampling was taken. The exclusion criteria were patients with insulin therapy and/or other oral antidiabetic drugs and patients with changes in therapy within one year of drug consumption based on data in the medical record. We calculated the minimum sample size using a calculation for mean comparison between the two groups with 5% of type 1 error (α) and 80% of the power of the test (1-β). The minimum sample size was 23 per group.

Blood samples were obtained from the patient's fingertips using a sterile lancet (General Care, Indonesia). The blood was picked by a capillary rod (Infopia, USA) to lipid profile test strip (LipidPro™ test strip, Infopia, USA) and HbA1c test cartridge (Afinion™ HbA1c Test Cartridge, Alere, USA). The blood samples were analyzed by a Lipid Profile Analyzer (LipidPro™ Testing Meter Infopia, USA) and an HbA1c analyzer (Alere Afinion™ AS100 Analyzer).

The lipid profile calculation used an analysis tool that applied the Friedewald formula. The Friedewald formula (FF) is one of the main methods for evaluating the amount of LDL-Cholesterol (LDL-C). In calculations using this formula, total cholesterol (TC), triglyceride (TG), and HDL-cholesterol (HDL-C) levels are needed. For each component, the calculations of TC, TG, and HDL-C are in units of mg/dL and not applicable for mmol/L units.

$$\text{LDL-C} = \text{TC} - \text{HDL-C} - (\text{TG}/5)$$

The data were then analyzed statistically. We selected covariates to be included in the multivariate model by conducting a bivariate analysis. Covariate that correlates with the outcomes

(Total Cholesterol, LDL, HDL, or triglyceride) with p<0.25 was included in the multivariate analysis.

RESULTS

The basic characteristics of the study subjects in the two groups were matched (Table 1). The proportion of gender, age, body weight, body height, BMI, the duration of diabetes mellitus, exercise habit, smoking habit, and the use of antihypertensive and antihyperlipidemic were not different between the two groups (p>0.05). However, the HbA1c level was significantly lower in the metformin group than the metformin-sulfonylurea group (Table 1).

Table 2 shows that patients taking metformin showed better lipid profile results than patients taking metformin-sulfonylurea, especially at the triglyceride levels, although not statistically significant. The ratio of LDL to HDL in the two study groups was more than 3:1, indicating a low HDL level (Table 2). The mean of total cholesterol level in subjects taking metformin and that of subjects taking metformin-sulfonylurea were within the normal limits (Table 2). However, the mean of HDL, triglyceride, and LDL levels in both groups were outside of the normal limits. The mean of total cholesterol, HDL, triglyceride, and LDL levels were better in the metformin group in relation compared to the metformin - sulfonylurea group although there were no significant difference (p>0.05) (Table 2). However, all parameters, except total cholesterol, were not in the normal range (Table 2).

Since there was a significant difference in the HbA1c level between the two groups, we did a stratified analysis according to the targeted HbA1c (≤7%)**.** We found better total cholesterol, LDL, HDL, and triglyceride levels in the HbA1c≤7% group although not statistically significant (Table 3).

We further conducted a multivariate analysis for each parameter. There were no significant differences in the metformin and metformin-sulfonylurea groups before and after adjusted by confounders (Table 4). Metformin-sulfonylurea showed a non-significant negative correlation with HDL and non-significant positive correlation with total cholesterol and triglyceride, even after adjusted by confounders (Table 4). However,

Table 1. Comparison of basic and clinical characteristics of the metformin group and metformin-sulfonylurea group.

Characteristic	Metformin (n=37)	Metformin–sulfonylurea (n=51)	*p*
Age (years)	64.19±7.71	61.12±7.79	0.070[a]
Gender			
Female (n)	25 (67.6)	44 (86.3)	0.065[c]
Male (n)	12 (32.4)	7 (13.7)	
Body mass index (kg/m^2)	24.30 ± 8.17	23.72 ± 4.73	0.936[b]
Duration of diabetes (years)	7.21± 5.15	8.95 ± 5.82	0.155[b]
Exercise habit (n)			
yes	24 (64.9)	28 (54.9)	0.472[c]
no	13 (35.1)	23 (45.1)	
Smoking (n)			
yes	0 (0.0)	1 (2.0)	1.000[c]
no	37 (100.0)	50 (98.0)	
Antihypertensive (n)			
yes	15 (40.5)	30 (58.8)	0.139[c]
no	22 (59.5)	21 (41.2)	
Antihyperlipidemic (n)			
yes	9 (24.3)	14 (27.5)	0.933[c]
no	28 (75.7)	37 (72.5)	
Blood Pressure			
Systolic (mmHg)	125.14±15.75	122.94±14.18	0.501[b]
Diastolic (mmHg)	76.22±5.94	77.06±6.72	0.636[b]
HbA1c (%)	7.75±1.34	9.04±1.82	**0.001[b*]**

Data presented in mean±SD or n (%); SD, standard deviation; *significant; [a]Independent T-Test; [b]Mann–Whitney Test; [c]Chi-Square Test

Table 2. Lipid profile according to the therapy groups.

	Cut-off values	Metformin (n=37)	Metformin–sulfonylurea (n=51)	*p*
Total Cholesterol (mg/dl)	<200	193.35±42.73	197.82±41.82	0.625[a]
LDL (mg/dl)	<100	125.49±40.70	125.98±44.33	0.958[a]
HDL (mg/dl)	>40	34.13±14.12	33.63±17.19	0.886[a]
Triglyceride (mg/dl)	<150	169.27±93.94	192.80±100.36	0.335[b]

Data presented in mean±SD; [a]Independent T-Test; [b]Mann–Whitney Test

Table 3. Lipid profile according to HbA1c level.

	Cut-off values	HbA1c≤7% (n=20)	HbA1c>7% (n=68)	*p*
Total Cholesterol (mg/dl)	<200	181.50±35.33	200.29±43.05	0.073[b]
LDL (mg/dl)	<100	112.75±43.11	129.60±41.99	0.120[b]
HDL (mg/dl)	>40	37.16±15.15	32.87±16.08	0.291[b]
Triglyceride (mg/dl)	<150	156.10±92.58	190.79±98.63	0.134[c]

Data presented in mean±SD; [b]Independent T-Test; [c]Mann–Whitney Test

for LDL, metformin-sulfonylurea showed a positive correlation with increased LDL but changed to a weak non-significant negative correlation after adjusted by confounders (Table 4). Additionally, only increased BMI contributed significantly to the increase in triglyceride levels (Table 4).

DISCUSSION

The distribution of female subjects dominates the total sample of each group (Table 1). Based on Indonesia Basic Health Research in 2013, the proportion of diabetes mellitus patients in Indonesia is greater in women than in men (Ministry of Health, 2013). The average BMI level in both groups did not exceed 25 kg/m^2, which was within the normal value (Nuttall F. Q, 2015). Based on data collection, gymnastic activity and daylight walk remain the most common exercise carried out frequently by the subjects. The proportion of subjects on antihypertensive and antihyperlipidemic medication did not differ significantly between the two groups. Diabetes mellitus, hypertension, and hyperlipidemia have the same clinical linkages through hyperinsulinemia (Tsimihodimos, Gonzalez-Villalpando, Meigs, & Ferrannini, 2018).

Chronic insulin resistance in diabetes mellitus patients may influence the subjects' lipid profiles. In type 2 diabetes mellitus patients, hyperinsulinemia, frequently insulin resistance, and β cell failure are related to dyslipidemia (Athyros et al., 2018). Insulin performs a role in lipolysis suppression and enhances the transport of triglycerides from blood vessels into adipose tissue for storage as well as inhibiting fatty acid oxidation. Therefore, the defect of the action of insulin on its receptors has an impact on the regulation of lipids in the body (Dimitriadis, Mitrou, Lambadiari, Maratou, & Raptis, 2011).

In this study, the total cholesterol level in both groups was still within the normal range (Table 2). It may be partially due to the use of metformin, which has an influence on lipid metabolism in the body (Kashi, Mahrooz, Kianmehr, & Alizadeh, 2016). Metformin may promote the lipid profile because of its mechanism of action in stimulating AMP-Kinase, which plays a role in liver lipogenesis (Madsen, Bozickovic, Bjune, Mellgren, & Sagen, 2015). Metformin enters the hepatocytes via Organic Cation Transporter 1(OCT1), a hepatic uptake transporter, and runs the interference of complex-1 in the mitochondria. The restrained complex-1 decreases the ATP/AMP ratio resulting in the activation of LKB1 (B1-liver kinase) and AMP-Kinase. The active AMP-Kinase phosphorylates HMG-CoA (3-hydroxy-3-methyl-glutaryl-coenzyme A) reductase, thus converting it into an inactive form (Madsen et al., 2015). HMG-CoA reductase is an enzyme that represents a role in cholesterol biosynthesis. Therefore, if a substance inhibits the action, i.e., metformin, the cholesterol level in the body will also decrease (Zhang et al., 2015).

On the other hand, a meta-analysis of randomized controlled trials (RCTs) which assess the effects of sulfonylureas, alone or in combination, showed that sulfonylurea increased the level of TC and LDL-c when compared to metformin and decreased HDL-c, which is in line with our results (Chen et al., 2015; Zhang et al., 2013). Sulfonylurea use is also reported to be potentially associated with a higher risk of cardiovascular diseases (Li et al., 2014; Middleton et al., 2017).

A study reported that apart from a reliable glycemic index, HbA1c can also be used as a predictor of dyslipidemia (Zhang et al., 2013). With further elevated HbA1c levels in diabetes mellitus patients, their lipid profile will also get worse (Zhang et al., 2013). We also found similar results in which all lipid parameters were better in HbA1c≤7% group (Table 3). Concerning the results, we also considered whether the variability of the HbA1c level resulted in bias in these study results. Therefore, we performed a multivariate analysis. However, in this study, we did not find HbA1c as a significant modifying variable (Table 4).

Table 4. Effect of therapy on lipid profile before and after controlling confounders.

Variable	R^2	Standardized coefficients (β)	*p*
Total Cholesterol			
Crude Model	0.003		
Therapy group		0.053	0.625
Adjusted Model	0.058		
Therapy group		0.025	0.822
Age (years)			0.311
Gender			0.598
Body Mass Index (BMI) (kg/m^2)			0.404
Smoking status			0.173
LDL cholesterol			
Crude Model	0.000		
Therapy group		0.006	0.958
Adjusted Model	0.051		
Therapy group		-0.073	0.531
Age (years)			0.263
HbA1c (%)			0.202
HDL cholesterol			
Crude Model	0.000		
Therapy group		-0.016	0.886
Adjusted Model	0.073		
Therapy group		-0.260	0.817
Age (years)			0.364
Gender			0.268
Body Mass Index (BMI) (kg/m^2)			0.176
Smoking status			0.640
Triglyceride			
Crude Model	0.014		
Therapy group		0.119	0.268
Adjusted Model	0.107		
Therapy group		0.046	0.689
Gender			0.161
Body Mass Index (BMI) (kg/m^2)		0.256	**0.016***
HbA1c			0.215

Therapy group is in ordinal scale (1=metformin, 2=metformin-sulfonylurea); gender is in nominal scale (0=female, 1=male); smoking status is in ordinal scale (0=not smoking, 1=smoking). The statistically significant different shown as *(p<0.05).

Optimal blood glucose control in combination therapy, such as metformin-sulfonylurea, could be maintained by the action mechanism of each drug. Metformin can lower blood glucose by inhibiting mechanisms of glucose production in the liver by suppressing gluconeogenesis, diminishing glucose uptake in the small intestine, and improving the utilization of glucose by skeletal muscle and adipose tissue. Metformin can also help strengthen cell sensitivity to insulin (Natali, A., & Ferrannini, E., 2006). The metformin mechanism is supported by sulfonylurea drugs because they can enhance insulin secretion as the hormone responsible for glucose uptake into cells that decrease blood glucose concentration (Sola et al., 2015). Blood glucose level affects the HbA1c level in the body because HbA1c describes blood glucose concentration for approximately 120 days (Hussain, A., Ali, I., Ijaz, M., & Rahim, A., 2017).

Our study subjects in the metformin-sulfonylurea group have a higher HbA1c level than the metformin group (Table 1). The results of this study were different from another study conducted on Afghani patients (Florkowski C., 2013) but similar to our previous study at the same study site (Chen et al., 2015). The cross-sectional nature of our study design and the relatively small

number of sample size are some of our limitations. However, we selected patients quite tightly, and all of the basic characteristics of the study groups were matched. Concerning compliance issues, we restricted data from the patient who uses the same medication routinely for more than one year without a switch or stop. Moreover, our study site implemented a national program, namely the Chronic Disease Management Program, to maintain adherence and monitor the clinical condition of study subjects. Nevertheless, HbA1c and some of the lipid parameters of the study subjects in the two groups did not reach the normal target, indicating the diabetes mellitus management therapy in the study site should be further evaluated.

CONCLUSION

This study presents no statistical differences in lipid profile after ≥1 year consumption of metformin and metformin-sulfonylurea combination.

Peer-review: Externally peer-reviewed.

Informed Consent: Written consent was obtained from the participants.

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

Ethics Committee Approval: This study has been approved by The Ethics Committee, Faculty of Medicine, Universitas Indonesia-Dr. Cipto Mangunkusumo Hospital (Number:016/UN2.F1/ETIK/2018).

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

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REFERENCES

- Adler, A., Shaw, E., Stokes, T., & Ruiz, F. (2009). Newer agents for blood glucose control in type 2 diabetes: summary of NICE guidance. *BMJ*, *338*, b1668-b1668. https://doi.org/10.1136/bmj.b1668
- Athyros, V. G., Doumas, M., Imprialos, K. P., Stavropoulos, K., Georgianou, E., Katsimardou, A., & Karagiannis, A. (2018). Diabetes and lipid metabolism. *Hormones (Athens, Greece)*, *17*(1), 61–67. https://doi.org/10.1007/s42000-018-0014-8
- Bangert, S. (2008). Hyperlipidaemia: Diagnosis and Management (3rd edn.). Annals of Clinical Biochemistry, 45(6), 619–619. https://doi.org/10.1258/acb.2008.200814
- Berkowitz, S. A., Krumme, A. A., Avorn, J., Brennan, T., Matlin, O. S., Spettell, C. M., Pezalla, E. J., Brill, G., Shrank, W. H., & Choudhry, N. ,. (2014). Initial choice of oral glucose-lowering medication for diabetes mellitus: a patient-centered comparative effectiveness study. *JAMA internal medicine*, *174*(12), 1955–1962. https://doi.org/10.1001/jamainternmed.2014.5294
- Brunton, L., Lazo J, Buxton I, Blumenthal D, Akil H, Amrein P. (2005). Goodman & Gilman's the pharmacological basis of therapeutics. 11th ed. New York: McGraw-Hill
- Busti, A. J. (2015). *The Mechanism for Metformin's (Glucophage) Improvement in the Lipid Profile Beyond its Glucose Lowering Effects in Diabetes Mellitus*. Ebmconsult.com. Retrieved 8 March 2018, from https://www.ebmconsult.com/articles/metformin-glucophage-diabetes-lipid-cholesterol-lowering.
- Chapman, M. J., Ginsberg, H. N., Amarenco, P., Andreotti, F., Borén, J., et al European Atherosclerosis Society Consensus Panel. (2011). Triglyceride-rich lipoproteins and high-density lipoprotein cholesterol in patients at high risk of cardiovascular disease: evidence and guidance for management. *European Heart Journal*, *32*(11), 1345–1361. https://doi.org/10.1093/eurheartj/ehr112
- Chen, Y. H., Du, L., Geng, X. Y., Peng, Y. L., Shen, J. N., Zhang, Y. G., Liu, G. J., & Sun, X. (2015). Effects of sulfonylureas on lipids in type 2 diabetes mellitus: a meta-analysis of randomized controlled trials. *Journal of Evidence-Based Medicine*, *8*(3), 134–148. https://doi.org/10.1111/jebm.12157
- Davies, M., D'Alessio, D., Fradkin, J., Kernan, W., Mathieu, C., Mingrone, G., Rossing, P., Tsapas, A., Wexler, D. J., & Buse, J. B. (2018). Management of hyperglycaemia in type 2 diabetes, 2018. A consensus report by the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). *Diabetologia*, *61*(12), 2461-2498. https://doi.org/10.1007/s00125-018-4729-5
- Dimitriadis, G., Mitrou, P., Lambadiari, V., Maratou, E., & Raptis, S. A. (2011). Insulin effects in muscle and adipose tissue. *Diabetes Research and Clinical Practice*, *93 Suppl 1*, S52–S59. https://doi.org/10.1016/S0168-8227(11)70014-6
- Florkowski C. (2013). HbA1c as a Diagnostic Test for Diabetes Mellitus - Reviewing the Evidence. *The Clinical Biochemist. Reviews*, *34*(2), 75–83.
- Hussain, A., Ali, I., Ijaz, M., & Rahim, A. (2017). Correlation between hemoglobin A1c and serum lipid profile in Afghani patients with type 2 diabetes: hemoglobin A1c prognosticates dyslipidemia. *Therapeutic Advances in Endocrinology and Metabolism*, *8*(4), 51–57. https://doi.org/10.1177/2042018817692296
- Indonesian Endocrinologist Association (PERKENI). (2015). The consensus in management and prevention of type 2 diabetes mellitus for 2015 Jakarta: Perkeni. https://pbperkeni.or.id/unduhan
- Jaiswal, M., Schinske, A., & Pop-Busui, R. (2014). Lipids and lipid management in diabetes. *Best Practice & Research Clinical Endocrinology & Metabolism*, *28*(3), 325-338. https://doi.org/10.1016/j.beem.2013.12.001
- Kashi, Z., Mahrooz, A., Kianmehr, A., & Alizadeh, A. (2016). The Role of Metformin Response in Lipid Metabolism in Patients with Recent-Onset Type 2 Diabetes: HbA1c Level as a Criterion for Designating Patients as Responders or Nonresponders to Metformin. *PloS one*, *11*(3), e0151543. https://doi.org/10.1371/journal.pone.0151543
- Laisupasin, P., Thompat, W., Sukarayodhin, S., Sornprom, A., & Sudjaroen, Y. (2013). Comparison of Serum Lipid Profiles between Normal Controls and Breast Cancer Patients. *Journal of Laboratory Physicians*, *5*(1), 38–41. https://doi.org/10.4103/0974-2727.115934
- Li, Y., Hu, Y., Ley, S. H., Rajpathak, S., & Hu, F. B. (2014). Sulfonylurea use and incident cardiovascular disease among patients with type 2 diabetes: prospective cohort study among women. *Diabetes care*, *37*(11), 3106–3113. https://doi.org/10.2337/dc14-1306
- Madsen, A., Bozickovic, O., Bjune, J.I., Mellgren. G., Sagen, J. V. (2015). Metformin inhibits hepatocellular glucose, lipid and cholesterol biosynthetic pathways by transcriptionally suppressing steroid receptor coactivator 2 (SRC-2). Sci Rep. doi: 10.1038/srep16430. PMID: 26548416; PMCID: PMC4637908.
- Middleton, T. L., Wong, J., Molyneaux, L., Brooks, B. A., Yue, D. K., Twigg, S. M., & Wu, T. (2017). Cardiac Effects of Sulfonylurea-Related Hypoglycemia. *Diabetes Care*, *40*(5), 663–670. https://doi.org/10.2337/dc16-1972

- Ministry of Health. (2013). Basic Health Research 2013. Jakarta: Ministry of Health Republic of Indonesia. https://www.litbang.kemkes.go.id/laporan-riset-kesehatan-dasar-riskesdas/
- Natali, A., & Ferrannini, E. (2006). Effects of metformin and thiazolidinediones on suppression of hepatic glucose production and stimulation of glucose uptake in type 2 diabetes: a systematic review. *Diabetologia*, *49*(3), 434–441. https://doi.org/10.1007/s00125-006-0141-7
- Nuttall, F. Q. (2015). Body Mass Index: Obesity, BMI, and Health: A Critical Review. *Nutrition today*, *50* (3), 117–128. https://doi.org/10.1097/NT.0000000000000092
- Rao, A. D., Kuhadiya, N., Reynolds, K., & Fonseca, V. A. (2008). Is the combination of sulfonylureas and metformin associated with an increased risk of cardiovascular disease or all-cause mortality?: a meta-analysis of observational studies. *Diabetes Care*, *31*(8), 1672–1678. https://doi.org/10.2337/dc08-0167
- Sauriasari, R., Andriany, R., Sekar, A., & Azizahwati, A. (2017). Comparison of Antioxidative Effect of Metformin and Combination of Metformin-Sulfonylurea in Type 2 Diabetes Mellitus Patients. *Journal of Young Pharmacists*, *9*(1), 39-42. https://doi.org/10.5530/jyp.2017.1s.10
- Sauriasari, R., Aristia H., Azizahwati (2020). Urine albumin-to-creatinine ratio and estimated glomerular filtration rate in matched groups of type 2 diabetes mellitus patients receiving metformin and metformin-sulfonylurea. *International Journal of Applied Pharmaceutics.* 12(Special Issue I), 257-259. DOI: 10.22159/ijap.2020.v12s1.FF056
- Schofield, J. D., Liu, Y., Rao-Balakrishna, P., Malik, R. A., & Soran, H. (2016). Diabetes Dyslipidemia. *Diabetes therapy: research, treatment and education of diabetes and related disorders*, *7*(2), 203–219. https://doi.org/10.1007/s13300-016-0167-x
- Shaw, R. J., Lamia, K. A., Vasquez, D., Koo, S. H., Bardeesy, N., Depinho, R. A., Montminy, M., & Cantley, L. C. (2005). The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science (New York, N.Y.)*, *310*(5754), 1642–1646. https://doi.org/10.1126/science.1120781
- Sola, D., Rossi, L., Schianca, G., Maffioli, P., Bigliocca, M., & Mella, R. et al. (2015). State of the art paper Sulfonylureas and their use in clinical practice. *Archives Of Medical Science*, *4*, 840-848. https://doi.org/10.5114/aoms.2015.53304
- Tsimihodimos, V., Gonzalez-Villalpando, C., Meigs, J., & Ferrannini, E. (2018). Hypertension and Diabetes Mellitus. *Hypertension*, *71*(3), 422-428. https://doi.org/10.1161/hypertensionaha.117.10546
- WHO. *Diabetes*. Who.int. (2021). Retrieved 10 November 2021, from http://www.who.int/news-room/fact-sheets/detail/diabetes.
- Zhang, F., Xiang, H., Fan, Y., Ganchuluun, T. A., Kong, W., Ouyang, Q., Sun, J., Cao, B., Jiang, H., & Nie, S. (2013). The effects of sulfonylureas plus metformin on lipids, blood pressure, and adverse events in type 2 diabetes: a meta-analysis of randomized controlled trials. *Endocrine*, *44*(3), 648–658. https://doi.org/10.1007/s12020-013-9970-6
- Zhang, X., Song, Y., Feng, M., Zhou, X., Lu, Y., Gao, L., Yu, C., Jiang, X., & Zhao, J. (2015). Thyroid-stimulating hormone decreases HMG-CoA reductase phosphorylation via AMP-activated protein kinase in the liver. *Journal of Lipid Research*, *56*(5), 963–971. https://doi.org/10.1194/jlr.M047654

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Original Article

Relationship of thiol/disulphide homeostasis with oxidative stress parameters in non-diabetic, prediabetic and type 2 diabetic Turkish women

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ABSTRACT

Background and Aims: Deterioration of thiol/disulphide homeostasis (TDH) is found in several diseases, including diabetes. This study aimed to examine the association between TDH and oxidative stress parameters in nondiabetics, prediabetics and newly diagnosed type 2 diabetics.
Methods: A total of 26 non-diabetic, 24 prediabetic and 19 type 2 diabetic women were involved in our study. They all applied to Zonguldak Bulent Ecevit University, Health Practice and Research Center, Endocrinology and Metabolism Diseases, Diabetes Polyclinic to be tested for type 2 diabetes mellitus. The demographic and laboratory data were collected from the patient files. Oxidative stress parameters and dynamic TDH were investigated using ELISA kits.
Results: Total oxidant status (TOS), total thiol and disulphide levels were significantly higher in type 2 diabetics than the non-diabetics (24.24±14.93 versus 14.14±12.19, 646.47±75.51 versus 470.88±180.85, and 179.32±51.24 versus 91.85±40.29, respectively). In type 2 diabetics, a positive correlation between TOS and native thiol, total thiol and disulphide levels was found (P=0.000). In prediabetics, a significant positive correlation was found between total antioxidant capacity and total thiol levels (P<0.05), and also between arylesterase and native and total thiol levels (P<0.05).
Conclusion: The elevation of oxidative stress and the deterioration of TDH might cause the formation of symptoms related to high blood glucose levels in type 2 diabetics.

Keywords: Sulfhydryl compounds; Diabetes mellitus, type 2; Prediabetic state; Oxidative stress

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is characterised by hyperglycaemia due to insufficient insulin oscillations. Its prevalence is increasing each day. In 2020, the WHO reported that about 422 million people have diabetes worldwide and each year 1.6 million deaths result from diabetes (Lovic et al., 2020). It has been known that insufficient insulin secretion from pancreatic beta cells causes metabolic syndromes before an individual is diagnosed as having type 2 diabetes. Prediabetes is a condition of having high blood sugar levels, but the whole range of symptoms of diabetes is not yet present. Therefore, prediabetes is considered as an underlying risk factor for T2DM (Khetan & Rajagopalan, 2018; Garber et al., 2019).

Cells can be damaged by the enhancement of free radicals, which are highly reactive chemicals. The antioxidant defence system protects the body against the hazardous effects of free radicals. Oxidative stress (OS) occurs when the equilibrium between

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the amount of free radicals and the amount of antioxidants deteriorates (Sies, 1997). Reactive oxygen species induce thiol groups (-SH) to form disulphide (RSSR) bonds. This reaction is reversible (Erel & Neselioglu, 2014). Under the conditions of OS, due to its cysteine residue, glutathione (GSH) becomes oxidized to glutathione disulphide (GSSG). NADPH-dependent glutathione reductase then reduces GSSG to GSH. The cellular redox state can be determined by measuring the GSH/GSSG ratio (Wu, Fang, Yang, Lupton & Turner, 2004). In many diseases, such as diabetes mellitus, hypertension, non-small cell lung cancer, familial Mediterranean fever (FMF), inflammatory bowel diseases, occupational diseases, gestational diabetes mellitus and preeclampsia, OS occurs and the dynamic thiol/disulphide homeostasis (TDH) deteriorates (Erel & Erdogan, 2020). OS is a crucial factor in the development of complications related to diabetes such as hyperglycaemia, insulin resistance, inflammation and dyslipidaemia due to high blood glucose levels (Hamamcioglu, 2017).

There are some studies that focus on detecting OS and dynamic TDH in diabetic patients. In a study performed by Ates et al (2015) with newly diagnosed prediabetics and healthy volunteers, a positive correlation between the disulphide and blood glucose and HbA1c levels was reported. After a year, the group published another study with type 1 diabetics and suggested that an elevation of thiol oxidation in type 1 diabetics is associated with hyperglycaemia and chronic inflammation present in these patients (Ates et al., 2016). In T2DM patients, the progression of diabetic retinopathy was found to be related with an increase in both TDH and ischemia-modified albumin (IMA) levels (Gulpamuk et al, 2018). In a study performed by Ergin et al (2020), three groups of T2DM patients were included (T2DM patients with complications, T2DM patients without complications and newly diagnosed T2DM patients). They reported a gradual increase in disulphide levels due to the severity of the disease. In children with type 1 DM, TDH was found to be deteriorated and shifted towards the disulphide direction. They proposed that this shift is due to damage in pancreatic β-cells. However, none of these studies examined newly diagnosed prediabetics together with newly diagnosed T2DM patients and healthy volunteers.

Paraoxonase 1 (PON1) is a hydrolase with a glycoprotein structure which demonstrates both paraoxonase and arylesterase (ARES) activities. It is known to be synthesised in the liver and then secreted into the bloodstream in association with HDL. It helps to protect lipoproteins from oxidation (Unal et al., 2012). It was stated previously that during diabetes mellitus, under the conditions of hyperglycaemia, PON1 is separated from HDL and therefore, it loses its protective property against the oxidation of lipoproteins. This was reported as a risk factor for the development of diabetes-induced coronary artery diseases (Rosenblat, Sapir, & Aviram, 2008).

Our study aimed to understand the balance between total oxidants and antioxidants in prediabetic and type 2 diabetic (T2D) Turkish women together with a novel OS marker, TDH, and to compare the differences between the groups. We also aimed to understand the activities of paraoxonase 1 (PON1) and arylesterase (ARES) in all three groups and to discuss the correlations between all parameters.

MATERIAL AND METHODS

Individuals who applied to Zonguldak Bulent Ecevit University, Health Practice and Research Centre, Endocrinology and Metabolism Diseases, Diabetes Polyclinic to be tested for T2DM between September 2018 and March 2019, who met the inclusion criteria and who signed an informed consent form were involved in the study. Non-diabetics (NDs) formed our control group; the individuals who were found to be prediabetic formed our prediabetic group (fasting blood glucose level was between 100-125 mg/dl, blood glucose level was between 140-199 mg/dl in the second hour of oral glucose tolerance test and the levels of HbA1c were between 5.9-6.4%) and the ones who were diagnosed as T2DM for the first time (fasting blood glucose level was above 130 mg/dl and HbA1c level was above 6.5%) formed our patient group. NDs, prediabetics and T2Ds had no other disease related to organ damage, were all above 18 years of age, were not pregnant or breastfeeding, were non-smokers and were non-alcoholic. None of the individuals was under any medication.

The study was approved by Zonguldak Bulent Ecevit University Ethics Committee adhering to the Declaration of Helsinki (approval number 2018-49-14/02) and informed consent forms were signed by all the individuals prior to the study. Out of 69 individuals, 19 were diagnosed as T2D, 24 were diagnosed as prediabetic and 26 were found to be ND. Blood samples (10 ml) were collected on their first visit to the hospital.

Biochemical data collection and laboratory analysis

Peripheral blood samples were drawn after an overnight fast. A total of 10 ml venous blood samples were collected and were centrifuged (1500 g at 4°C for 10 min) to separate sera. The samples were stored at -80°C until usage. Fasting blood glucose levels, lipid profiles (HDL, LDL, cholesterol, and triglycerides), HbA1c and creatinine levels were all measured in a standard fashion at the Zonguldak Bulent Ecevit University Hospital Biochemistry laboratory, and demographic data such as height, weight, age, etc. were collected from the patients' files.

Total antioxidant capacity (TAC)

TAC levels of the sera were determined using ELISA kits (Relassay, Turkiye). The principle of the method depends on the decolorising of a characteristic colour produced by ABTS (2, 2′- Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)). The reaction was measured spectrophotometrically at 660 nm. The data were expressed as mmol Trolox equivalent per litre (Erel, 2004).

Total oxidant status (TOS)

ELISA kits developed by Erel (2005) were also used to detect serum TOS levels (Relassay, Turkiye). Generally, the principle depends on the formation of ferric ions by the oxidants present in the sample. Glycerol molecules present in the reaction medium enhanced the oxidation reaction. A coloured complex formed by ferric ions and xylene was measured at 530 nm..

Hydrogen peroxide was used to calibrate the assay and, therefore, "µmol hydrogen peroxide equivalent per litre" was used to express data.

Oxidative stress index (OSI)

Oxidative stress index (OSI) was calculated by dividing TOS to TAC values and was expressed in Arbitrary Units (AU) (Harma, Harma & Erel, 2003; Kosecik, Erel, Sevinc & Selek, 2005; Yumru et al, 2009).

Thiol/disulphide homeostasis (TDH)

TDH assay was developed by Erel and Neselioglu (2014). According to the principle of the assay, free thiol groups were formed by the reduction of disulphide bonds in the presence of sodium borohydride. To determine the amount of disulphides, the amount of native thiols were subtracted from the amount of total thiols and the result was divided into two. Percentages of the ratio of disulphide to total thiol and native thiol, as well as the ratios of native thiol to total thiol, were reported.

PON1 and ARES activities

PON1 and ARES activities in the sera samples were detected using ELISA kits (Relassay, Turkiye). Paraoxon was used as a substrate for PON1 activity and phenyl acetate for arylesterase activity. The enhancement of absorbance at 412 nm and 37°C were used to determine PON1 activity in terms of international units per 1 litre of sera (U/L). ARES activity was expressed in terms of kilo units per 1 litre of sera (KU/L) and measured at 270 nm and 37°C (Eckerson, Wyte, & La Du, 1983; Aldemir et al, 2015; Kilinc et al., 2016).

Statistical analysis

Data were analysed using the Statistical Package for Social Sciences (SPSS) version 22.0 for Windows (SPSS Inc., Chicago, IL, USA). The Kolmogorov-Smirnov test was used to understand the distribution of our data. Numerical variables having normal distribution were described as mean + standard deviation and those not having normal distribution were described with mean values. Student t test was used to compare the NDs with prediabetics and T2Ds. For continuous variables with non-normal distribution, the Mann Whitney U test was used to compare the clinical features of the NDs, prediabetics and T2Ds.The relationships between numeric parameters were determined through Pearson Spearman correlation analysis. P <0.05 was considered significant.

RESULTS

Our study consisted of 26 ND, 24 prediabetic and 19 T2D women. The demographic data and laboratory findings of NDs, prediabetics and T2Ds involved in our study are included in Table 1. Body mass index, fasting blood glucose and HbA1c levels were higher in T2Ds than the other groups, as estimated. Triglyceride levels were lower in NDs than prediabetics and T2Ds (P <0.05). HDL levels were higher in the NDs than the T2Ds (P<0.05). Even though total cholesterol and LDL levels were higher in T2Ds than the other groups, this difference was not found to be significant. In addition, T2Ds were older than NDs and prediabetics (55.68 + 10.91 versus 33.00 + 10.10 and 55.68 + 10.91 versus 42.83 + 11.18, respectively).

When the OS parameters were evaluated, no significant difference in the TAC values was found between NDs and the prediabetics nor the T2Ds. However, there was a significant reduction (P<0.05) in the TOS levels of prediabetics and a significant increase (P<0.005) in the TOS levels of T2Ds compared to NDs. TOS values of prediabetics were significantly lower (P<0.001) than the TOS values of T2Ds. When the OSI values were calculated, a non-significant decrease in prediabetics compared to NDs was found. OSI values of T2Ds increased significantly compared to the NDs and prediabetics (P<0.05 and P<0.001 respectively) (Table 2).

Both total thiol and native thiol levels decreased in prediabetics and increased in T2Ds compared to NDs. An elevation of

Table 1. Comparison of the demographic data and laboratory findings among the groups.

Variables	Non diabetics (n=26)	Prediabetics (n=24)	Type 2 diabetics (n=19)	p value
n (%)	26 (37.7)	24 (34.8)	19 (27.5)	
Age (years)	33.00±10.10	42.83±11.18	55.68±10.91	<0.005†, <0.001‡, =0.001§
BMI (kg/m2)	25.92±4.63	29.83±3.69	31.63±2.34	<0.005†, <0.001‡
FBG (mg/dL)	93.62±4.68	108.04±6.85	141.95±65.12	<0.001†, <0.001‡, <0.005§
HbA1c (%)	5.24±0.28	5.59±0.29	7.51±1.84	<0.001†, <0.001‡, <0.001§
Creatinine (mg/dL)	0.70±0.092	0.76±0.15	0.76±0.13	
Triglyceride (mg/dL)	125.81±60.92	149.67±69.49	200.89±67.70	<0.001‡, <0.05§
Total cholesterol (mg/dL)	193.19±46.55	204.96±38.89	214.26±46.40	
HDL (mg/dL)	54.23±13.22	49.25±10.36	45.58±6.95	<0.05‡
LDL (mg/dL)	117.27±36.68	125.79±32.43	127.95±36.41	

BMI: Body Mass Index, FBG: Fasting Blood Glucose, HbA1c: haemoglobinA1c, HDL: High-density Lipoprotein, LDL: Low-density Lipoprotein. The variables are expressed as mean±standard deviation. † shows a statistically significant difference between non-diabetics and prediabetics. ‡ shows a statistically significant difference between non-diabetics and type 2 diabetics. § shows a statistically significant difference between prediabetics and type 2 diabetics.

Table 2. Comparison of oxidative stress parameters, thiol/disulphide homeostasis parameters, and antioxidant enzymes (PON1 and ARES) among the groups.

Variables	Non diabetics (n=26)	Prediabetics (n=24)	Type 2 diabetics (n=19)	p value
Total thiol (mmol/L)	470.88±180.85	413.95±56.58	646.47±75.51	<0.001‡,<0.001§
Native thiol (mmol/L)	379.04±157.46	241.71±30.23	467.16±56.86	<0.005†, <0.001§
Disulphide (mmol/L)	91.85±40.29	172.25±43.26	179.32±51.24	<0.001†, <0.001‡,
Disulphide/ Total Thiol (%)	20.69±8.50	41.25±6.15	27.51±6.39	<0.001†, <0.005‡, <0.001§
Disulphide/ Native Thiol (%)	27.65±15.30	72.01±18.12	38.94±11.97	<0.001†, <0.005‡, <0.001§
Native Thiol/ Total Thiol (%)	79.31±8.50	58.75±6.15	72.49±6.39	<0.001†, <0.005‡, <0.001§
TAC (mmolTrolox equivalent/L)	2.41±0.41	2.38±0.49	2.60±0.57	
TOS (μmol H2O2 equivalent/L)	14.14±12.19	6.92±1.65	24.24±14.93	<0.05†, <0.005‡,<0.001§
OSI	0.65±0.67	0.31±0.11	1.04±0.76	<0.05‡, <0.001§
PON1	303.88±212.93	368.75±186.74	170.21±123.85	<0.05‡, <0.001§
ARES	264.92±140.81	228.54±47.44	175.42±92.31	<0.05‡,<0.05§

TAC: Total Antioxidant Capacity, TOS: Total Oxidant Status, OSI: Oxidative Stress Index, PON: Paraoxonase, ARES: Arylesterase.
The variables are expressed as mean±standard deviation. † shows a statistically significant difference between non-diabetics and prediabetics. ‡ shows a statistically significant difference between non-diabetics and type 2 diabetics.§ shows a statistically significant difference between prediabetics and type 2 diabetics.

total thiol levels in T2Ds was significant (P<0.001) compared to NDs. A reduction of native thiol levels in prediabetics was significant (P<0.005) compared to NDs. The level of total thiol and native thiol decreased significantly in prediabetics compared to T2Ds (P<0.001). Disulphide levels increased significantly in prediabetics and T2Ds when compared to the NDs (P<0.001) (Table 2).

Antioxidant enzymes, PON1 and ARES, both decreased significantly (P <0.05) in T2Ds when compared to the control group. PON1 levels showed a slight, non-significant increase in prediabetics. On the other hand, ARES levels of prediabetics decreased when compared to NDs. The levels of both PON1 and ARES reduced significantly in T2Ds compared to prediabetics (P<0.001 and P <0.05, respectively). (Table 2).

Correlation analysis

In T2Ds, a positive significant correlation was determined between the HbA1c levels and native thiol, total thiol and disulphide levels (r=0.351, P <0.05; r=0.543, P =0.000; and r=0.624, P =0.000 respectively). Similar to this, a significant strong positive correlation was also noticed between TOS and native thiol, total thiol and disulphide levels (r=0.814, P =0.000; r=0.828, P =0.000 and r=0.546, P =0.000, respectively). A positive correlation between triglyceride levels and total thiol and disulphide was also found (r=0.340, P<0.05 and r=0.310, P<0.05, respectively). No correlation was found between the antioxidant enzymes (PON1, and ARES) and TDH parameters (Table 3).

In prediabetics, both triglyceride and total cholesterol levels were found to correlate with disulphide ratio to both native thiol and total thiol, as well as the ratio of native thiol to total thiol. A significant positive correlation was found between TAC and total thiol levels (r=0.457, P<0.05). A strong positive correlation between ARES and native thiol as well as total thiol levels was also determined (r=0.706, P =0.000 and r=0.525, P <0.05, respectively) (Table 4).

DISCUSSION

Insufficient insulin effects and/or insulin release causes T2DM and is characterized by hyperglycaemia. It is a chronic disease where micro and macro complications occur due to the deterioration of carbohydrate, lipid and protein metabolisms.

OS has been defined by an imbalance between the levels of free radicals and antioxidants within the body. This causes molecular and cellular dysfunction. The effects of OS have been investigated by several researchers in diabetics, mostly in T2Ds (Sozer et al., 2014; Eljaoudi et al., 2017; Nair & Nair, 2017).

Thiols, also known as mercaptans, are organic compounds with a sulfhydryl group. They serve as the component of a mitochondrial antioxidant defence mechanism. Under OS conditions, the thiol groups of the aminoacids with sulphur groups such as cysteine and methionine form reversible disulphide bonds with the low molecular weight thiol groups. This is known as dynamic TDH and has been investigated in several diseases such as Graves' disease (Agan et al., 2019), childhood iron deficiency anemia (Topal, et al., 2019), Welders' lung disease (Karatas et al., 2019), gestational diabetes (Aktun, Aykanat, Erel, Neselioglu, & Olmuscelik, 2018), neonatal sepsis (Aydogan et al., 2021), urolithiasis (Sonmez et al., 2019), etc. The abnormalities in dynamic TDH may have a role in the pathogenesis of diabetes mellitus. Therefore, we aimed to detect dynamic TDH in NDs, prediabetics and T2Ds.

PON1 and ARES are calcium-dependent antioxidant enzymes. They are both encoded by the same gene. The activities of both PON1 and ARES are shown to be reduced in several diseases. In patients with renal cell carcinoma, levels of PON-1 in advanced

Table 3. Correlation analysis of type 2 diabetics (Findings related to thiol/disulphide homeostasis).

	Native Thiol		Total Thiol		Disulphide		Disulphide/ Native Thiol		Disulphide/ Total Thiol		Native Thiol/ Total Thiol	
	r	p value	r	p value	r	p value	r	p value	r	p value	r	p value
BMI	0.256	0.089	0.375	0.011	0.367	0.013	0.190	0.211	0.190	0.211	-0.190	0.211
FBG	0.040	0.792	0.207	0.173	**0.376**	**0.011***	**0.394**	**0.007****	**0.394**	**0.007****	**0.394**	**0.007****
HbA1c	**0.351**	**0.018***	**0.543**	**0.000****	**0.624**	**0.000****	**0.322**	**0.031***	**0.322**	**0.031***	**-0.322**	**0.031***
Creatinine	-0.067	0.660	0.015	0.920	0.046	0.765	0.062	0.684	0.062	0.684	-0.062	0.684
Triglyceride	0.275	0.068	**0.340**	**0.022***	**0.310**	**0.038***	0.167	0.272	0.167	0.272	-0.167	0.272
Total cholesterol	0.110	0.471	0.160	0.293	0.199	0.189	0.160	0.292	0.160	0.292	-0.160	0.292
HDL	-0.174	0.253	-0.129	0.399	-0.144	0.344	-0.040	0.793	-0.040	0.793	0.040	0.793
LDL	0.032	0.836	0.069	0.651	0.141	0.356	0.129	0.399	0.129	0.399	-0.129	0.399
TAC	-0.265	0.078	-0.197	0.194	-0.016	0.917	0.056	0.716	0.056	0.716	-0.056	0.716
TOS	**0.814**	**0.000****	**0.828**	**0.000****	**0.546**	**0.000****	-0.039	0.799	-0.039	0.799	0.039	0.799
ARES	-0.181	0.459	-0.321	0.180	-0.169	0.488	-0.123	0.616	-0.123	0.616	-0.123	0.616
PON1	-0.133	0.589	-0.196	0.422	-0.035	0.886	0.004	0.986	0.004	0.986	0.004	0.986

BMI: Body Mass Index, FBG: Fasting Blood Glucose, HbA1c: Haemoglobin A1c, HDL: High-density Lipoprotein, LDL: Low-density Lipoprotein, TAC: Total Antioxidant Capacity, TOS: Total Oxidant Status, ARES: Arylesterase, PON: Paraoxonase.* means p<0.05, ** means p<0.01.

Table 4. Correlation analysis of prediabetics (Findings related to thiol/disulphide homeostasis).

	Native Thiol		Total Thiol		Disulphide		Disulphide/Native Thiol		Disulphide/Total Thiol		Native Thiol/Total Thiol	
	r	p value	r	p value	r	p value	r	p value	r	p value	r	p value
BMI	-0.386	0.062	0.095	0.659	**0.461**	**0.024***	**0.548**	**0.006****	**0.548**	**0.006****	**0.548**	**0.006****
FBG	-0.236	0.268	-0.003	0.987	0.168	0.434	0.234	0.271	0.234	0.271	0.234	0.271
HbA1c	-0.201	0.346	0.204	0.340	0.267	0.207	0.281	0.184	0.281	0.184	0.281	0.184
Creatinine	0.354	0.090	**0.423**	**0.040***	0.323	0.123	0.210	0.325	0.210	0.325	0.210	0.325
Triglyceride	0.110	0.608	**0.471**	**0.020***	**0.566**	**0.004****	**0.452**	**0.027***	**0.452**	**0.027***	**0.452**	**0.027***
Total cholesterol	-0.172	0.423	0.157	0.464	0.371	0.074	**0.408**	**0.048***	**0.408**	**0.048***	**0.408**	**0.048***
HDL	-0.243	0.252	-0.315	0.134	-0.182	0.393	-0.047	0.826	-0.047	0.826	-0.047	0.826
LDL	-0.189	0.377	0.094	0.663	0.265	0.211	0.310	0.140	0.310	0.140	0.310	0.140
TAC	0.370	0.075	**0.457**	**0.025***	0.403	0.051	0.147	0.494	0.147	0.494	0.147	0.494
TOS	-0.025	0.908	-0.172	0.421	-0.303	0.149	-0.294	0.163	-0.294	0.163	-0.294	0.163
ARES	**0.706**	**0.000****	**0.525**	**0.008****	0.048	0.823	0.066	0.759	-0.279	0.187	0.279	0.187
PON1	-0.362	0.082	-0.261	0.218	-0.110	0.609	-0.279	0.187	0.066	0.759	-0.066	0.759

BMI: Body Mass Index, FBG: Fasting Blood Glucose, HbA1c: Haemoglobin A1c, HDL: High-density Lipoprotein, LDL: Low-density Lipoprotein, TAC: Total Antioxidant Capacity, TOS: Total Oxidant Status, ARES: Arylesterase, PON: Paraoxonase.* means p<0.05, ** means p<0.

stage were found to be significantly lower than the lower stage patients. In contrast, ARES levels were found to correlate with nuclear grade in renal cell carcinoma patients (Aldemir et al., 2015). In localized scleroderma patients, decreased ARES levels were reported (Kilinc et al., 2016). In *pityriasis rosea* patients, TAC levels and ARES activities were found to be significantly lower in T2Ds than the NDs (Emre et al., 2016). In our study, ARES levels were also found to be lower than NDs when compared to prediabetics and T2Ds ($p<0.05$, Table 2). In prediabetics, TAC levels were also lower than both the NDs and T2Ds, but this was not significant. This may suggest a relationship between TAC and ARES values. Similar to our findings, ARES activity in the heart and liver homogenates was found to be significantly lower in the diabetic control group than the normal control group in rats ($P<0.01$) (Zarei et al., 2016). In another study, it was also stated that PON1 and ARES activities were decreased in streptozocin-induced diabetic rats and that vitamin B6 supplementation improved PON1 and ARES activities (Tas, Sarandol, & Dirican, 2014). In our study, PON1 levels of T2Ds significantly decreased compared to the NDs ($P<0.05$, Table 2). PON-1 levels were also found to be decreased in diabetics with periodontitis, but in contrast to our high levels of PON1 in prediabetics, they did not find any impaired PON1 status in prediabetics (Noack et al., 2013).

To our knowledge, this study firstly investigates TDH together with TAC, TOS, OSI and enzymatic parameters (PON1 and ARES) in NDs as well as in newly diagnosed prediabetics and T2Ds. There are not many studies investigating OS in prediabetics. In one study, OS biomarkers were detected in elderly (> 65 years of age) prediabetics (Dziegielewska-Gesiak et al., 2014) and in another study, only nine prediabetics were involved and an elevation in lipid peroxidation and superoxide dismutase activity in T2Ds were evaluated (Bandeira et al., 2012). Prediabetics were involved in this study to understand whether the OS conditions also occur in individuals with slightly elevated blood glucose levels without diabetic complications and whether TDH was also affected. A shift towards oxidised thiols is found to be a lot higher in prediabetics than T2Ds (Table 2). In a study performed by Ates et al. (2016), they also found dynamic TDH shifted towards disulphide form in type 1 diabetic patients. Also, for the first time in our study, a positive and significant ($P<0.005$) correlation was found between ARES levels and both native thiol and total thiol levels in prediabetics. In T2Ds no correlation was found between enzymatic antioxidants (PON1 and ARES) and thiol groups.

A strong positive correlation between TOS and native thiol, total thiol as well as disulphide levels in T2Ds was also found ($P<0.001$, Table 3). However, in prediabetics this correlation was negative and insignificant (Table 4).

The main limitation of our study was its cross-sectional design. The individuals' blood was taken at the time they came to the hospital to be checked for T2DM. For our newly diagnosed T2Ds, the onset of the disease is not known. In addition, our study is designed as a pilot study with a small sample size. Another limitation was not being able to evaluate the other enzymatic and non-enzymatic parameters of OS and, therefore, not being able to make comparisons with TDH. During the study period (between September 2018 and March 2019), mostly women (69 women and only 5 men) applied to the diabetes polyclinic to be tested for T2DM. Due to the low number, men were excluded from the study in order to provide a homogenous group in terms of gender.

In conclusion, the present study demonstrated that TDH is weakened in newly diagnosed T2Ds when compared to NDs and the balance shifted towards disulphide formation. In addition, related to an increase in TOS levels, oxidized thiols were also found to be increased significantly in T2Ds. Therefore, we believe that the occurrence of diabetic symptoms due to high blood sugar levels is related to the increase in TOS levels and, consequently, to TDH. This study revealed that TDH is an independent risk factor for T2Ds. OS has major effects in type 2 diabetes ethiopathogenesis. We believe that our study will enlighten the current literature as well as extended further studies that will help with the prognosis and cure of T2Ds.

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REFERENCES

- Agan, V., Celik, H., Eren, M. A., Agan, F. Z., Erel, O., Neselioglu, S. … Gonel, A. (2019). An investigation of oxidative stress and thiol/disulphide homeostasis in graves' disease. *Medicina (Kaunas)*, *55*, 275-287. http://dx.doi.org/10.3390/medicina55060275.
- Aktun, L. H., Aykanat, Y., Erel, O., Neselioglu, S., & Olmuscelik, O. (2018). A study over thiol disulphide homeostasis in cord blood in women with gestational diabetes. *Jounal of Family and Reproductive Health*, *12*, 217-222.
- Aldemir, M., Karaguzel, E., Okulu, E., Gudeloglu, A., Ener, K., Ozayar, A., & Erel, O. (2015). Evaluation of oxidative stress status and antioxidant capacity in patients with renal cell carcinoma. *Central European Journal of Urology*, *68*, 415-420. http://dx.doi.org/10.5173/ceju.2015.656.
- Ates, I., Kaplan, M., Inan, B., Alisik, M., Erel, O., Yilmaz, N., & Guler, S. (2015). How does thiol/disulphide homeostasis change in prediabetic patients? *Diabetes Research and Clinical Practice, 110*(2), 166–171. https://doi.org/10.1016/j.diabres.2015.09.011.
- Ates, I., Kaplan, M., Yuksel, M., Mese, D., Alisik, M., Erel, O. … Guler, S. (2016). Determination of thiol/disulphide homeostasis in type 1 diabetes mellitus and the factors associated with thiol oxidation. *Endocrine*, *51*, 47-51. http://dx.doi.org/10.1007/ s12020-015-0784-6.

- Aydogan, S., Akduman, H., Dilli, D., Koyuncu, E., Citli, R., Erel, O … Zenciroglu A. (2021). The role of thiol-disulphide homeostasis in neonatal sepsis. *The Journal of Maternal-Fetal & Neonatal Medicine*, *34*, 1522-1528. http://dx.doi.org/10.1080/14767058.2019.1638904.
- Bandeira, S., Guedes, G., da Fonseca, L. J., Pires, A. S., Gelain, D. P., Moreira, J. C. … Goulart, M. O. (2012). Characterization of blood oxidative stress in type 2 diabetes mellitus patients: increase in lipid peroxidation and SOD activity. *Oxidative Medicine and Cellular Longevity 2012*, 819310. https://doi.org/10.1155/2012/819310.
- Dziegielewska-Gesiak, S., Wysocka, E., Michalak, S., Nowakowska-Zajdel, E., Kokot, T., & Muc-Wierzgon M. (2014). Role of lipid peroxidation products, plasma total antioxidant status, and Cu-, Zn-superoxide dismutase activity as biomarkers of oxidative stress in elderly prediabetics. *Oxidative Medicine and Cellular Longevity*, *2014*, 987303. https://doi.org/10.1155/2014/987303.
- Eckerson, H.W., Wyte, C.M., & La Du, B.N. (1983). The human serum paraoxonase/arylesterase polymorphism. *The American Journal of Human Genetics, 35*, 1126–1138.
- Eljaoudi, R., Elomri, N., Laamarti, M., Cherrah, Y., Amezyane, T., Ghafir D, & Ibrahimi, A. (2017). Antioxidants status in type 2 diabetic patients in Morocco. *Turkish Journal of Medical Sciences*, *47*, 782-788. http://dx.doi.org/10.3906/sag-1512-110.
- Emre, S., Akoglu, G., Metin, A., Demirseren, D.D., Isikoglu, S., Oztekin, A. & Erel, O. (2016). The oxidant and antioxidant status in pityriasis rosea. *Indian Journal of Dermatology*, *61*, 118-138. http://dx.doi.org/10.4103/0019-5154.174073.
- Erel, O. (2004). A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clinical Biochemistry*, *37*, 277-285. http://dx.doi.org/10.1016/j.clinbiochem.2003.11.015.
- Erel, O. (2005). A new automated colorimetric method for measuring total oxidant status. *Clinical Biochemistry*, *38*, 1103-1111. http://dx.doi.org/10.1016/j.clinbiochem.2005.08.008.
- Erel, O., & Neselioglu, S. (2014). A novel and automated assay for thiol/disulphide homeostasis. *Clinical Biochemistry*, *47*, 326–332. http://dx.doi.org/10.1016/j.clinbio chem.2014.09.026.
- Erel, O., & Erdogan, S. (2020). Thiol-disulphide homeostasis: an integrated approach with biochemical and clinical aspects. *Turkish Journal of Medical Sciences, 50*(SI-2), 1728-1738. http://dx.doi.org/10.3906/sag-2003-64.
- Ergin, M., Aydin, C., Yurt, E.F., Cakir, B., Erel, O. (2020). The variation of disulphides in the progression of type 2 diabetes mellitus. *Experimental and Clinical Endocrinology & Diabetes, 128* (2), 77-81. http://dx.doi.org/10.1055/s-0044-100376.
- Garber, A.J., Abrahamson, M.J., Barzilay, J.I., Blonde, L., Bloomgarden, Z.T., Bush, M.A. … Umpierrez,G.E. (2019). Consensus Statement by The American Association of Clinical Endocrinologists and American College of Endocrinology on the Comprehensive Type 2 Diabetes Management Algorithm - 2019 Executive Summary. *Endocrinology Practice*, *25*, 69-100. http://dx.doi.org/10.4158/CS-2018-0535.
- Gulpamuk, B., Tekin, K., Sonmez, K., Inanc, M., Neselioglu, S… .Yilmazbas, P. (2018). The significance of thiol/disulphide homeostasis and ischemia- modified albumin levels to assess the oxidative stress in patients with different stages of diabetes mellitus. *Scandinavian Journal of Clinical Laboratory Investigation, 78*, 136-142. http://dx.doi.org/10.1080/003655132017.
- 1422540.
- Hamamcioglu, A.C. (2017). The role of oxidative stress and antioxidants in diabetes mellitus. *Turkish Journal of Diabetes and Obesity*, *1*, 7-13. http://dx.doi.org/10.25048/tjdo.2017.2.
- Harma, M., Harma, M., & Erel, O. (2003). Increased oxidative stress in patients with hydatidiform mole. *Swiss Medical Weekly*, *133*, 563-566.
- Karatas, M., Ozis, T. N., Buyuksekerci, M., Gunduzoz, M., Ozakıncı, O. G., … Erel, O. (2019). Thiol-disulphide homeostasis and ischemia-modified albumin levels as indicators of oxidative stress in welders' lung disease. *Human & Experimental Toxicology*, *38*, 1227-1234. http://dx.doi.org/10.1177/0960327119871093.
- Khetan, A. K., & Rajagopalan, S. (2018). Prediabetes. *Canadian Journal of Cardiology*, *34*, 615-623. http://dx.doi.org/10.1016/j.cjca.2017.12.030
- Kilinc, F., Sener, S., Akbas, A., Metin, A., Kirbas, S., Neselioglu S., & Erel, O. (2016). Oxidative stress parameters in localized scleroderma patients. *Archives of Dermatological Research*, *308*, 625-629. http://dx.doi.org/10.1007/s00403-016-1682-3.
- Kosecik, M., Erel, O., Sevinc, E., & Selek, S. (2005). Increased oxidative stress in children exposed to passive smoking. *International Journal of Cardiology*, *100*, 61–64. http://dx.doi.org/10.1016/j.ijcard.2004.05.069.
- Lovic, D., Piperidou, A., Zografou, I., Grassos, H., Pittaras, A., & Manolis, A. (2020). The growing epidemic of diabetes mellitus. *Current Vascular Pharmacology*, *18*, 104-109. http://dx.doi.org/10.2174/1570161117666190405165911.
- Nair, A., & Nair, B. J. (2017). Comparative analysis of the oxidative stress and antioxidant status in type II diabetics and nondiabetics: A biochemical study. *Journal of Oral and Maxillofacial Pathology*, *21*, 394-401. http://dx.doi.org/10.4103/jomfp. JOMFP_56_16.
- Noack, B., Aslanhan, Z., Boué, J., Petig, C., Teige, M., Schaper, F. … Hannig, C. (2013). Potential association of paraoxonase-1, type 2 diabetes mellitus, and periodontitis. *Journal of Periodontology*, *84(5)*, 614–623. https://doi.org/10.1902/jop.2012.120062.
- Rosenblat, M., Sapir, O., & Aviram, M. (2008). The paraoxonases: their role in disease development and xenobiotic metabolism. In B. Mackness, M. Mackness, M. Aviram & G. Paragh (Eds.), Glucose inactivates Paraoxonase 1 (PON1) and displaces it from high density lipoprotein (HDL) to a free PON1 form (pp.35-51). New York, USA: Springer.
- Sies, H. (1997). Oxidative stress: oxidants and antioxidants. *Experimental Physiology*, *82*, 291-295. http://dx.doi.org/10.1113/expphysiol.1997.sp004024.
- Sonmez, M. G., Kozanhan, B., Deniz, C. D., Iyisoy, M. S., Kilinc, M. T., Ecer, G. … Erel, O. (2019). Dynamic thiol/disulphide homeostasis as a novel indicator of oxidative stress in patients with urolithiasis. *Investigative and Clinical Urology*, *60*, 258-266. http://dx.doi.org/10.4111/icu.2019.60.4.258.
- Sozer, V., Himmetoglu, S., Korkmaz, G. G., Kaya, S., Aydin, S., Yumuk, V. … Uzun, H. (2014). Paraoxonase, oxidized low density lipoprotein, monocyte chemoattractant protein-1 and adhesion molecules are associated with macrovascular complications in patients with type 2 diabetes mellitus. *Minerva Medica*, *105*, 237-244.
- Tas, S., Sarandol, E., & Dirican, M. (2014). Vitamin B6 supplementation improves oxidative stress and enhances serum paraoxonase/arylesterase activities in streptozotocin-induced diabetic rats. *Scientific World Journal,2014*, 351598. https://doi.org/10.1155/2014/351598.
- Topal, I., Mertoglu, C., Surucu Kara, I., Gok, G., & Erel, O. (2019). Thiol-disulphide homeostasis, serum ferroxidase activity, and serum ischemia modified albumin levels in childhood iron deficiency anemia. *Fetal and Pediatric Pathology*, *38*, 484-489. http://dx.doi.org/10.1080/15513815.2019.1627626.
- Unal, E., Eris, C., Kaya, B., Uzun, H., Cavdar, F., …,Titiz, I. (2012). Paraoxonase and arylesterase activities, lipid profile, and oxidative damage in experimental ischemic colitis model. *Gastroenterology Research and Practice*, *2012*, 2012-2017. https://doi.org/10.1155/2012/979506.
- Wu, G., Fang, Y-Z., Yang, S., Lupton, J.R., & Turner, N.D. (2004). Glutathione Metabolism and Its Implications for Health. *The Journal of Nutrition*, *134*(3), 489–492. https://doi.org/10.1093 /jn/134.3.489.

- Yumru, M., Savas, H. A., Kalenderoglu, A., Bulut, M., Celik, H., & Erel, O (2009). Oxidative imbalance in bipolar disorder subtypes: a comparative study. *Progress in Neuropsychopharmacology & Biological Psychiatry*, *33*, 1070-1074. http://dx.doi.org/10.1016/j.pnpbp.2009.06.005.
- Zarei, M., Fakher, S., Tabei, S. M., Javanbakht, M. H., Derakhshanian, H., …Djalali, M. (2016). Effects of vitamin A, C and E, or omega-3 fatty acid supplementation on the level of paraoxonase and arylesterase activity in streptozotocin-induced diabetic rats: an investigation of activities in plasma, and heart and liver homogenates. *Singapore Medical Journal*, 57, 153-156. https://doi.org/10.11622/smedj.2015102.

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Original Article

Effects of the CASR rs104893706 (A843E) gain-of-function mutation on bone mineral density in postmenopausal women by advanced age and smoking

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ABSTRACT

Background and Aims: The G protein-coupled calcium-sensing receptor (CaSR) plays an important role in extracellular calcium homeostasis and regulation of parathyroid hormone (PTH) secretion. There are more than 300 activating/inactivating mutations in the *CASR* gene. However, the effects of both CaSR protein and *CASR* gene on bone mineral density (BMD) have not been investigated enough. The aim of this study was therefore to determine the effect of rs104893706 (A843E, (Ala to Glu at codon843)), a gain-of-function mutation of the *CASR* gene, on BMD in postmenopausal women.
Methods: We studied the CASR A843E variation using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism methods in 180 postmenopausal women. Statistical analyses were performed using SPSS software package (version 21.0 SPSS Inc.,Chicago,IL,U.S.A.).
Results: No minor A allele, homozygous (AA) or heterozygous (CA), was observed in the study population. In other words, the frequency of the *CASR* A843E common CC genotype was 100%. BMD levels of the lumbar spine (L1-L4), femoral neck, and total hip were 1.03±0.13g/cm^2, 0.87±0.11g/cm^2, and 0.93±0.11g/cm^2, respectively. Although the femoral neck (p=0.017), upper neck (p=0.040), lower neck (p=0.011), and Ward's triangle BMD values (p=0.005) were found significantly higher in younger postmenopausal women (age<55 years) compared to older postmenopausal women (age>=55 years), there were no significant differences on BMD value of the lumbar spines, trochanter, and total hip between the age groups (p>0.05).
Conclusion: Our findings confirm the effects of advanced age in favor of decreased BMD in postmenopausal women. This study suggests that the *CASR* A843E gain-of-function mutation may not be associated with bone mineral density and osteoporosis risk since we did not detect the A843E variation in Turkish postmenopausal women.

Keywords: Osteoporosis, Calcium-sensing receptor, Gain-of-function mutation, Bone mineral density, Smoking

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INTRODUCTION

Osteoporosis is characterized by decreased bone mineral density (BMD) and a high risk of fractures. It is especially prevalent among postmenopausal women due to estrogen deficiency (North American Menopause Society, 2010). Human bones are composed of bone minerals and bone matrix, the main components of which are calcium and collagen, respectively. Collagen degradation and a decrease in levels of calcium are key contributors to osteoporosis development (Li, Zhao, Tang, & Qu, 2010; NIH Consensus Development Panel on Osteoporosis, 2001). Additionally, advanced age, genetic factors, and smoking are associated with osteoporosis development (Bjarnason & Christiansen, 2000; Rapuri, Gallagher, Balhorn, & Ryschon, 2000).

It is crucial to maintain calcium balance for the structural integrity of the bone, however, the plasma calcium level is highly affected by environmental and physiological conditions (North American Menopause Society, 2010). The total body calcium balance is regulated by the absorption of calcium from the intestine and its excretion in the urine. When calcium levels increase in blood, the calcium-sensing receptor (CaSR) inhibits the secretion of parathyroid hormone (PTH) and reabsorption of calcium in renal tubules (Brown, 1999; Brown & MacLeod, 2001). Calcium level is decreased by the inhibition of PTH secretion, increasing urinary calcium excretion, and increasing calcitonin secretion due to the effect of extracellular calcium on CaSR. (Brown & Hansen, 2005; Hannan et al., 2018). CaSR, a seven-transmembrane-spanning extracellular G-protein-coupled receptor, is primarily expressed in the parathyroids and the kidney. It can also monitor the extracellular calcium in the body and regulate calcium metabolism (Brown, Gamba, & Riccardi, 1993; Garrett et al., 1993). There are three domains in CaSR; i) a large extracellular domain that interacts with Ca^{+2}, ii) a transmembrane domain with seven helixes among the membrane, and iii) an intracellular domain with carboxyl-terminal that activates some signal pathways of the cell (Garrett et al., 1993; Aida, Koishi, Tawata, & Onaya, 1995). After binding Ca^{2+} to CaSR, phospholipase C is activated by Gq/11. Second messengers such as diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) are produced. IP3 releases Ca^{2+} from intracellular stores and DAG activates protein kinase C (PKC) and the mitogen-activated protein kinase (MAPK) pathway. Also, the CaSR stimulates the Gi/o protein and then cAMP production is inhibited. These signaling pathways give rise to a reduction in PTH secretion and a decrease in renal tubular Ca^{2+} reabsorption (Hannan et al., 2018).

More than 300 *CASR* gene mutations have been identified related to the activation or inactivation of the CaSR receptor (Hend, Guarnieri, & Canaff, 2009). There are more than 95 different *CASR* mutations called activating mutations (gain-of-function). These mutations generally exist in the extracellular domain .However, they can also occur in the transmembrane domain. Some activated *CASR* mutations have been associated with autosomal dominant hypocalcemia (ADH) and Bartter Syndrome Type V (Thakker, 2004; Regala, Cavaco, Domingues, Limbert, & Lopes, 2015). A novel missense heterozygous *CASR* gene mutation, a leucine substitution for serine at codon 123 (p.Leu123Ser), was identified in a male with ADH (Regala et al., 2015). rs104893706, a constitutive A843E mutation of the *CASR* gene in exon7 is a substitution that involves a cytosine to adenine at nucleotide 2528, resulting in a substitution of Alanine (A, with C allele) to Glutamic acid (E, with A allele) in codon 843 (Zhao, Hauache, Goldsmith, Collins, & Spiegel, 1999; Sato et al., 2002). This mutation results in conformational changes in transmembrane domain 7 and gives a novel function to the receptor. A843E mutation in the *CASR* gene has been associated with high basal activity in absence of calcium and the ligand. The A843E mutation most likely stabilizes the active state of the receptor (Zhao et al., 1999).

There are numerous investigations focusing on the association of *CASR* gene variations with hyperparathyroidism (Yamauchi, 2001; Jeong et al., 2016), chronic kidney disease (Guha et al., 2015), hypertension (Jung et al., 2009), and breast cancer (Li et al., 2014). Many studies have focused on A986S loss-of-function mutation in exon 7 of the *CASR* gene (Lorentzon, Lorentzon, Lerner, & Nordstrom, 2001; Takács et al., 2002; Young et al., 2003), which is an alanine substitution to serine in codon 986. The association of the S allele with increased serum calcium levels due to decreased urinary calcium excretion indicates that this polymorphism may affect extracellular calcium levels (Young et al., 2003). There are only a few studies focusing on the association between *CASR* A986S loss-of-function mutation and osteoporosis (Lorentzon et al., 2001; Takács et al., 2002; Young et al., 2003). In a study conducted on premenopausal and postmenopausal Iraqi women, A986S was reported to be associated with osteoporosis risk and it was also reported to have an effect on mineral levels (Al-Azzawie, 2001). In contrast, no association was found between BMD values and *CASR* A986S genotypes between osteoporosis and control groups in Hungarian postmenopausal women (Takács et al., 2002). However, the effects of the *CASR* gain-of-function mutations on osteoporosis development have not been well studied in the literature. For this reason, our aim in this study was to investigate the effect of the *CASR* A843E gain-of-function mutation on osteoporosis and to determine the effects of this variation on BMD comparatively depending on the presence of the postmenopausal period.

MATERIAL AND METHODS

Selection and description of participants

One hundred and eighty Turkish postmenopausal women (57.53±7.44 mean age) were included in the study. Participants were selected from the Uskudar State Hospital in Istanbul, Turkey. Inclusion criteria for the selection of the study group were determined as being in the post-menopausal period with the absence of a menstruation period for at least a year. All participants underwent a standard inquiry including queries about the risk factors for osteoporosis (such as menopausal condition and age, smoking, alcohol consumption, family history of osteoporosis, drugs, and other medical conditions). Demographic and morphometric features were also noted. Participants having conditions or medications known to affect metabolisms of bone, such as malignities, endocrine diseases (hyper-

thyroidism, hypo/hyperparathyroidism, Cushing's syndrome), drastic liver or gastroenteric diseases, bone diseases (arthritis, Paget's disease, osteomalacia, and osteogenesis imperfecta), corticosteroids, androgenic-anabolic steroids, female steroid sex hormones, estrogen-related molecules or anticonvulsives were excluded from the study. The study protocol was approved by the Local Ethical Committee of Istanbul University, Istanbul Medical Faculty Protocol No: 2006/2145). Informed written permission was obtained from all participants before the collection of blood samples.

BMD measurement

During the scanning procedure, participants were stationed motionlessly in the scanner per standard procedures. BMD values of the lumbar spine (L1–L4) and hip (femoral neck and total hip) were evaluated by GE-Lunar DPX Pro (GE Healthcare, Madison, WI, USA) Pencil Beam DXA densitometer. All DEXA scans were analyzed using software (encore version 2005, 9.30.044) provided by the manufacturer.

PCR-Based Detection of the *CASR* A843E mutation

Genomic DNA was extracted from the blood by salting-out procedure (Miller et al., 1998). A843E (rs104893706) C>A in exon 7 of *CASR*, an activating mutation, was determined by carrying out a polymerase chain reaction (PCR) at a thermal cycler (Biorad T100 Thermal Cycler). The specific primers for rs104893706 C>A mutation (the forward primer sequence: 5'-GCCTTCAAGTCCCGGAAGCTGC-3', and reverse primer sequence: 5'-CGGTGCTGCAACGCACCTCCTC-3') were designed by in silico method and used in PCR. PCR reactions were carried out in a total volume of 25 µl containing 150-200 ng genomic DNA, sterile deionized water, 10xTaq polymerase buffer with $(NH4)_2SO_4$ (MBI Fermentas), 25mM $MgCl_2$ (MBI Fermentas), 5 mM deoxynucleotide triphosphates (MBI Fermentas), 50 pmol/µl of each primer (IDT DNA Technologies) and 1.5 U Taq DNA polymerase (MBI Fermentas). PCR cycling conditions were as follows: an initial denaturing step for 5 minutes at 94°C, an amplification step including 35 cycles of denaturation (45 seconds at 94°C), annealing (45 seconds at 61°C), and extension (45 seconds at 72°C), followed by a final extension step for 5 minutes at 72°C. The A843E PCR products (259 bp) were directly digested with BglI restriction enzyme (10units/µl) (Promega), and the digested DNA fragments were characterized on 3% agarose gel in 1X Tris-borate-EDTA buffer by gel electrophoresis. Results were analyzed considering that a single fragment of 259 bp was the A allele, and two fragments of 154 bp and 105 bp were the C allele.

Statistics

Statistical analyses were performed by the SPSS software package (version 21.0 SPSS Inc., Chicago, IL, U.S.A.). Clinical parameters were given as mean ± SD. Mean values were compared between the groups using the unpaired Student's t-test. Values of $p<0.05$ were accepted as statistically significant.

RESULTS

The characteristics and bone mineral density (BMD) values of the subjects are shown in Table 1. In our study group consisting of postmenopausal women, the frequency of the *CASR* A843E common CC genotype was 100%, that is, rare A allele (AA and CA genotypes) was not observed.

Table 1. Characteristics and BMD values of the study population.

	Study population (n=180)	Min.	Max.
Age (year)	57.53±7.44	44	80
Age of menopause (year)	47.10±4.56	30	56
BMI (kg/m^2)	30.75±4.92	20.17	46.11
Lumbarspine L1 BMD (g/cm^2)	0,95±0.14	0.71	1.56
Lumbarspine L2 BMD (g/cm^2)	1.02±0.13	0.76	1.75
Lumbarspine L3 BMD (g/cm^2)	1.07±0.15	0.71	1.76
Lumbarspine L4 BMD (g/cm^2)	1.06±0.16	0.68	1.66
Lumbarspine L1-L2 BMD (g/cm^2)	0.98±0.13	0.74	1.67
Lumbarspine L1-L3 BMD (g/cm^2)	1.03±0.13	0.75	1.70
Lumbarspine L1-L4 BMD (g/cm^2)	1.03±0.13	0.76	1.69
Lumbarspine L2-L3 BMD (g/cm^2)	1.04±0.14	0.76	1.76
Lumbarspine L2-L4 BMD (g/cm^2)	1.05±0.14	0.74	1.72
Lumbarspine L3-L4 BMD (g/cm^2)	1.06±0.15	0.70	1.71
Femoral neck BMD (g/cm^2)	0.87±0.11	0.61	1.26
Upper neck BMD (g/cm^2)	0.72±0.11	0.51	1.09
Lower neck BMD (g/cm^2)	1.01±0.11	0.79	1.42
Ward's triangle BMD (g/cm^2)	0.70±0.12	0.44	1.08
Trochanter BMD (g/cm^2)	0.76±0.11	0.50	1.12
Shaft BMD (g/cm^2)	1.01±0.17	0.27	1.57
Total hip BMD (g/cm^2)	0.93±0.11	0.69	1.27

BMI: Body mass index, BMD: Bone mineral density. The values were given as means ± SD.

The percentage of smokers in the study group was 8.88% . The mean age of the smokers was 52.69±4.54, while the mean age of the nonsmokers was 58.00±7.51 (p=0.001). The percentage of smokers was 43.8% in the subgroup of postmenopausal women aged 55 years or over, while that of smokers in the subgroup of postmenopausal women younger than 55 years old was 56.3% (p=0.136) (Data not shown).

The subjects were placed into two groups according to their age (classified as age≥55 vs. ages<55) and smoking status (smoking vs. non-smoking). It was observed that the BMD values of the femoral neck (p=0.017), upper neck (p=0.040), lower neck (p=0.011), and Ward's triangle (p=0.005) were significantly higher in postmenopausal women younger than 55 years old compared to postmenopausal women aged 55 and older. However, there was no significant difference between age groups in terms of BMD values of lumbar vertebra, trochanter, and total hip (p>0.05). Also, there were no differences in BMD values between the smoking and non-smoking groups (Table 2).

Table 2. Effects of smoking and age on critical BMD values in postmenopausal women

	AGE			SMOKING		
	Age<55	**Age≥55**	**p-value**	**(-)**	**(+)**	**p-value**
L1 BMD (g/cm²)	0.96±0.12	0.94±0.14	0.271	0.95±0.14	0.93±0.12	0.685
L2 BMD (g/cm²)	1.03±0.13	1.00±0.14	0.347	1.01±0.13	1.04±0.14	0.489
L3 BMD (g/cm²)	1.08±0.14	1.06±0.15	0.624	1.07±0.15	1.10±0.12	0.401
L4 BMD (g/cm²)	1.06±0.15	1.06±0.16	0.978	1.06±0.16	1.04±0.14	0.668
L1-L2 BMD (g/cm²)	0.99±0.12	0.98±0.13	0.265	0.98±0.13	0.99±0.13	0.864
L1-L3 BMD (g/cm²)	1.03±0.13	1.00±014	0.373	1.01±0.13	1.03±0.12	0.671
L1-L4 BMD (g/cm²)	1.04±0.13	1.02±0.14	0.452	1.03±0.14	1.03±0.12	0.862
L2-L3 BMD (g/cm²)	1.05±0.13	1.04±0.14	0.482	1.04±0.14	1.07±0.13	0.452
L2-L4 BMD (g/cm²)	1.05±0.13	1.04±0.15	0.674	1.05±0.14	1.06±0.12	0.760
L3-L4 BMD (g/cm²)	1.07±0.14	1.06±0.15	0.809	1.06±0.15	1.07±0.12	0.848
Femoral neck BMD (g/cm²)	0.89±0.12	0.85±0.10	**0.017***	0.87±0.11	0.85±0.10	0.534
Upper neck BMD (g/cm²)	0.74±0.13	0.70±0.10	**0.040***	0.72±0.11	0.70±0.10	0.489
Lower neck BMD (g/cm²)	1.04±0.12	1.00±0.10	**0.011***	1.01±0.11	1.01±0.10	0.948
Ward's triangle BMD (g/cm²)	0.73±0.12	0.68±0.12	**0.005***	0.70±0.13	0.68±0.09	0.478
Trochanter BMD (g/cm²)	0.76±0.12	0.76±0.09	0.897	0.76±0.10	0.73±0.13	0.236
Shaft BMD (g/cm²)	1.14±0.18	1.10±0.17	0.128	1.12±0.07	1.06±0.18	0.288
Total hip BMD (g/cm²)	0.95±0.13	0.92±0.09	0.193	0.93±0.11	0.91±0.12	0.410

BMD: Bone Mineral Density. BMD values were given as Mean ± SD.

DISCUSSION

Bone mineral density (BMD), which is regulated by fine-tuned metabolic control, is also under genetic control (Li et al., 2010; Zhang et al., 2016; Kuo, Chang, Chi, & Chu,2008). It is known that there is a positive correlation between decreased BMD levels and increased fracture risk (Hend et al., 2009). Acquired bone mass in adolescence and young adulthood partially determines the risk of developing osteoporosis and suffering from resulting fractures (Lorentzon et al., 2001). Although 60-80% of the age-specific changes in BMD are linked with genetic factors, the specific genes affecting BMD have not yet been clearly described (Lorentzon et al., 2001). Many studies have investigated the genes responsible for vitamin D receptor (VDR), the estrogen receptor (ER), and the collagen receptor (COL1A1) as candidates for osteoporosis (Ioannidis et al., 2002; Willing et al., 1998; Xie et al., 2015). Among these, the variations of the VDR gene affecting calcium levels were found to be associated with BMD levels and osteoporosis (Kurt et al., 2012). Another receptor that affects calcium metabolism is the calcium-sensing receptor (CaSR) (Wang et al., 2006). It was hypothesized that mutations in the *CASR* gene were associated with BMD levels and osteoporosis risk, however, more evidence is needed for confirmation as the results of existing studies examining certain variations were contradictory.

More than three hundred mutations/polymorphisms associated with loss/ gain-of-function in the *CASR* gene have been identified (Hend et al., 2009). These variations have been investigated in different patient groups having different diseases including hypoparathyroidism/hyperparathyroidism, chronic kidney disease, and hypertension (Yamauchi et al., 2001; D'Souza-Li, 2006; Jung et al., 2009; Guha et al., 2015; Jeong et al., 2016). Heterozygous activating mutations of the *CASR* gene have been described in autosomal dominant hypocalcemia (ADH), which is associated with hypoparathyroidism or Bartter syndrome subtype V (Egbuna & Brown, 2008). On the other hand, heterozygous inactivating mutations of the *CASR* gene have been associated with familial hypocalciuric hypercalcemia type 1. Additionally, homozygous inactivating *CASR* mutations have been associated with hypercalcemia in neonatal severe primary hyperparathyroidism (Egbuna & Brown, 2008).

The investigation of the relationship between the *CASR* gene and BMD values has mostly been limited to the inactivating A986S variation. Even though there are controversial findings related to the relationship between *CASR* A986S genotypes and osteoporosis risk in humans (Cetani et al., 2003; O'Seaghdha et al., 2010; Eller-Vainicher et al., 2014), many studies have implied an association between *CASR* A986S genotypes and BMD (Lorentzon et al., 2001; Takács et al., 2002; Young et al., 2003; Donáth et al., 2004). In a study on healthy adolescent girls, it was reported that the A986S polymorphism of the *CASR* gene was associated with circulating calcium levels and BMD (Lorentzon et al., 2001). Eller-Vainicher et al. (2014) suggested that the probability of vertebral fracture increased more than four times in Caucasian primary hyperparathyroidism patients with *CASR* 986S genotype, after adjusting for factors including age, lumbar spine, and serum calcium levels (Eller-Vainicher et al., 2014). In a genome-wide association study (GWAS) conducted on this subject, it was found that the *CASR* A986S polymorphism was associated with lower lumbar spine BMD level, but not with the femoral neck (O'Seaghdha et al., 2010). On the contrary, the *CASR* gene A986S variation was not associated with BMD and osteoporotic fractures in Italian and Hungarian postmenopausal women (Takács et al., 2002; Cetani et al., 2003). This finding may be explained by the fact that the CaSR does not play an important role in the regulation of osteoblast function (Takács et al., 2002). Additionally, an osteoblastic extracellular cation-sensing mechanism, different than CaSR, has also been identified (Pi, Garner, Flannery, Spurney, & Quarles, 2000). It is important to note that the A986S effect on bone could be modified by the osteoblastic CaSR-like receptor (Takács et al., 2002).

While the association of BMD with the loss of function mutations has been examined in many studies, the BMD relationship with gain-of-function mutations has not been studied extensively. The most common gain-of-function mutations in the *CASR* gene are R990, A843E, S122C, P569H, and I839T. The gain-of-function *CASR* mutations such as L125P, C131W, and A843E have been identified in patients with Bartter Syndrome (Pi et al., 2000) and the functional studies show that these mutations resulted in more pronounced receptor activation than other known gain-of-function mutations (Brown & MacLeod, 2001). To the author's knowledge, the present study is the first article investigating whether there is an association between gain-of-function mutation A843E of the *CASR* gene and osteoporosis.

Watanabe et al. (2002) reported that the A843E mutation caused inhibition of renal external medullary potassium channel activity in two patients with hypocalcemia, insufficient parathyroid hormone secretion, and Bartter syndrome. Kinoshita et al. (2014) showed that the PTH and serum Mg^{+2} levels were lower, and fractional excretion of Mg^{+2} was increased in 12 autosomal dominant hypocalcemia patients with A843E mutations. Since we did not observe the *CASR* A843E mutation in our study group, we could not analyze the combined effects of the mutation on both osteoporosis risk and risk factors in postmenopausal women.

BMD values of the femoral upper, lower neck, and Ward's triangle were higher in younger postmenopausal women compared to older women. It was reported that calcium absorption may be defective in smokers (Zhang et al., 2016). However, the mechanisms related to the negative effect of smoking on bone mass have not yet been identified (Zhang et al., 2016). Both the status and duration of smoking have been reported to have deleterious effects on the bone mineral density of the lumbar spine (Brown & MacLeod, 2001). It was reported that women who smoke have lower bone mass and tend to lose bone faster than non-smokers. Also, postmenopausal women who smoked were reported to have significantly higher fracture rates than non-smokers (Zhang et al., 2016). Our findings did not confirm the negative effects of smoking on bone density. This different finding could be related to the low rate of smoking in the study group.

An age-dependent decrease in BMD in women occurs mostly after menopause (Brown & MacLeod, 2001). Menopause related to estrogen deficiency stimulates the expedition of bone loss due to age (Nuti et al., 2019). Age is a notable risk factor for fracture, specifically hip fracture. Hip fracture risk increases fourfold between ages 55 and 85 depending on BMD (North American Menopause Society, 2010). We therefore divided our study group accordingly into two subgroups: younger (aged under 55 years old) and older (aged 55 years old or over) women. In the present study, BMD values of the femoral neck, upper neck, lower neck, and Ward's triangle were lower in older than in younger postmenopausal women. There was no significant difference found between the age groups and the BMD values of the total hip, lumbar spines, and trochanter ($p>0.05$). Our findings showed that age was a risk factor for osteoporosis in postmenopausal women. It is important to note that hormonal changes may also affect osteoporosis along with age, especially in postmenopausal terms.

A843E mutation (rs104893706, C>A) of the *CASR* gene is responsible for alanine to glutamate substitution which affects the inactive conformation of the CaSR. A843E was also associated with high basal activity, and the activation of the receptor in the absence of calcium (Zhao et al.,1999). Mechanistically, receptor activation due to gain-of-function mutation resulted in a decrease in calcium reabsorption and an increase in calcium excretion. In the NCBI Alfa Allele Frequencies database (https://www.ncbi.nlm.nih.gov/snp/rs104893706?horizontal_tab=true#frequency_tab), the minor allele frequency (MAF) of the rs104893706 is very low in different populations around the world (A=0.00001 (1/78682, PAGE_STUDY) and A=0.000 (0/660, ALFA)). In the present study, our findings related to *CASR* A843E gain-of-function mutation were consistent with the reported findings in the NCBI Alfa Allele Frequencies database (https://www.ncbi.nlm.nih.gov/snp/rs104893706?horizontal_tab=true#frequency_tab). Our findings detected a similar allele distribution of the rs104893706 in the Turkish population as in other populations. The limitation was the small number of samples (n=180). Because allele frequencies were so low, the findings of this study offer new information for the Turkish population.

CONCLUSION

In conclusion, since the *CASR* gene A843E mutation was not observed in the selected population, our findings showed that BMD

levels were affected by age. However, the present study suggests that the variations in the *CASR* gene should be further investigated by DNA sequencing which will lead to a comprehensive understanding of the *CASR* gene as it relates to the risk of osteoporosis and BMD levels in Turkish postmenopausal women.

Ethics Committee Approval: The study protocol was approved by the Local Ethical Committee of Istanbul University, Istanbul Medical Faculty Protocol No: 2006/2145). Informed written permission was obtained from all participants before the collection of blood samples.

Peer-review: Externally peer-reviewed.

Informed Consent: Written consent was obtained from the participants.

Author Contributions: Conception/Design of Study- Ö.K.G.; Data Acquisition- Ö.K.Ş., M.U., A.C., A.B.T., F.Y., E.I.A.; Data Analysis/Interpretation- Ö.KG., H.Y.A.; Drafting Manuscript- Ö.K.G.; Critical Revision of Manuscript- Ö.K.G., H.Y.A.; Final Approval and Accountability- Ö.K.Ş., M.U., A.C., A.B.T., F.Y., E.I.A., Ö.K.G., H.Y.A.

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REFERENCES

- Aida, K., Koishi, S., Tawata, M., & Onaya, T. (1995). Molecular cloning of a putative Ca2+-sensing receptor cDNA from human kidney. *Biochemical and Biophysical Research Communications,* 214(2), 524–529.
- Al-Azzawie, A. F. (2001). Molecular assessment of calcium-sensing receptor gene polymorphism rs1801725 in Iraqi women with osteoporosis, *Asian Journal of Agriculture and Biology*, *3*, 1-9. https://doi.org/10.35495/ajab.2020.04.252
- Bjarnason, N. H., & Christiansen, C. (2000). The influence of thinness and smoking on bone loss and response to hormone replacement therapy in early postmenopausal women. *Journal of Clinical Endocrinology & Metabolism,* 85(2), 590–596. https://doi.org/10.1210/jcem.85.2.6364
- Brown, E. M., Gamba, G., & Riccardi, D. (1993). Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. *Nature*, 366, 575-580.
- Brown, E. M., & Hansen, J. T. (2005). The calcium-sensing receptor in normal physiology and pathophysiology: a review. *Critical Reviews in Clinical Laboratory Sciences*, 42(1), 35–70.
- Brown, E. M., & MacLeod, R. J. (2001). Extracellular calcium sensing and extracellular calcium signaling. *Physiological Reviews*, 81(1), 239-297.
- Brown, E. M. (1999). Physiology and pathophysiology of the extracellular calcium-sensing receptor. *American Journal of Medicine*, 106(2), 238-253.
- Cetani, F., Pardi, E., Borsari, S., Vignali, E., Dipollina, G., Braga, V., … Marcocci, C. (2003). Calcium-sensing receptor gene polymorphism is not associated with bone mineral density in Italian postmenopausal women. *EuropeanJournal of Endocrinology,148*, 603–607.
- D'Souza-Li, L. (2006). The calcium-sensing receptor and related diseases. *Arquivos Brasileiros de Endocrinologia e Metabologia*, 50, 628–636. doi:10.1111/j.1365-2036.2011.04905.x
- Donáth, J., Speer, G., Poór, G., Gergely, P., Tabák, Jr. A., Lakatos, P. (2004). Vitamin D receptor, oestrogen receptor-α and calcium-sensing receptor genotypes, bone mineral density and biochemical markers in Paget's disease of bone. *Rheumatology (Oxford)*, *43* (6), 692-695. doi: 10.1093/rheumatology/keh162
- Egbuna, O. I., & Brown, E. M. (2008). Hypercalcaemic and hypocalcaemic conditions due to calcium-sensing receptor mutations. *Best Practice & Research: Clinical Rheumatology, 22*(1), 129–148. doi: 10.1016/j.berh.2007.11.006.
- Eller-Vainicher, C., Battista, C., Guarnieri, V., Muscarella, S., Palmieri, S., Salcuni, A. S., … Scillitani A. (2014). Factors associated with vertebral fracture risk in patients with primary hyperparathyroidism. *European Journal of Endocrinology,* 171(3): 399-406. doi: 10.1530/EJE-14-0343.
- Garrett, J. E., Capuano, I. V., Hammerland, L. G., Hung, B. C., Brown, E. M., Hebert, S. C., Nemeth, E. F., Fuller, F. (1995). Molecular cloning and functional expression of human parathyroid calcium receptor cDNAs. *Journal of Biological Chemistry*, 270(21), 12919–12925.
- Guha, M., Bankura, B., Ghosh, S., Pattanayak, A. K., Ghosh, S., Pal, D.K., … Das M. (2015). Polymorphisms in CaSR and CLDN14 genes associated with increased risk of kidney stone disease in patients from the eastern part of india. *PLoS One,*10(6): e0130790
- Hannan, F. M., Kallay, E., Chang, W., Brandi, M. L., Thakker, R. V. (2018). The calcium-sensing receptor in physiology and in calcitropic and noncalcitropic diseases. *Nature Reviews Endocrinology*, 15(1): 33-51. doi: 10.1038/s41574-018-0115-0.
- Hend, G. N., Guarnieri, V., Canaff, L. (2009). Calcium-sensing receptor and associated diseases. *Progress in Molecular Biology and Translational Science, 89*, 31-95. doi: 10.1016/S1877-1173(09)89003-0.
- Ioannidis, J. P., Stavrou, I., Trikalinos, T. A., Zois, C., Brandi, M. L., Gennari, L., … Tsatsoulis, A. (2002). ER-alpha genetics meta-analysis. Association of polymorphisms of the estrogen receptor alpha gene with bone mineral density and fracture risk in women: a meta-analysis. *Journal of Bone and Mineral Research, 17*, 2048–2060.
- Jeong, S., Kim, I. W., Oh, K. H., Han, N., Joo, K. W., Kim, H. J., Oh, J. M. (2016). Pharmacogenetic analysis of cinacalcet response in secondary hyperparathyroidism patients. *Drug Design, Development and Therapy*, 10, 2211-2225. doi: 10.2147/DDDT.S103370.
- Jung, J., Foroud, T. M., Eckert, G. J., Flury-Wetherill, L., Edenberg, H. J., Xuei, X., …Pratt, J. H. (2009). Association of the calcium-sensing receptor gene with blood pressure and urinary calcium in African-Americans. *Journal of Clinical Endocrinology & Metabolism,94*(3), 1042-1048. doi: 10.1210/jc.2008-1861.
- Kinoshita, Y., Hori, M., Taguchi, M., Watanabe, S., Fukumoto, S. (2014). Functional activities of mutant calcium-sensing receptors determine clinical presentations in patients with autosomal dominant hypocalcemia. *Journal of Clinical Endocrinology & Metabolism, 99*, 363–368. doi:10.1210/jc.2013-3430
- Kuo, CW., Chang, T. H., Chi, W. L., Chu, T. C. (2008). Effect of Cigarette Smoking on Bone Mineral Density in Healthy Taiwanese Middle-Aged Men. *Journal of Clinical Densitometry*, *11*(4), 518-524. doi: 10.1016/j.jocd.2008.07.005.
- Kurt, O., Yilmaz-Aydogan, H., Uyar, M., Isbir, T., Seyhan, M., Can, A. (2012). Evaluation of ERα and VDR gene polymorphisms in relation to bone mineral density in Turkish postmenopausal women. *MolecularBiologyReports,39*(6), 6723-6730. doi: 10.1007/s11033-012-1496-0.
- Li, X., Kong, X., Jiang, L., Ma, T., Yan, S., …, Yang, Q. (2014). A genetic polymorphism (rs17251221) in the calcium-sensing receptor is associated with breast cancer susceptibility and prognosis. *Cellular Physiology and Biochemistry, 33*(1), 165-172. doi: 10.1159/000356659.
- Li, Y., Zhao, Y., Tang, R., Qu, X. (2010). Preventive and therapeutic effects of antlercollagen on osteoporosis in ovariectomized rats.

African Journal of Biotechnology, 9(38): 6437-6441. https://doi.org/10.5897/AJB10.251

- Lorentzon, M., Lorentzon, R., Lerner, U. H., Nordstrom, P. (2001). Calcium sensing receptor gene polymorphism, circulating concentrations and bone mineral density in healthy adolescent girls. *European Journal of Endocrinology, 144*: 257–261.
- Miller, S. A., Dykes, D. D., Poleskey, H. S. (1988). A simple salting out procedure for extracting DNA from human nucleated cells, *Nucleic Acids Research*, 16: 1215.
- NIH consensus development panel on osteoporosis. (2001) Prevention diagnosis and therapy osteoporosis prevention, diagnosis, and therapy. *Journal of theAmericanMedicalAssociation,* 285, 785–795. doi:10.1001/jama.285.6.785
- North American Menopause Society. (2010). NAMS continuing medical education activity. *Menopause*, 17(1), 25–54. https://doi.org/10.1097/gme.0b013e3181cdd4a7
- Nuti, R., Brandi, M. L., Checchia, G., Di Munno, O., Dominguez, L., Falaschi, P., …, Isaia, G. C. (2019). Guidelines for the management of osteoporosis and fragility fractures. *Internal and Emergency Medicine,* 14(1), 85-102. doi: 10.1007/s11739-018-1874-2.
- O'Seaghdha, C. M., Yang, Q., Glazer, N. L., Leak, T. S., Dehghan, A., Smith, A. V., …, Köttgen, A. (2010). Common variants in the calcium-sensing receptor gene are associated with total serum calcium levels. *Human Molecular Genetics, 19*, 4296–4303. doi:10.1093/hmg/ddq342
- Pi, M., Garner, S. C., Flannery, P., Spurney, R. F., Quarles, L. D. (2000). Sensing of extracellular cations in CaSR-deficient osteoblasts. Evidence for a novel cation sensing mechanism. *Journal of Biological Chemistry,* 275, 3256–3263.
- Rapuri, P. B., Gallagher, J. C., Balhorn, K. E., Ryschon, K. L. (2000). Smoking and bone metabolism in elderly women. *Bone*, 27, 429–436. http://dx.doi.org/10.1016/S8756-3282(00)00341-0
- Regala, J., Cavaco, B., Domingues, R., Limbert, C., Lopes, L. (2015). Novel mutation in the CASR gene (p.Leu123Ser) in a case of autosomal dominant Hypocalcemia. *Journal of Pediatric Genetics,* 4(1), 29-33. doi:10.1055/s-0035-1554979
- Sato, K., Hasegawa, Y., Nakae, J., Nanao, K., Takahashi, I., Tajima, T., …, Fujieda, K. (2002). Hydrochlorothiazide effectively reduces urinary calcium excretion in two Japanese patients with gain-of-function mutations of the calcium-sensing receptor gene. *Journal of ClinicalEndocrinology & Metabolism*, 87(7), 3068-3073.
- Takács, I., Speer, G., Bajnok, É., Tabák, Á., Nagy, Z., Horváth, C., …, Lakatos, P. (2002). Lack of association between calcium-sensing receptor gene "A986S" polymorphism and bone mineral density in Hungarian postmenopausal women. *Bone*, 30(6), 849–852. doi: http://dx.doi.org/10.1016/S8756-3282(02)00741-X
- Thakker, R. V. (2004). Diseases associated with the extracellular calcium-sensing receptor. *Cell Calcium*, 35(3), 275-82
- Wang, Y. B., Guo, J. J., Liu, Y. J., Deng, F. Y., Jiang, D. K., Deng, H. W. (2006). The human calcium-sensing receptor and interleukin-6 genes are associated with bone mineral density in Chinese. *Acta Genetica Sinica*, 33(10), 870-880.
- Watanabe, S., Fukumoto, S., Chang, H., Takeuchi, Y., Hasegawa, Y., Okazaki, R., …, Fujita, T. (2002). Association between activating mutations of calcium-sensing receptor and Bartter's syndrome. *Lancet*, 360(9334), 692-694.
- Willing, M., Sowers, M., Aran, D., Clark, M. K., Burns, T., Bunten, C. (1998). Bone mineral density and its change in white women: estrogen and vitamin D receptor genotypes and their interaction. *Journal of Bone and Mineral Research,* 13, 695–705.
- Xie, P., Liu, B., Zhang, L., Chen, R., Yang, B., Dong, J., Rong, L. (2015). Association of *COL1A1* polymorphisms with osteoporosis: a meta-analysis of clinical studies. *International Journal of Experimental and ClinicalMedicine,*8(9), 14764–14781.
- Yamauchi, M., Sugimoto, T., Yamaguchi, T., Yano, S., Kanzawa, M., Kobayashi, A., Chihara, K. (2001). Association of polymorphic alleles of the calcium-sensing receptor gene with the clinical severity of primary hyperparathyroidism. *Clinical Endocrinology (Oxford)*,55, 373–379.
- Young, R., Wu, F., Van de Water, N., Ames, R., Gamble, G., Reid, I.R. (2003). Calcium sensing receptor gene A986S polymorphism and responsiveness to calcium supplementation in postmenopausal women. *Journal of ClinicalEndocrinology & Metabolism,* 88(2), 697-700. doi: 10.1210/jc.2002-020355.
- Zhang, J., Jameson, K., Sayer, A. A., Robinson, S., Cooper, C., Dennison, E. (2016). Accumulation of risk factors associated with poor bone health in older adults. *Archives of Osteoporosis*, 11, 3. doi: 10.1007/s11657-015-0250-3.
- Zhao, X. M., Hauache, O., Goldsmith, P. K., Collins, R., Spiegel, A. M. (1999). A missensemutation in the seventh transmembrane domain constitutively activates the human Ca^{2+}receptor. *FEBS Letters*, 448(1), 180–184.

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Original Article

Assessment of genotoxic effects of organophosphate and carbamate pesticides by comet assay

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ABSTRACT

Background and Aims: Pesticide poisoning is the most widespread occupational hazard for agricultural workers in the developing world, due to the extensive presence of pesticides in the environment. The aim of this study was to investigate the cytotoxicity and DNA damaging effects of organophosphosphate and carbamate pesticides.
Methods: In the present study, the cytotoxicity of chlorpyrifos methyl, azinphos ethyl, [(O-Ethyl O-(p-nitrophenyl) phenylphosphonothioate] (EPN), aldicarb sulfone, and ethiofencarb were assessed by the trypan blue dye exclusion method. An alkaline comet assay was performed to assess the genotoxic effects of applied pesticides in human peripheral blood lymphocytes.
Results: We demonstrated the cytotoxic effect of EPN following 30 and 120 min exposure at 100 µg/mL concentration. Although chlorpyrifos-methyl and azinphos ethyl seem to be safer concerning cytotoxicity compared to other pesticides, significantly higher DNA damage levels were determined after exposure of these pesticides for 120 min at 100 µg/mL concentration by in vitro comet assay. The potential DNA-damaging effects of these pesticides were sorted from high to low, as chlorpyrifos-methyl, aldicarb sulfone, EPN, and azinphos ethyl after 30 min of exposure, and were sorted as chlorpyrifos-methyl, azinphos ethyl, aldicarb sulfone, and EPN after 120 min of exposure. Our results revealed that these pesticides tend to increase DNA damage in a dose- and time-dependent manner.
Conclusion: The genotoxic effects of these widely used pesticides may cause prominent and serious health risks for human populations; hence, the DNA-damaging potential of pesticides can lead to genotoxic risk and adverse health effects like cancer.

Keywords: Carbamate pesticides, Organophosphate pesticides, In vitro comet assay, Genotoxicity

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INTRODUCTION

Pesticides are physical, chemical, and biologically active substances that are widely used in agriculture to improve the efficiency of food production processes, lower food costs, and ensure high-quality produce. Weeds, pests, and diseases cause over 40% of global food production to be lost each year (Jamil, Shaik, Mahboob, & Krishna, 2004; Suratman, Edwards, & Babina, 2015). In addition, pesticides help to reduce the spread of infectious diseases. Despite the advantageous effects of pesticides, the residues of these chemicals in the soil, air, water, and food may pose a threat to human health and the natural environment (van der Werf, 1996; Ahmed, 2001). As a result, the balance of the ecological system may be disturbed, and they may cause acute and chronic poisoning in the immediate environment. Additionally, humans in the developing world are exposed to these chemicals in various job processes such as spraying, handling, manufacturing and packing. Besides eradicating insects or weeds, pesticides can show their toxic effects on other non-target organisms like birds, fish, beneficial insects, plants, and humans (Mohanty, Mohanty, Jena, & Dutta, 2011). The toxic effects of pesticides, including bioaccumulation, biomagnification, chronic toxicity, acute immune response, hypersensitivity reactions, and mutagenic, carcinogenic, and teratogenic responses are propounded due to exposure (Ecobichon, 2001).

Organophosphates (OPs), synthetic pyrethroids, and carbamates are the most widely used pesticides in the world today for the control of agricultural and domestic insects; as a result, these substances come into direct contact with humans. OPs in particular have been manufactured since 1943 and until today, more than one hundred OP compounds have been identified and utilized worldwide (Suratman et al., 2015). OPs and carbamate insecticides are toxic to insects and mammals by virtue of their ability to deactivate the acetylcholinesterase enzyme (AChE), which catalyzes the hydrolysis of acetylcholine (ACh), a neurotransmitter. Whereas OP insecticides inhibit the acetylcholinesterase enzyme irreversibly, carbamate insecticides inhibit it reversibly. This inhibition leads to an excess of ACh accumulating and overstimulating cholinergic neurons. If the concentration of pesticide is high, in some instances even death can occur in a matter of minutes (Munoz-Quezada et al., 2016).

Human exposure to pesticides and the genotoxic effects of these chemicals has remained a global concern over the last decade. *In vitro* and *in vivo* genotoxicity studies have shown that DNA damage and oxidative stress are the underlying causes of pesticide toxicity, including OPs and carbamates (Muniz et al. 2008; Mohanty et al. 2011). Micronucleus (MN) tests, sister chromatid exchange (SCE), chromosome aberrations (CA) and the alkaline comet assay (single cell gel electrophoresis assay, SCGE) were utilized to assess these compounds' genotoxicity *in vitro*. The comet assay is a well-known genotoxicity assay for determining DNA damage at the individual cell level. It identifies DNA strand breaks, alkali-labile sites, and inadequate excision repair processes in individual cells (Singh, McCoy, Tice, & Schneider, 1988; McKelvey-Martin et al., 1993). As a result of these characteristics, it becomes possible to evaluate physiologically relevant levels of oxidative DNA damage. Up to now, insufficient information has been obtained concerning *in vitro* genotoxic influence of pesticides on human peripheral blood lymphocytes. The purpose of this study was to assess potential genotoxic effects of aldicarb sulfone, ethiofencarb from the carbamate class, and azinphos ethyl, chlorpyrifos methyl, EPN from the organophosphate class of pesticides.

MATERIAL AND METHODS

Pesticide exposure

Five milliliters of heparinized blood samples from two healthy non-smoking female volunteers, aged 30-32 years were collected in heparinized syringes. Volunteers provided informed consent. The study protocol was conducted in accordance with the Declaration of Helsinki. The samples were used immediately for the determination of viability by trypan blue dye exclusion assay and DNA damage by comet assay.

In our preliminary study, the genotoxic effects of certain carbamates (aminocarb, carbaryl, methiocarb, promecarb and propoxur) were evaluated in Maden-Darby Canine Kidney (MDCK) cell lines and human blood lymphocytes. Pesticides were applied to MDCK cell lines at several concentrations. The studied pesticide concentrations and incubation time in the current study were determined based on our preliminary study. Histopaque 1077 separating solution was used to isolate lymphocytes, which were then rinsed with PBS. Cell concentrations were adjusted to around $2x10^5$ per mL in the buffer. Isolated lymphocytes were incubated with 10, 50 and 100 µg/mL concentrations of pesticides (aldicarb sulfone, azinphos ethyl, chlorpyrifos methyl, ethiofencarb, EPN) for 30 min and 120 min at 37°C. In parallel with the pesticide standards, negative controls were established by incubating lymphocytes with the solvent DMSO at a final concentration of 1% at the same temperature and exposure duration as the pesticide standards. As a positive control, the cells were treated with 30% H_2O_2 at 100 µM and incubated for 5 min at 37°C. Blood samples from the same donor were taken at different time periods in triplicate tests. Positive and negative controls were used in each experiment.

Cell proliferation assay

The cell viability was assessed using the trypan blue dye exclusion method. The lymphocytes were washed in PBS after pesticide exposure. Subsequently, the cell suspension was mixed with the 0.04% trypan blue solution at a ratio of 1:1, and living cells were counted manually using a hemacytometer in duplicate. The mean percentage of living cells was calculated.

Comet assay

The alkaline version of the comet assay was used in this investigation, and it was modified slightly from Singh et al. (1988) method. At the end of the incubation period, pesticide-treated and control cells were mixed with 0.7% low melting agarose (LMA) and spread on microscope slides covered with 0.7% normal melting agarose. The slides were submerged in a lysing solution (10 mM Tris, 100 mM Na_2EDTA, 2.5 M NaCl pH 10 with 10% DMSO and 1% Triton X-100) for at least 1 h at +4 °C after

the LMA had solidified. To enable for DNA unwinding and the appearance of alkali labile damage, the slides were submerged in electrophoresis buffer (0.3 M NaOH, 1mM EDTA, pH 13) for 20 min. Afterwards, the DNA was electrophoresed for 30 min at 300 mA and 15 V. The cells were neutralized with 0.4 M Tris buffer, pH 7.5 and stained with 50 µL ethidium bromide (EtBr - 20 µg/mL). The stained DNA images were examined using a fluorescent microscope with a 200x objective (Olympus BX51 microscope, Tokyo, Japan). To calculate DNA damage, 100 cells were chosen at random from each sample and visually examined for comet appearance. The degree of DNA migration in the cells was classified into five categories by eye. Five classes, ranging from class 0 (no DNA damage) to class 4 (maximum DNA damage), provided sufficient declaration (Collins, 2014).

The parameter total comet score was used to assess DNA damage (TCS). Total comet score (TCS) was then calculated according to the formula:

TCS= 0 (n)+1(n)+2(n)+3(n)+4(n), where "n" indicated the number of cells in each class.

Statistical analysis

The SPSS software (ver. 22.0. Chicago: SPSS Inc.) was used for statistical analysis. The results were statistically compared using the non-parametric Kruskal-Wallis test and the Mann Whitney *U* test as the *post hoc* analysis of differences between the groups with the least significant difference test. All results were expressed as mean±SD and a *p*-value less than 0.05 was determined to be statistically significant.

RESULTS

The cytotoxic effects of the pesticides on the cells were determined by the trypan blue dye exclusion method. According to the data obtained from three separate experiments, the cytotoxicity was increased in a concentration- and time-dependent manner after the incubation of the pesticides. The highest toxicity was observed with EPN at 50 and 100 µg/mL concentrations after 30 min incubation (p>0.05). The cell viability percentage of lymphocytes after treatment with chlorpyrifos methyl was found to be statistically higher than the other tested pesticides (*p>0.05*). After 120 min incubation of the tested compounds, the toxicity of EPN at 100 µg/mL concentration was determined as statistically significant (p>0.05). Chlorpyrifos methyl and azinphos ethyl at 50 µg/mL concentrations were found to be safer than other tested pesticides, while cell viability percentages were over 60% in lymphocytes treated with azinphos ethyl at 100 µg/mL concentration. Cell viability percentages of lymphocytes after exposure to increasing concentrations of pesticides for 30 min and 120 min are shown in Figure 2 and 3.

After visualizing the comet tail using a fluorescent microscope, the length of DNA migration in the comet tail was determined as comet tail length, which is an estimate of DNA damage for each cell. It was observed that the comet tail length extended with increasing concentrations of pesticides. Pesticides caused DNA damage at all concentrations compared to the negative control; however, responses of DNA damage varied. The highest toxicity was observed with 100 µg/mL exposure after 120 min incubation for chlorpyrifos methyl (TCS: 222±85, p<0.05). An increase in DNA damage after OPs (chlorpyrifos methyl, azinphos ethyl and EPN) exposure at 50 µg/mL concentration and carbamates (aldicarb sulfone) exposure at 100 µg/mL concentration for 30 min incubation was found statistically significant (*p<0.05*). Figures 4 and 5 show the mean TCS distribution

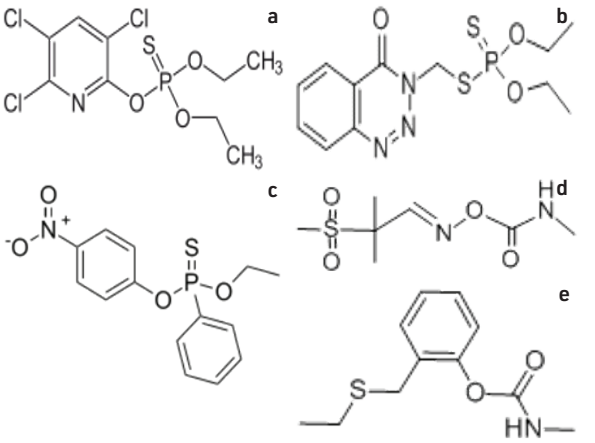

Figure 1. Chemical structures of tested pesticides (a. chlorpyrifos methyl; b. azinphos ethyl; c. EPN; d. aldicarb sulfone; e. ethiofencarb)

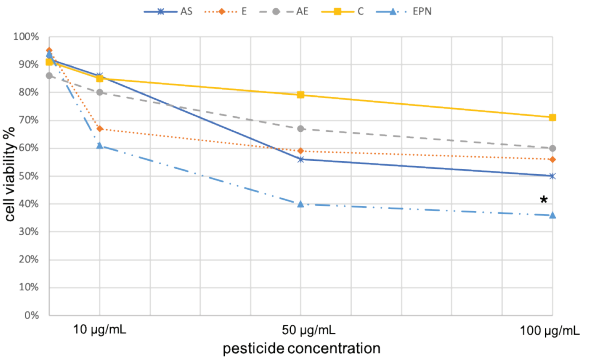

Figure 2. Cell viability percentage in lymphocytes after exposures to increasing concentrations of pesticides for 30 min (AS: aldicarb sulfone; E: ethiofencarb; AE: azinphosethy; C: chlorpyrifos methyl) (*$p<0.05$).

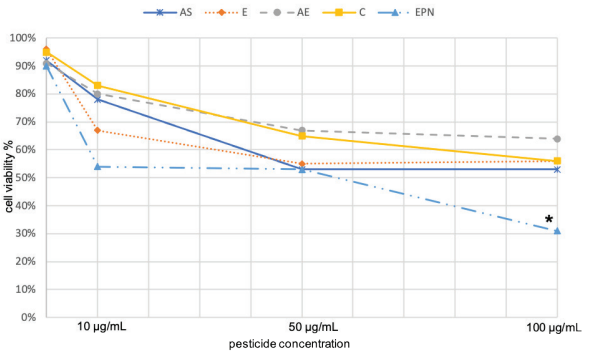

Figure 3. Cell viability percentage in lymphocytes after exposures to increasing concentrations of pesticides for 120 min. (AS: aldicarb sulfone; E: ethiofencarb; AE: azinphosethy; C: chlorpyrifos methyl) (*$p<0.05$).

of DNA damage in lymphocytes after pesticide treatments at various concentrations for 30 and 120 min. 50 µg/mL of chlorpyrifos methyl, azinphos ethyl, and 100 µg/mL of EPN aldicarb sulfone significantly increased DNA damage after 120 min exposure. On the other hand, ethiofencarb did not induce DNA damage in a dose- and incubation time-dependent manner. The potential DNA damaging effects of these pesticides were sorted from high to low, as chlorpyrifos methyl, aldicarb sulfone, EPN, azinphos ethyl after 30 min of exposure. After 120 min of exposure, they were sorted as chlorpyrifos, azinphos ethyl, aldicarb sulfone, EPN.

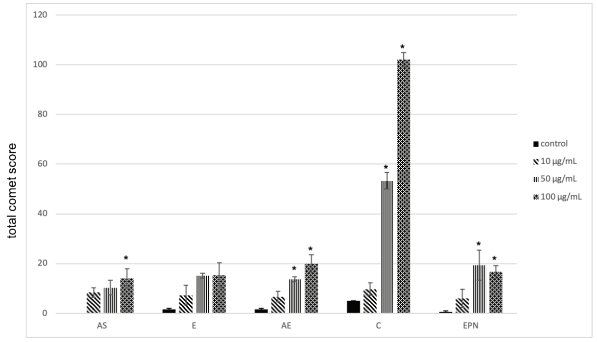

Figure 4. cDNA damage levels in lymphocytes after exposures of pesticides at different concentrations for 30 min (*p<0.05). (AS: aldicarb sulfone; E: ethiofencarb; AE: azinphosethy; C: chlorpyrifos methyl) (*p<0.05).

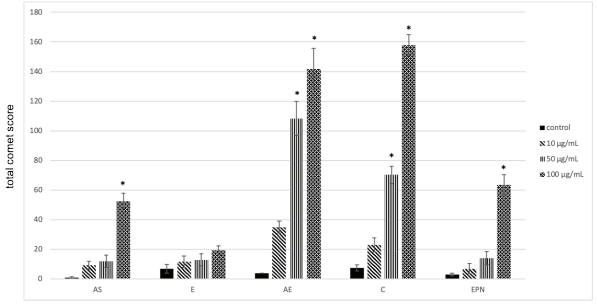

Figure 5. DNA damage levels in lymphocytes after exposures of pesticides at different concentrations for 120 min (*p<0.05). (AS: aldicarb sulfone; E: ethiofencarb; AE: azinphosethy; C: chlorpyrifos methyl) (*p<0.05).

Table 1. Total comet scores in lymphocytes after exposures of pesticides at different concentrations for 30 min and 120 min.

Pesticides	Concentrations (µg/mL)	TCS[a] (Mean±SD)	
		30 min	120 min
Chlorpyriphos methyl	10	6.67±2.5	23.01±4.7
	50	53.31± 3.4*	70.32±5.8*
	100	101.99±2.8*	157.66±7.2*
Azinphos ethyl	10	6.64±2.1	34.98±4.1
	50	13.67±1.2*	108.32±11.5*
	100	20.01±3.6*	141.67±14.2*
EPN	10	6.01±3.6	6.99±3.5
	50	19.34±6.2*	14±4.4
	100	16.66±2.5*	63.67±6.7*
Aldicarb sulfone	10	8.33±2.3	9.32±2.6
	50	10.34±3.4	11.99±4.2
	100	13.98±4.1*	52.65±5.3*
Ethiofencarb	10	7.31±4.3	11.66±4.3
	50	15.02±1.4	12.97±4.5
	100	15.31±5.2	19.33±3.2

[a]Totalcometscore: 0 x No Migration (NM) + 1 x Low Migration (LM) + 2 x Medium Migration (MM) + 3 x High Migration (HM) + 4 x Extensive Migration (EM). *P <0.05.

DISCUSSION

Pesticide poisoning is the most widespread occupational hazard for agricultural workers in the developing world, due to the extensive presence of pesticides in the environment. Therefore, the mechanisms of action of these chemicals are still being investigated *in vitro* and *in vivo* (Gaikwad, Karunamoorthy, Kondhalkar, Ambikapathy, & Beerappa, 2015). Investigations which were performed using *in vivo* and *in vitro* comet assays have shown that long-term exposure to OPs is associated with increased DNA damage (Ündeğer & Başaran, 2002; Shadnia et al., 2005; Muniz et al., 2008). The trypan blue exclusion test is commonly used to assess cell viability as a part of *in vitro* genotoxicity studies conducted by comet analysis (Vigreux et al., 1998; Das, Shaik, & Jamil, 2007). In our preliminary study, the genotoxic effects of certain carbamates (aminocarb, carbaryl, methiocarb, promecarb and propoxur) were evaluated in Maden-Darby Canine Kidney (MDCK) cell lines and human blood lymphocytes. Pesticides were applied to MDCK cell lines at several concentrations (3, 10, 30 and 100 µg/mL) for 48 hours at 37ºC. The DNA damage of lymphocytes treated with pesticides at 30 µg/mL concentration for 30 min or 16 h were analyzed with a comet assay. The study revealed that the highest damage was observed in cells treated with carbaryl and methiocarb, which showed the correlation with toxicity on MDCK cells. Lower DNA damage levels were determined in MDCK cells treated with pesticides in contrast to lymphocytes. No significant difference was observed in cell growth in the presence of pesticides at 10 µg/mL concentration (data not shown). Therefore, in the present study, we demonstrated the cytotoxic effect of EPN in human peripheral blood lymphocytes following 30 and 120 min exposure at 100 µg/mL concentration by the trypan blue exclusion test. Although chlorpyrifos methyl and azinphos ethyl seem to be safer regarding cytotoxicity compared to other pesticides, significantly higher DNA damage levels were determined after exposure of these pesticides for 120 min at 100 µg/mL concentration by *in vitro* comet assay. The toxicity mechanisms of these pesticides vary according to their chemical structures or their fate in the biological system.

Previous investigations on OPs revealed their potential genotoxic effect; however, the results were inconsistent (Ojha & Srivastava, 2014). The present study showed that chlorpyrifos methyl led to enhanced DNA damage after 30 and 120 min treatment at the highest concentration when compared to the control. This result is in accordance with the findings of other researchers (Rahman, Mahboob, Danadevi, Banu, & Grover, 2002; Mehta, Verma, & Srivastava, 2008; Sandal & Yilmaz, 2011). These authors showed that chlorpyrifos exposure increased DNA damage in the liver and brain tissues of rats in a dose-dependent manner and their leukocytes and lymphocytes. Abuwarda et al. investigated the aneuploidy-inducing effect of chlorpyrifos in human peripheral blood lymphocyte cultures by using the fluorescence *in situ* hybridization (FISH) (Abuwarda, Alashi, & Sharif, 2021). They demonstrated that this compound has shown acceptable levels of cytotoxicity; however, frequencies of aneuploidy, chromosome loss, and chromosome gain were enhanced after exposure. The DNA damage evaluation by the comet assay of chlorpyrifos, as well as reports on bone marrow micronucleus assay of chlorpyrifos in rats, was in agreement with the DNA damage results of chlorpyrifos in lymphocytes (Okonko, Ikpeme, & Udensi, 2016). Jamil et al. (2004) studied the genotoxic effects of organophosphorus (monochrotophos, chlorpyrifos, dimethoate) and organochlorine (endosulfan) pesticides by using the comet assay (Jamil et al., 2004). They reported that two pesticides; monochrotophos, chlorpyrifos were 10 times more toxic than dimethoate.

Azinphos ethyl is highly toxic to mammals and readily absorbed by dermal exposure, inhalation of dust or spray and swallowin (Petroianu, Nurulain, Hasan, Kuca, & Lorke, 2015). There is no evidence of the genotoxicity of azinphos ethyl in human peripheral blood lymphocytes. This study presents the first report about the genotoxicity of azinphos ethyl with the comet assay. Hence, the genotoxic potential of azinphos methyl has been compared to azinphos ethyl, which is the pesticide residue found most commonly in the houses of both farm workers and growers (McCauley et al., 2001). Azinphos methyl and azinphos ethyl are in the same chemical form, both of them have phosphorodithioate linkage. Although azinphos ethyl showed a positive genotoxic effect *in vitro* micronucleus assay in Chinese hamster lung cells, the DNA damaging effects of *in vivo* micronucleus assay in mice were reported to be negative (Ni, Li, Liu, Tang, & Pang, 1993). The lack of consistency in the response between the *in vivo* and *in vitro* cytogenetic tests can be explained by the fact that the two azinphos compounds are metabolized rather quickly *in vivo* before they can induce any genotoxic effects. Kisby et al (2009) showed that cultures of human lymphocytes exposed with azinphos methyl for 24 h induced oxidative DNA damage. DNA damage is assumed to be a main underlying mechanism for the toxicity of pesticides by disrupting the function of cells (Kisby et al., 2009). The long-term exposure of OPs causes enhanced release of cytochrome c from mitochondria to cytosol and the activation of caspase-3 and results in a disrupted cellular antioxidant defense system, which causes DNA damage (Hodgson & Levi, 1996; Kaur, Radotra, Minz, & Gill, 2007; Kisby et al., 2009).

EPN [(O-Ethyl O-(p-nitrophenyl) pheriylphosphonothioate] is a nonsystemic organophosphorus insecticide and acaricide. It is highly toxic to birds and mammals through acute oral exposure (Smith, 1987). EPN was not classified as a human carcinogen, but there is no evidence of the genotoxic effects of EPN. Herein, we present the first report about the genotoxic effect of EPN in human blood lymphocytes. In our study, exposure of EPN at 100 µg/mL concentration for 120 min induces cytotoxicity in human lymphocytes, moreover led to a significant increase in DNA damage.

Carbamates, which are potent cholinesterase inhibitors as organophosphates, constitute another important class of pesticides, which have also been revealed to be mutagenic in various test systems (Proença et al., 2004; Mohanty et al., 2011). Das et al (2007) assessed the damage caused by pesticides (organophosphate, organochlorine and carbamate) and their combinations on humans by cytotoxicity and genotoxicity assays (Das et al, 2007). High doses of certain pesticides (0.5-4.0 µM) produced considerable DNA damage, as evidenced by apparent tail lengths, according to the authors. Carbamate pesticides caused significant DNA damage in lymphocytes. However, besides direct strand breakage, DNA damage may be caused by inhibition of some other metabolic pathways and cell death. Aldicarb is a carbamate used in agriculture as an insecticide and nematicide. It is metabolized to aldicarb sulfoxide and aldicarb sulfone. Aldicarb sulfoxide is a more potent inhibitor of acetylcholinesterase than aldicarb. The genotoxic potential of aldicarb determined by genotoxicity tests (comet assay, SCE and micronucleus assay) has been previously reported (Cid & Matos, 1984; Sun et al., 2010). Sun et al (2010) investigated the genotoxicity of aldicarb and methomyl at different concentrations by micronucleus test, Ames test and comet assay. According to the results of the comet assay, high concentrations of aldicarb were observed to cause DNA damage at different levels in human peripheral blood lymphocytes (Sun et al., 2010). However, studies on genotoxicity caused by aldicarb sulfone are very limited. In our study, DNA damage was observed after treatment with 100 µg/mL of aldicarb sulfone. Venkat et al (1995) evaluated the mutagenic potential of 47 pesticides, including aldicarb sulfone using a modified SOS microplate assay (Venkat et al., 1995). Aldicarb sulfone was found as one of the ten most active pesticides. Canna-Michaelidou&Nicolaou (1996) studied the genotoxic effect of aldicarb sulfone with the Mutatox™ test, both directly and after exogenous activation with the S9 hepatic enzyme (Canna-Michaelidou & Nicolaou, 1996). The genotoxic effect of aldicarb sulfone was categorized as 'suspect genotoxic' both directly and after S9-activation in mutatox. The findings obtained from these studies have demonstrated that aldicarb sulfone significantly damages DNA.

Ethiofencarb, which is used frequently as a systemic insecticide, acts through contact and oral route. It is almost completely absorbed in mammals and excreted rapidly as metabolites, mainly in the urine (Al-Samarraie et al., 2009). There is no evidence of the genotoxic effects of ethiofencarb *in vivo* and *in vitro* mammalian test systems. This is the first data to evaluate that ethiofencarb is not cytotoxic and does not cause DNA damage in a comet assay at studied concentrations and incubation times. Further studies are required to better understand the genotoxic effects of ethiofencarb.

CONCLUSION

OPs and carbamate pesticides cause significant DNA damage in lymphocytes by inhibition of some other metabolic pathways and cell death. Genotoxic damage is considered a relevant biomarker for carcinogenic risk. These widely used agricultural pesticides should be handled cautiously since low-level, long-term exposure to pesticides can lead to genotoxic risk and adverse health effects like cancer. Further investigations on genotoxic thresholds and susceptibility to pesticide-related pathologies in human populations are required.

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

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Ethics Committee Approval: The blood samples were collected from authors of the study (S.K. and E.F.İ.D.). The study was carried out between February 2008 and May 2009 prior to the publication of the Regulation on Clinical Trials on August 19, 2011 (https://www.resmigazete.gov.tr/eskiler/2011/08/20110819-9.htm). As a result, even though being conducted in accordance with the Helsinki Declaration, the study protocol was unable to obtain approval from an Ethics Committee.

REFERENCES

- Abuwarda, H. N., Alashi, S. F., & Sharif, F. A. (2021). Investigating aneuploidy-inducing effect of Nemacur, Rogor, and Dursban in human peripheral blood lymphocyte cultures. *Environmental and Molecular Mutagenesis, 62*(5), 328-334. doi:10.1002/em.22445
- Ahmed, F. E. (2001). Analyses of pesticides and their metabolites in foods and drinks. *TrAC Trends in Analytical Chemistry, 20*(11), 649-661.
- Al-Samarraie, M. S. J., Karinen, R., Rognum, T., Hasvold, I., Stokke, M. O., & Christophersen, A. S. (2009). Lethal Poisoning with Ethiofencarb and Ethanol. *Journal of Analytical Toxicology, 33*(7), 389-392. doi: 10.1093/jat/33.7.389
- Canna-Michaelidou, S., & Nicolaou, A. S. (1996). Evaluation of the genotoxicity potential (by Mutatox test) of ten pesticides found as water pollutants in Cyprus. *Science of the Total Environment, 193*(1), 27-35. doi:10.1016/s0048-9697(96)05322-3
- Cid, M. G., & Matos, E. (1984). Induction of sister-chromatid exchanges in cultured human lymphocytes by aldicarb, a carbamate pesticide. *Mutation Research/Genetic Toxicology, 138*(2), 175-179.
- Collins, A. R. (2014). Measuring oxidative damage to DNA and its repair with the comet assay. *Biochimica et Biophysica Acta, 1840*(2), 794-800. doi:10.1016/j.bbagen.2013.04.022
- Das, P., Shaik, A. P., & Jamil, K. (2007). Genotoxicity induced by pesticide mixtures: in-vitro studies on human peripheral blood lymphocytes. *Toxicology and Industrial Health, 23*(8), 449-458.
- Ecobichon, D. J. (2001). Pesticide use in developing countries. *Toxicology, 160*(1-3), 27-33. doi: 10.1016/S0300-483x(00)00452-2
- Gaikwad, A. S., Karunamoorthy, P., Kondhalkar, S. J., Ambikapathy, M., & Beerappa, R. (2015). Assessment of hematological, biochemical effects and genotoxicity among pesticide sprayers in grape garden. *Journal of Occupational Medicine and Toxicology, 10*(1), 11.
- Hodgson, E., & Levi, P. E. (1996). Pesticides: an important but underused model for the environmental health sciences. *Environmental Health Perspectives, 104*(Suppl 1), 97.
- Jamil, K., Shaik, A. P., Mahboob, M., & Krishna, D. (2004). Effect of organophosphorus and organochlorine pesticides (monochrotophos, chlorpyriphos, dimethoate, and endosulfan) on human lymphocytes in-vitro. *Drug& Chemical Toxicology, 27*(2), 133-144. doi:10.1081/dct-120030725
- Kaur, P., Radotra, B., Minz, R. W., & Gill, K. (2007). Impaired mitochondrial energy metabolism and neuronal apoptotic cell death after chronic dichlorvos (OP) exposure in rat brain. *Neurotoxicology, 28*(6), 1208-1219.
- Kisby, G. E., Muniz, J. F., Scherer, J., Lasarev, M. R., Koshy, M., Kow, Y. W., & McCauley, L. (2009). Oxidative stress and DNA damage in agricultural workers. *Journal of Agromedicine, 14*(2), 206-214.
- McCauley, L. A., Lasarev, M. R., Higgins, G., Rothlein, J., Muniz, J., Ebbert, C., & Phillips, J. (2001). Work characteristics and pesticide exposures among migrant agricultural families: a community-based research approach. *Environmental Health Perspectives, 109*(5), 533.
- McKelvey-Martin, V. J., Green, M. H., Schmezer, P., Pool-Zobel, B. L., De Meo, M. P., & Collins, A. (1993). The single cell gel electrophoresis assay (comet assay): a European review. *Mutation Research, 288*(1), 47-63. doi:10.1016/0027-5107(93)90207-v
- Mehta, A., Verma, R. S., & Srivastava, N. (2008). Chlorpyrifos-induced DNA damage in rat liver and brain. *Environmental and Molecular Mutagenesis, 49*(6), 426-433.
- Mohanty, G., Mohanty, J., Jena, S. D., & Dutta, S. (2011). Genotoxicity Testing in Pesticide Safety Evaluation. In M. Stoytcheva (Ed.) *Pesticides in the Modern World-Pests Control and Pesticides Exposure and Toxicity Assessment* (pp. 403-426). Rijeka, Croatia: InTech.
- Muniz, J. F., McCauley, L., Scherer, J., Lasarev, M., Koshy, M., Kow, Y., . . . Kisby, G. (2008). Biomarkers of oxidative stress and DNA damage in agricultural workers: a pilot study. *Toxicology and Applied Pharmacology, 227*(1), 97-107.
- Munoz-Quezada, M. T., Lucero, B. A., Iglesias, V. P., Munoz, M. P., Cornejo, C. A., Achu, E., . . . Villalobos, M. (2016). Chronic exposure to organophosphate (OP) pesticides and neuropsychological functioning in farm workers: a review. *International Journal of Occupational and Environmental Health, 22*(1), 68-79. doi:10.1080/10773525.2015.1123848
- Ni, Z., Li, S., Liu, Y., Tang, Y., & Pang, D. (1993). Induction of micronucleus by organophosphorus pesticides both in vivo and in vitro. *Hua xi yi ke da xue xue bao= Journal of West China University of Medical Sciences= Huaxi yike daxue xuebao/[bian ji zhe, Hua xi yi ke da xue xue bao bian wei hui], 24*(1), 82-86.
- Ojha, A., & Srivastava, N. (2014). In vitro studies on organophosphate pesticides induced oxidative DNA damage in rat lymphocytes. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 761*, 10-17.
- Okonko, L., Ikpeme, E., & Udensi, O. (2016). Genotoxic Effect of Chlorpyrifos and Cypermethrin in Albino Rats. *Research Journal of Mutagenesis, 6*(1), 31.

- Petroianu, G. A., Nurulain, S. M., Hasan, M. Y., Kuca, K., & Lorke, D. E. (2015). Reversible cholinesterase inhibitors as pre-treatment for exposure to organophosphates: assessment using azinphos-methyl. *Journal of Applied Toxicology, 35*(5), 493-499. doi:10.1002/jat.3052
- Proença, P., Teixeira, H., De Mendonça, M., Castanheira, F., Marques, E. P., Corte-Real, F., & Vieira, D. N. (2004). Aldicarb poisoning: one case report. *Forensic Science International, 146*, S79-S81.
- Rahman, M., Mahboob, M., Danadevi, K., Banu, B. S., & Grover, P. (2002). Assessment of genotoxic effects of chloropyriphos and acephate by the comet assay in mice leucocytes. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 516*(1), 139-147.
- Sandal, S., & Yilmaz, B. (2011). Genotoxic effects of chlorpyrifos, cypermethrin, endosulfan and 2, 4-D on human peripheral lymphocytes cultured from smokers and nonsmokers. *Environmental Toxicology, 26*(5), 433-442.
- Shadnia, S., Azizi, E., Hosseini, R., Khoei, S., Fouladdel, S., Pajoumand, A., . . . Abdollahi, M. (2005). Evaluation of oxidative stress and genotoxicity in organophosphorus insecticide formulators. *Human & Experimental Toxicology, 24*(9), 439-445.
- Singh, N. P., McCoy, M. T., Tice, R. R., & Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research, 175*(1), 184-191. doi:10.1016/0014-4827(88)90265-0
- Smith, G. J. (1987). Pesticide Use and Toxicology in Relation to Wildlife: Organophosphorus and Carbamate, Compounds. All U.S. Government Documents (Utah Regional Depository).
- Sun, X., Jin, Y., Wu, B., Wang, W., Pang, X., & Wang, J. (2010). Study on genotoxicity of aldicarb and methomyl. *Huan jing ke xue= Huanjing kexue, 31*(12), 2973-2980.
- Suratman, S., Edwards, J. W., & Babina, K. (2015). Organophosphate pesticides exposure among farmworkers: pathways and risk of adverse health effects. *Reviews on Environmental Health, 30*(1), 65-79. doi:10.1515/reveh-2014-0072
- Ündeğer, Ü., & Başaran, N. (2002). Assessment of DNA damage in workers occupationally exposed to pesticide mixtures by the alkaline comet assay. *Archives of Toxicology, 76*(7), 430-436.
- van der Werf, H. M. (1996). Assessing the impact of pesticides on the environment. *Agriculture, Ecosystems & Environment, 60*(2-3), 81-96.
- Venkat, J. A., Shami, S., Davis, K., Nayak, M., Plimmer, J. R., Pfeil, R., & Nair, P. P. (1995). Relative genotoxic activities of pesticides evaluated by a modified SOS microplate assay. *Environmental and Molecular Mutagenesis, 25*(1), 67-76. doi:10.1002/em.2850250110
- Vigreux, C., Poul, J., Deslandes, E., Lebailly, P., Godard, T., Sichel, F., . . . Gauduchon, P. (1998). DNA damaging effects of pesticides measured by the single cell gel electrophoresis assay (comet assay) and the chromosomal aberration test, in CHOK1 cells. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 419*(1), 79-90.

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Original Article

The practice of dividing tablets: An uncertain act

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ABSTRACT

Background and Aims: Tablets can be split by patients for a number of reasons, using various instruments. Tablets can be scored or unscored; if scored, they may be split into pieces, and in spite of guidelines to do so patients are still at risk of resultant drug dose fluctuations, or being exposed to toxic or subtherapeutic doses. The aim of this study was to investigate differences in weight between halves of tablets, split by different populations and with different devices.
Methods: 3-factor full factorial design (3 runs) was used with participants: patients, caregivers, nurses, medical doctors, and pharmacists; instruments: scissors, tablet cutters, knives, hand; drugs: losartan, clonidine, metoprolol, and warfarin. The risk of unequal tablet splitting was estimated and analyzed for each factor and their interaction with linearized generalized models.
Results: Differences in weight were found to be above 15% and 25% of the theoretical weight as in general, the highest weight variations after splitting were found in clonidine with patients using scissors. The overall risk of non-equal tablet splitting was 22.5% for deviations > 15% and for > 25%.
Conclusion: In this study, no tablet was split into halves of equal weight; based on these findings, splitting tablets is a questionable practice.

Keywords: Splitting tablets, Equal weight, Amount, Patients, Risk

INTRODUCTION

The practice of splitting tablets is a widespread activity in hospitals, nursing homes and private homes, and is done with various tools, such as pill cutters, scissors, knives, scalpels, and hands (Arnet & Hersberger, 2010; Verrue et al., 2011). Some patients report using their teeth to break the tablet into two pieces. Some tablets come with a line or bisect, frequently referred to as a score; these scores are usually a sign that those tablets can be split, supposedly guaranteeing that each piece of the tablet will contain the same amount of active principle or just weigh exactly the same as the other half. However, not all scores are a sign of this; some of these bisects have an aesthetic purpose (Rowley F, s. f., 2006; Thompson, 2012), which can be misleading and people end up splitting a tablet that will not render two equal parts, with identical weight and amount of drug. State agencies, like the U.S. Food and Drug Admistration (FDA, 2013), even give advice as to how to split a tablet as "adequately" as possible. The purpose of this study is to investigate whether people who split various medicines using different tools can effectively split tablets into equal halves.

MATERIAL AND METHODS

Knives and scissors were purchased in a supermarket while tablet cutters, brand warfarin and metoprolol, and generic clonidine and losartan were acquired from a local pharmacy. Subjects participated in this study after complying with inclusion criteria

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(medical doctors, nurses, pharmacists, patients, and caregivers) and exclusion criteria (mental illness, Parkinson's, and any neuromotor disease), and were enrolled after signing the informed consent. There were three subjects for each profession. Care givers' mean age was 45 (two females and one male); patients' mean age was 75 (two males and one female); medical doctors' mean age was 50 (two males and one female); nurses' mean age was 32 (all females); pharmacists' mean age was 30 (one male and two females). All splitting was done in triplicate using an instrument and an active principle.

The tablets were all round. Metoprolol and warfarin were scored and had one score line each to split them into two halves. The insert had no information as to whether this score was aesthetical or functional. Only the metoprolol was flat. The amount for each drug is as follows: clonidine: 0.15 mg, losartan and metoprolol: 50 mg, and warfarin: 5 mg. These drugs were selected because previously, different hospitals were contacted by phone asking them which drugs they split more often and these four drugs were the most split in these institutions.

For each tablet piece or "half," the deviation from the theoretical weight and the weight loss were calculated as follows: theoretical weight = weight of the tablet before splitting/2; deviation (%) from theoretical weight = (weight of the tablet piece - theoretical weight)/theoretical weight x 100; weight loss = weight of the tablet before splitting - sum of 2 tablet halves (Verrue et al., 2011). The limits for deviation from theoretical weight were set on 15% and 25%, as these are the reference values in industry guidelines when testing for content uniformity (United States Pharmacopeial Convention Inc., 2018).

Statistical analysis

Statistical analyses were performed with the generalized linear model, binomial family, and logarithmic link function using cluster robust estimation of variance for replications. The risk of inappropriate tablet splitting was obtained from marginal estimations for deviation over 15% and 25%. To analyze the influence of each factor in inappropriate splitting, risk ratios (RR) with 95% confidence intervals (95%CI) were calculated for each factor independently and for their interaction. Statistical analyses were performed using the Stata 16.1 software (College Station, TX).

Ethical considerations

As the population manipulated sharp objects in order to split the tablets, this research was labeled with a minimum risk to participants according to local regulations. This investigation was approved by CES university ethical committee, code 721 and only the subjects that signed an informed consent were enrolled in the study.

RESULTS

Table 1 displays the risk of splitting tablets into two unequal halves for the drug regardless of instrument and population, and the same applies for instrument (regardless of drug and population) and population (regardless of drug and instrument). Table 1 provides an overview of which drug, tool, and population are most prone to splitting unevenly. When splitting tablets, none of the subjects except one mentioned the score line, stating that its purpose is to make an even split easier; also, a nurse and a patient split tablets using the scissors' blade as a knife. The nurses and patients had the most difficulty when splitting tablets by

Table 1. Risk and risk ratio of nonequal pill splitting according to drug, instrument, and role.

	Deviation >15%				Deviation >25%			
	Risk	RR	CI95%	*p*-value	Risk	RR	CI95%	*p*-value
Drug								
Clonidine	37.09				26.55			
Warfarin	14.08	0.38	(0.28 - 0.51)	0.000	5.55	0.21	(0.11 - 0.38)	0.000
Metoprolol	13.46	0.36	(0.25 - 0.52)	0.000	2.79	0.10	(0.05 - 0.22)	0.000
Losartan	24.80	0.67	(0.47 - 0.96)	0.027	14.74	0.56	(0.34 - 0.9)	0.018
Instrument								
Scissors	36.00				18.16			
Pill cutter	16.16	0.45	(0.3 - 0.66)	0.000	2.80	0.15	(0.07 - 0.36)	0.000
Knife	19.55	0.54	(0.36 - 0.81)	0.003	18.37	1.01	(0.71 - 1.44)	0.950
Hand	17.71	0.49	(0.35 - 0.7)	0.000	10.30	0.57	(0.32 - 1.01)	0.056
Role								
Patient	28.85				11.38			
Caregiver	20.63	0.71	(0.49 - 1.04)	0.083	14.42	1.27	(0.77 - 2.07)	0.347
Nurse	22.09	0.77	(0.53 - 1.11)	0.159	13.58	1.19	(0.72 - 1.97)	0.488
MD	18.42	0.64	(0.43 - 0.94)	0.023	9.76	0.86	(0.52 - 1.42)	0.551
Pharmacist	21.79	0.76	(0.47 - 1.21)	0.243	12.90	1.13	(0.57 - 2.26)	0.723

RR: risk ratio, CI: confidence interval

hand, and in some cases they used the butt of the knife to hit the tablet and then proceeded to break the tablet into two pieces with their hands; this was seen with clonidine and metoprolol. All subjects agreed that the clonidine tablet was the most challenging to split since it has no score line and it has a small size. With a deviation of more than 15% of theoretical weight, patients were 28.85% of the population, which tended to split all tablets more unevenly, followed by nurses, pharmacists, caregivers, and medical doctors. The instrument for splitting tablets that rendered the most unequal weights was scissors, with a rate of 36%, followed by knife, hand, and pill cutter. It was no surprise to find that clonidine was the tablet with the highest variations in the halves' weight differences after splitting due to its smaller size and lack of a score line. The second highest variation was that of losartan, followed by warfarin and metoprolol. In deviations greater than 25%, the higher differences were seen with the scissors and knife. The pill cutter had a lower risk of splitting unequally. Also, for deviations greater than 25%, caregivers had greater differences in weight followed closely by pharmacists and patients, as opposed to the findings for deviations greater than 15% mentioned above. The scored tablets were easier to split but not equally. Halves were not the same weight in all cases in this study.

Table 2 focuses on the differences between drugs and instruments. The findings show that these two variables have the highest probability of splitting unequally, exhibiting greater differences in weight deviation. In this table, the reference taken was clonidine and scissors; this combination has the highest risk of splitting unequally (55.56% for a deviation greater than 15%) and for deviations beyond 25%, the clonidine and knife combination was the highest but not statistically significant. The case of metoprolol and hand is the lowest. Scissors was the instrument with the highest weight variations for losartan, as well.

Table 3 shows the mean of weight loss for drug, instrument, and subject because it has been seen that after splitting a tablet not only do the two halves have an unequal weight but also one or both halves lose mass. It can be seen that after splitting (regardless of instrument and subject), clonidine was the drug that lost more weight. In some cases, the mass lost was not big, but in some others (almost one half) the majority of their mass was lost. Taking this into consideration, the risk of patients not receiving the needed amount is too high. Warfarin lost an average of 0.003 g out of 0.005 g. That is more than half of the drug's intended dose. Once again, this weight loss becomes a serious risk if the therapeutic index of the drug is considered.

Weight loss for any drug was higher when scissors or knives were used (a mean of 0.010 g for scissors and 0.011 g for knives). If we consider just clonidine or warfarin, the amount of drug lost surpasses that of the drug amount. In contrast, the pill cutter lost an average weight lower than any other instrument; these losses are very high and might endanger patients' safety. As for weight loss related to subjects, this was higher for medical doctors and nurses, and lower for pharmacists, caregivers, and patients.

Table 2. Risk and risk ratio of nonequal pill splitting regarding drug and instrument.

Drug	Instrument	Deviation >15%				Deviation >25%			
		Risk	RR	95%CI	*p*- value	Risk	RR	95%CI	*p*- value
Clonidine	Scissors	55.56	1.00			35.56			
	Pill cutter	35.56	0.64	(0.43 - 0.96)	0.032	8.89	0.25	(0.1 - 0.63)	0.003
	Knife	28.89	0.52	(0.28 - 0.98)	0.044	37.78	1.06	(0.69 - 1.63)	0.783
	Hand	33.33	0.60	(0.38 - 0.95)	0.030	26.67	0.75	(0.36 - 1.57)	0.444
Warfarin	Pill cutter	0.00	NE			0.00	NE		
	Scissors	24.44	0.44	(0.25 - 0.78)	0.005	11.11	0.31	(0.12 - 0.82)	0.019
	Knife	13.33	0.24	(0.12 - 0.46)	0.000	4.44	0.13	(0.03 - 0.48)	0.002
	Hand	17.78	0.32	(0.17 - 0.6)	0.000	6.67	0.19	(0.06 - 0.55)	0.002
Metoprolol	Pill cutter	4.44	0.08	(0.02 - 0.3)	0.000	0.00	NE		
	Scissors	24.44	0.44	(0.26 - 0.74)	0.002	6.67	0.19	(0.06 - 0.55)	0.002
	Knife	13.33	0.24	(0.11 - 0.54)	0.001	2.22	0.06	(0.01 - 0.43)	0.005
	Hand	11.11	0.20	(0.09 - 0.42)	0.000	2.22	0.06	(0.01 - 0.43)	0.005
Losartan	Pill cutter	22.22	0.40	(0.19 - 0.85)	0.017	2.22	0.06	(0.01 - 0.43)	0.005
	Scissors	42.22	0.76	(0.55 - 1.04)	0.088	22.22	0.63	(0.33 - 1.17)	0.141
	Knife	24.44	0.44	(0.2 - 0.95)	0.037	28.89	0.81	(0.46 - 1.44)	0.479
	Hand	8.89	0.16	(0.07 - 0.38)	0.000	4.44	0.125	(0.03 - 0.48)	0.002

RR: risk ratio, CI: confidence interval

Table 3. Mean and mean difference of weight loss.

	Mean (g)	Diff.	95%CI		*p*-value
Drug					
Clonidine	0.014	0.000			
Warfarin	0.003	-0.009	-0.020	0.002	0.105
Metoprolol	0.004	-0.008	-0.018	0.003	0.174
Losartan	0.009	-0.003	-0.016	0.009	0.599
Instrument					
Scissors	0.010	0.000			
Pill cutter	0.002	-0.004	-0.007	-0.002	0.003
Knife	0.011	0.002	-0.002	0.005	0.350
Hand	0.007	-0.002	-0.008	0.004	0.497
Role					
Patient	0.004	0.000			
Care giver	0.004	0.000	-0.001	0.001	0.497
Nurse	0.011	0.006	-0.003	0.016	0.198
MD	0.015	0.010	-0.004	0.025	0.167
Pharmacist	0.003	0.000	-0.001	0.001	0.638

CI: confidence interval

DISCUSSION

One out of five tablets was unequally split with a deviation >15%. This should be looked at carefully because it is higher than the 15% recommended by quality control in USP Pharmacopeia (United States Pharmacopeial Convention Inc., 2018). Furthermore, all tablets' halves lost weight and none of them weighed the same as the other half. In this study, deviations higher than 25% were also seen. Regardless of statistics, all tablets' halves did not weigh the same and did not weigh the theoretical weight. They lost either excipients or drug amount. This is relevant because the quantity lost might lead to therapeutic failure or, if one half weighs more, as it is the case of clonidine in Table 2, this could end up having toxic effects. Other papers have paid little attention to the distribution of the active ingredient in the tablet (Cook et al., 2004; Elliott et al., 2014). It is relevant to recall that in addition to the drug, excipients are also added to the formulation (Haywood & Glass, 2011; Palcsó & Zelkó, 2018), and as they are mixed together it makes it difficult to know the active ingredient distribution in the tablet (Shah et al., 2010). This raises the question of whether the patients are taking mostly the drug or the excipients. When splitting a losartan tablet, if one half of the tablet is bigger and weighs more, this might pose a threat to the therapeutic goals and to the safety of the patient. As was mentioned above, the active ingredient does not distribute homogenously in the tablet, making the splitting practice riskier (Shah et al., 2010; Veronin & Youan, 2004).

The therapeutic index is something to consider, too. In this study, two narrow-range drugs (clonidine and warfarin) (Johnson, 2012; Spiller et al., 2005) were studied. The toxic effects could even be lethal given that a very high amount or the total amount of these active ingredients might be present in just one half. The clonidine tablets used in our assay came in 0.00015g amounts, however the tablets weighed around 0.12 g. This clearly shows that 0.11985 g are excipients. Given these amounts, the active ingredient may be in just one part of the tablet or unevenly distributed. Now, if warfarin is considered, the active ingredient weight is 0.005g and the whole tablet is around 0.2g. The content of the tablet is 97.5% excipients. The probability of active ingredient uneven distribution is higher, as is the risk of the patient not receiving the required dose.

CONCLUSION

There are some uncertainty factors regarding the practice of splitting tablets. First, some factors are dependent on the tablet itself, such as if they are scored or unscored (the latter being more difficult to split and with a higher risk of splitting unequally), active ingredient heterogeneous distribution, and therapeutic index. Second, some are dependent on the subject doing the splitting (age and disease have an impact on the practice of tablet splitting). Third, some are dependent on the instrument used to split the tablet. These many variables make it seem unreasonable to instruct a patient to split a tablet. The risk of not getting the right amount for their therapy is too high, and this means that toxic effects or therapy failure might happen. Both pose a problem, therefore it is not worthwhile to run the risk of splitting. Other alternatives, such as different formulations or extended release tablets, should be considered.

Ethics Committee Approval: This investigation was approved by CES university ethical committee, code 721 and only the subjects that signed an informed consent were enrolled in the study.

Peer-review: Externally peer-reviewed.

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REFERENCES

- Arnet, I., & Hersberger, K. E. (2010). Misleading score-lines on tablets: Facilitated intake or fractional dosing? *Swiss Medical Weekly*, *140*(7-8), 105-110. https://doi.org/smw-12953
- Cook, T. J., Edwards, S., Gyemah, C., Shah, M., Shah, I., & Fox, T. (2004). Variability in tablet fragment weights when splitting unscored cyclobenzaprine 10 mg tablets. *Journal of the American Pharmacists Association: JAPhA*, *44*(5), 583-586. https://doi.org/10.1331/1544-3191.44.5.583.cook
- Elliott, I., Mayxay, M., Yeuichaixong, S., Lee, S. J., & Newton, P. N. (2014). The practice and clinical implications of tablet splitting in international health. *Tropical Medicine & International Health: TM & IH*, *19*(7), 754-760. https://doi.org/10.1111/tmi.12309
- FDA. (2013). *Best Practices for Tablet Splitting*. https://www.fda.gov/drugs/ensuring-safe-use-medicine/best-practices-tablet-splitting
- Haywood, A., & Glass, B. D. (2011). Pharmaceutical excipients – where do we begin? *Australian Prescriber*, *34*(4), 112-114. https://doi.org/10.18773/austprescr.2011.060
- Johnson, J. A. (2012). Warfarin pharmacogenetics: A rising tide for its clinical value. *Circulation*, *125*(16), 1964-1966. https://doi.org/10.1161/CIRCULATIONAHA.112.100628
- Palcsó, B., & Zelkó, R. (2018). Different types, applications and limits of enabling excipients of pharmaceutical dosage forms. *Drug Discovery Today. Technologies*, *27*, 21-39. https://doi.org/10.1016/j.ddtec.2018.04.002
- Rowley F. (s. f.). Minimize Bisect Risk: Part I. *Oct 06, 2006*. https://www.pharmamanufacturing.com/articles/2006/188/
- Shah, R. B., Collier, J. S., Sayeed, V. A., Bryant, A., Habib, M. J., & Khan, M. A. (2010). Tablet splitting of a narrow therapeutic index drug: A case with levothyroxine sodium. *AAPS PharmSciTech*, *11*(3), 1359-1367. https://doi.org/10.1208/s12249-010-9515-8
- Spiller, H. A., Klein-Schwartz, W., Colvin, J. M., Villalobos, D., Johnson, P. B., & Anderson, D. L. (2005). Toxic clonidine ingestion in children. *The Journal of Pediatrics*, *146*(2), 263-266. https://doi.org/10.1016/j.jpeds.2004.09.027
- Thompson, C. A. (2012). New term will distinguish tablets known to split in half. *American Journal of Health-System Pharmacy: AJHP: Official Journal of the American Society of Health-System Pharmacists*, *69*(19), 1619-1621. https://doi.org/10.2146/news120071
- United States Pharmacopeial Convention Inc. (2018). USP 41-NF 36. En *United States Pharmacopeia and National Formulary*. https://www.deutscher-apotheker-verlag.de/shop/produkt/9783769270228/usp-41-nf-36-the-united-states-pharmacopeia-and-national-formulary-2018
- Veronin, M. A., & Youan, B.-B. C. (2004). Medicine. Magic bullet gone astray: Medications and the Internet. *Science (New York, N.Y.)*, *305*(5683), 481. https://doi.org/10.1126/science.1097355
- Verrue, C., Mehuys, E., Boussery, K., Remon, J.-P., & Petrovic, M. (2011). Tablet-splitting: A common yet not so innocent practice. *Journal of Advanced Nursing*, *67*(1), 26-32. https://doi.org/10.1111/j.1365-2648.2010.05477.x

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Original Article

Determination of the chemical composition, antioxidant potential of *Sambucus ebulus* L. (dwarf elder) fruit extracts and investigation of antimicrobial activity on *Trichophyton rubrum* (Castell.) Sabour and some microorganisms

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ABSTRACT

Background and Aims: *Sambucus ebulus* L. is one of the medicinal plants well known in the traditional medicine of Anatolia since ancient times. The present study was aimed to investigate the antifungal potential of *S. ebulus* fruit extracts against *Trichophyton rubrum* (Castell.) Sabour as well as phytochemical composition, antibacterial, and antioxidant activities based on traditional usage.

Methods: Two extracts were prepared from *S. ebulus* fruits. The phytochemical composition of *S. ebulus* fruit extracts was identified by LC-MS/MS. The antimicrobial activity was examined by using the broth microdilution method against a panel of microorganisms. In addition to this, *S. ebulus* extracts were evaluated for their *in vitro* antifungal activity against three yeast and *T. rubrum* by disc diffusion method.

Results: The major compounds were determined in dried fruit methanol extract (DFM) as hederagenin (5.38±0.4949 µg/g) and fumaric acid (3.06±0.0275µg/g). The fumaric acid (3.97±0.0357µg/g) was detected as the abundant compound in the fresh fruit juice (FFJ). Acacetin, chrysin, eupatilin, hederagenin, isosakuranetin, myricitrin, and rhamnocitrin were detected in the extracts for the first time. DFM showed moderate activity against *E. coli* (MIC: 625 mg/L) and *Candida tropicalis* (MIC: 312.5 mg/L). Both extracts possessed weak activity against *Proteus mirabilis* and *Staphylococcus aureus*,which had the MIC values 1250 mg/L. *T. rubrum* was found resistant to both extracts. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical cleaning method was used to measure the antioxidant capacity of the extracts. DFM and FFJ exhibited strong antioxidant activity against DPPH radicals with IC50 value of 5.941±0.236 µg/mL and 7.893±0.939 µg/mL, respectively.

Conclusion: As a conclusion, although *S. ebulus* fruits are used to treat nail fungus (*onychomycosis*) by local folk, our results showed that it could not be useful to use in the antifungal topical formulations. In addition to this, the antibacterial activity result is parallel to the results of studies in this particular so far.

Keywords: *Sambucus ebulus*; LC-MS/MS; antifungal activity; *Trichophyton rubrum*; antioxidant activity

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INTRODUCTION

The genus *Sambucus* belongs to the Adoxaceae family, which comprises of 30 species all over the world, including out of which two, namely *Sambucus nigra* and *Sambucus ebulus*, have been recorded being from Turkey (Scopel et al., 2007; Senica, Stampar, & Mikulic-Petkovsek, 2019). *S. ebulus* L., whose common name is elderberry or dwarf elder, a kind of shrub, is widely distributed in southern and central Europe and southwest Asia (especially in Iran and Turkey) (Shokrzadeh & Saravi, 2010). The rhizomes, stem barks, aerial parts, leaves, flowers, and fruits of *S. ebulus* have long been used to treat different diseases such as colds and coughs, arthritis, edema, rheumatic diseases, constipation, infected wounds, eczema, burns, urticaria, hemorrhoids, and bee stingsin Turkish traditional medicine (Demirci & Özhatay, 2012; Kültür, 2007; Tuzlacı & Tolon, 2000; Yesilada, 1997). According to their extensive usage in treatment, *S. ebulus* is called "hekimana," which means "the mother of the physician" by Anatolian folk (Jabbari, Daneshfard, Emtiazy, Khiveh, & Hashempur, 2017; Yesilada, Gürbüz, & Toker, 2014). Traditionally, uses of *S. ebulus* fruits to treat nail fungus infections were reported for the first time in this study (Demirci & Özhatay, 2012). We were therefore inspired to organize the current study because of this remarkable knowledge obtained from the countryside in Kahramanmaras, Turkey.

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

Aiming to reveal the new pharmaceutical applications of these plants, numerous studies have been focused on investigating their biological activities. Among them, antiinflammatory (Ahmadiani, Fereidoni, Semnanian, Kamalinejad, & Saremi, 1998; M. Ebrahimzadeh, Mahmoudi, & Salimi, 2006; Yesilada, 1997), antinociceptive (Ahmadiani et al., 1998; M. Ebrahimzadeh, Mahmoudi, Saiednia, Pourmorad, & Salimi, 2006), antimicrobial (Rodino et al., 2015; Salehzadeh, Asadpour, Naeemi, & Houshmand, 2014), antiherpes simplex (Zahmanov et al., 2015), antiulcerogenic (Yesilada et al., 2014), antioxidant (Cvetanović, 2020; Hashemi, Ebrahimzadeh, & Khalili, 2019), antihypoxic(Kaveh, Mohamadyan, & Ebrahimzadeh, 2019), hypolipidemic (Ivanova, Tasinov, & Kiselova-Kaneva, 2014), and wound healing (Süntar et al., 2010) activities have been demonstrated.

Incidentally, besides the studies that investigated the chemical compositions and biological activities of *S. ebulus*, some ethnopharmacological studies were also carried out by scientists. One of them reported that the leaves and fruits of *S. ebulus* were traditionally used to handle stomach pain, snake bites, and coughs (Kültür, 2007). Another study conducted by Demirci and Ozhatay indicated that the fruits of *S. ebulus* are used for the treatment of hemorrhoids, rheumatism, and nail fungus in Kahramanmaraş, Southern Turkey (Demirci & Özhatay, 2012).

Traditional uses of *S. ebulus* fruits to treat nail fungus infections were reported for the first time in this study (Demirci & Özhatay, 2012). We were therefore inspired to organize the current study because of this remarkable knowledge obtained from the countryside in Kahramanmaras, Turkey.

Nail fungus, also called *onychomycosis* or *tinea unguium*, is a fungal infection of the nail caused mainly by *Trichophyton rubrum*. It may cause pain, discomfort, cosmetic problems, and daily and social life limitations, and, consequently, reducesquality of life (Lipner & Scher, 2019). In the study conducted by Demirci and Ozhatay (2012), it was reported that the locals had been applying the crushed fresh fruits on the infected nail for the treatment of onychomycosis (Demirci & Özhatay, 2012). They repeat the procedure every two or three days until the nail is fully recovered. Therefore, the goal of the current study is to identify the chemical composition, antibacterial, antifungal, and antioxidant activities of *S. ebulus* fruits collected from Kahramanmaras, Turkey based on traditional uses in that area.

It has been suggested that some cases of *onychomycosis* are associated with a periungual inflammation while others may be associated with low-grade systemic inflammation (Duhard, 2014; Shi et al., 2016; Sinikumpu et al., 2018). The inflammatory response is the mechanism of injury involving oxidative stress (Balea, Pârvu, Pop, Marín, & Pârvu, 2018). Reactive oxygen species (ROS) can circulate freely in the cell by passing through the membranes. Then, ROS damage DNA, RNA, proteins, and lipids. As a result, it causes local or systemic damage (Andreicuț et al., 2018). Moreover, untreated onychomycosis can lead to further infection (Gupta, Versteeg, & Shear, 2017). Given these circumstances, oxidative stress should also be considered as a secondary target in onychomycosis treatments (Pârvu et al., 2019).

The therapeutic properties of plants are derived from chemical compounds isolated from extracts and essential oils that can scavenge free radicals (Granato et al., 2018; Morais et al., 2013). Extracts and essential oils are frequently used as antimicrobial agents in traditional medicine due to their antiseptic and antioxidant effects (Bakkali, Averbeck, Averbeck, & Idaomar, 2008; Nogueira et al., 2020).

MATERIAL AND METHODS

Plant material

S. ebulus fruits were collected from Andırın, Kahramanmaraş in June 2018. The voucher specimen was deposited at the Faculty of Pharmacy of Cukurova University Herbarium (CUEF 1671).

Extraction and fractionation

The fresh fruits were divided into two parts, then the first part was dried at room temperature in shade, and then the dried material was extracted with methanol: water (50:50; v/v) by using a shaker at 25 °C for 24 hours. The procedure was repeated 4 times until the samples were exhausted. After filtration, the solvent was removed by rotary, and the water was removed by lyophilization. The extract (DFM) was stored at -20 °C until the analysis. The other part (fresh fruits) was squeezed.

LC-MS/MS analysis

A Thermo Orbitrap Q-Exactive instrument in ESI Source was used for LC-HRMS (Liquid Chromatography High-Resolution Mass Spectrometry) measurements. The validated method was used for the analysis, and validation parameters of the method were reported by Gülçin et al. 2010 (Gülçin, Bursal, Şehitoğlu, Bilsel, & Gören, 2010; Han, Yilmaz, & Gulcin, 2018).

Determination of Minimum Inhibitory Concentrations (MIC)

Antimicrobial activities against standard microorganisms are listed in Table 1. Antimicrobial activity was identified by the broth microdilution technique using the Clinical Laboratory Standards Institute (CLSI) recommendations (CLSI, 1997; CLSI, 2020). For antimicrobial activity, Mueller–Hinton broth (Oxoid) and for antifungal activity, RPMI-1640 (Applichem, Darmstadt, Germany) medium were used as the medium. The extract by making serial twofold dilutions ranging from 2500 mg/L to 1.2 mg/L was produced in the media. The inoculum was made to give a final concentration of 5×10^5 CFU/mL for bacteria and 0.5×10^3 to 2.5×10^3 CFU/mL for yeast in the 96 well plate. The 96 well plates were covered and placed in plastic bags to prevent evaporation. The microplates were incubated at 35ºC for 18–20 h for bacteria while the microplates were incubated at 35ºC for 46–50 h for yeast strains. The MIC was defined as the lowest concentration of compound giving complete inhibition of visible growth. As a control, the antimicrobial effects of the solvents were investigated against test microorganisms. The results were evaluated according to the values of the controls.

Table 1. The quality control strains used to test with the extracts of *Sambucus ebulus* fruits.

Tested microorganisms
Staphylococcus aureus ATCC 29213
Staphylococcus epidermidis ATCC 12228
Escherichia coli ATCC 25922
Klebsiella pneumoniae ATCC 4352
Pseudomonas aeruginosa ATCC 27853
Proteus mirabilis ATCC 14153
Enterococcus faecalis ATCC 29212
Candida albicans ATCC 10231
Candida parapsilosis ATCC 22019
Candida tropicalis ATCC 750

ATCC: American Type Culture Collection, 12301, Parklawn Drive, Rockville, MD 20852, USA.

Antifungal susceptibility testing by disc diffusion method

A standard *Trichophyton rubrum* (ATCC 28188) isolate was applied for *in vitro* antifungal evaluation by using the CLSI M38-A guidelines. RPMI 1640 medium (Applichem, Darmstadt, Germany) was buffered to pH 7.0 with morpholinopropanesulfonic acid (MOPS; Applichem, Darmstadt, Germany) and filtered with a membrane filter for sterilization. A 2% glucose and 2% agar were suspended in 200 mL distilled water and autoclaved for sterilization. The RPMI 1640 medium and the solution of agar were combined in a water bath at 45–50°C. The mixture was passed onto plastic petri dishes and cooled at room temperature. The inoculum was prepared according to the CLSI M38-A criteria *T. rubrum* suspension was diluted to reach conidium concentration as $1–5x10^6$. The inoculum was dispersed to the area of 2% glucose RMPI 1640 agar plates and desiccated for 15 min. A hole (diameter; 2 mm, height; 4 mm) was opened in the middle of the agar plates. The extracts by making serial twofold dilutions ranging from 10000 mg/L to 1.2 mg/L were put in this hole with a sterile spatula and forceps. Plates were kept at 26°C for 4–7 days and investigated for the proliferation of fungus and zone diameter of inhibition. Ketoconazole (10 µL Liofilchem) was used as a reference drug for evaluating the inhibition zone diameter according to CLSI criteria (CLSI, 1997; CLSI, 2020).

2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals scavenging method

The antioxidant activity of *S. ebulus* extracts was measured by the DPPH radicals scavenging method, which was established by Blois (1958) and Brand-Williams et al. (1995) and was adapted for 96-well plates (Blois, 1958; Brand-Williams, Cuvelier, & Berset, 1995). Extracts were dissolved in ultra pure water, and dilutions were made with methanol. Ascorbic acid (Aldrich, St. Louis MO, USA) was used as a positive control. The negative control was without any *S. ebulus* extracts. 50 µL of 0.1 mM DPPH (Aldrich, St. Louis MO, USA) radical solution, which was freshly made in methanol (Aldrich, St. Louis MO, USA), was added to 150 µL of extracts or standards in 96-well plates. The plates were shaken 1 min with the microplate reader (Thermo Scientific™, Multiskan™ Sky Microplate Spectrophotometer, Waltham, MA, USA) and were incubated 45 min in the dark at room temperature at 517 nm % of DPPH scavenging activity was calculated according to % DPPH Scav. Act. = [(AControl – ASample)/AControl] × 100 formula.

Statistical analysis

Data were expressed as mean ± standard deviation (mean ± SD) of experiments. The experiment was repeated four times (n=4). The data were analyzed by Microsoft Office Excel and analyzed by an analysis of variance ($p<0.05$). The statistical evaluation of data was performed using a one-way analysis of variance (ANOVA) and Tukey multiple comparisons.

RESULTS AND DISCUSSION

LC-MS/MS analysis

In the current study, phytochemical compositions of the dried fruits methanol extract (DFM) and fresh fruits juice (FFJ) of *S. ebulus* collected from Kahramanmaraş, Turkey were determined by LC-MS/MS. The results of the LC-MS/MS analysis are shown in Table 2. In the DFM extract, 10 compounds were present. The most abundant secondary metabolites in the DFM were hederagenin (5.38±0.4949 µg/g) and fumaric acid (3.06±0.0275 µg/g). Additionally, hederagenin was only found in the DFM extract. Other compounds that were detected in DFM were acacetin (0.13±0.0001 µg/g), chrysin (0.13±0.0001 µg/g), eupatilin (0.14±0.0001 µg/g), hyperoside (0.25±0.0075 µg/g), isosakuranetin (0.01±0.0001 µg/g), myricitrin (0.09±0.0027 µg/g), naringenin (0.11±0.0046 µg/g), and rutin (0.11±0.0004 µg/g). In the FFJ extract of *S. ebulus*, 9 compounds were determined. FFJ is the richest extract in terms of one of the dicarboxylic acid derivates, fumaric acid

Table 2. Quantitative determination (µg/g) of 23 phytochemicals in the extracts of *Sambucus ebulus* fruits.

	Content of the extracts (µg/g)		
Compounds	**DFM**	**FFJ**	**U % (k=2)**
(-)-Epicatechin	<LOD	<LOD	3.6
(-)-Epigallocatechin gallate	<LOD	0.01±0.0002	2.7
(+)-Trans Taxifolin	<LOD	<LOD	3.0
Acacetin	0.13±0.0001	0.13±0.0001	1.5
Caffeic acid	<LOD	<LOD	2.4
Chrysin	0.12±0.0001	0.13±0.0001	1.2
Dihydrokaempferol	<LOD	<LOD	3.8
Eupatilin	0.14±0.0001	0.11±0.0001	1.4
Fumaric Acid	3.06±0.0275	3.97±0.0357	0.9
Hederagenin	5.38±0.4949	<LOD	9.2
Herniarin	<LOD	<LOD	2.4
Hispidulin	<LOD	<LOD	1.7
Hyperoside	0.25±0.0075	0.63±0.0189	3.0
Isosakuranetin	0.01±0.0001	<LOD	1.2
Myricitrin	0.09±0.0027	0.46±0.0142	3.1
Naringenin	0.11±0.0046	<LOD	4.2
Nepetin-7-glucoside	<LOD	<LOD	4.4
Orientin	<LOD	<LOD	3.8
Quercitrin	<LOD	<LOD	4.8
Rhamnocitrin	<LOD	0.01±0.0003	3.2
Rutin	0.11±0.0004	0.34±0.0153	4.5

DFM: Dried fruit methanol extract; FFJ: Fresh fruit juice; LOD: Limit of detection

(3.97±0.0357µg/g). Also, (-)-Epigallocatechin gallate (0.01±0.0002 µg/g), acacetin (0.13±0.0001 µg/g), chrysin (0.13±0.0001 µg/g), eupatilin (0.11±0.0001 µg/g), hyperoside (0.63±0.0189 µg/g), myricitrin (0.46±0.0142 µg/g), rhamnocitrin (0.01±0.0003 µg/g), and rutin (0.34±0.0153 µg/g) were identified in the extract.

A few numbers of studies have been conducted on the chemical composition of *S. ebulus* fruits. In a previous study, acetone extract of the ripe fruits was analyzed using an LC-PDA-MS method. According to the results of the study, epicatechin (0.84 mg/100 g FW: fresh weight), quercetin (0.15 mg/100 g FW), and kaempferol (0.05 mg/100 g FW) were identified in the extract (Vankova, Todorova, Kisselova-Kaneva, & Galunska, 2019). Another study was also carried on to determine the secondary metabolites in water extract of *S. ebulus* fruits by HPLC. The results show that gallic acid (868.98 mg/mL), protocatechuic acid (39.16 mg/mL), chlorogenic acid (36.82 mg/mL), caffeic acid (17.21 mg/mL), ferulic acid (3.38 mg/mL), naringin (1.97 mg/mL), and rutin (6.53 mg/mL) were identified in the fruit water extract of *S. ebulus* (Cvetanović et al., 2018). In another study conducted by Mikulic-Petkovsek et al, *S. ebulus* fruit extract was analyzed using HPLC-DAD-MS; according to the result, quercetin-3-rutinoside (421.14±5.15 mg/kg FW) was the major compound in the extract. The other compounds were reported as quercetin-3-glucoside (42.30±1.37 mg/kg FW), quercetin-3-galactoside (79.54±2.75 mg/kg FW), quercetin-hexosidepentoside (77.08±1.83 mg/kg FW), kaempferol-3-rutinoside (77.22±1.19 mg/kg FW), isorhamnetin-3-rutinoside (145.75±1.49 mg/kg FW), and isorhamnetin-hexoside (29.05±0.79 mg/kg FW) (Mikulic-Petkovsek, Ivancic, Todorovic, Veberic, & Stampar, 2015). As reported by Cvetanovic et al. (2016), 13 compounds were detected in *S. ebulus* fruits by using HPLC-DAD (Cvetanovic et al., 2016). The study result showed that rutin (6.453 µg/mL), quercetin (1.407 µg/mL), and synaptic acid (1.291 µg/mL) were contained as a major compound in the fruits. The other compounds were determined as *p*-hydroxybenzoic acid (0.430 µg/mL), vanillic acid (0.506 µg/mL), syringic acid (0.378 µg/mL), *p*-coumaric acid (0.241 µg/mL), ferulic acid (0.212 µg/mL), rosmarinic acid (0.241 µg/mL), luteolin (0.134 µg/mL), naringenin (0.164 µg/mL), kaempferol (0.407 µg/mL), and apigenin (0.262 µg/mL).

Evaluation of the *in vitro* antimicrobial activity

In the present study, four Gram-negative bacteria (*P. aeruginosa, E. coli, P. mirabilis,* and *K. pneumoniae*), three Gram-positive bacteria (*S. aureus, S. epidermidis,* and *E. faecalis*) for antibacterial activity, and three yeast (*C. albicans, C. tropicalis,* and *C. parapsilosis*) for antifungal activity were used to preliminarily screen their antibacterial and antifungal activity employing the standard drugs using the broth microdilutions method according to the Clinical Laboratory Standards Institute (CLSI) recommendations (CLSI, 1997; CLSI,

2020). The MIC values are summarized in Table 3. Taken together, with MIC values, the results identified that most of the tested compounds showed weak activity against the studied microorganisms. The test-cultures *P. aeruginosa, E. faecalis, K. pneumoniae,* and *S. epidermidis* in addition to *C. albicans* and *C. parapsilosis* appeared as resistant to all the studied extracts. However, all the studied extracts possessed activity against *P. mirabilis* and *S. aureus* which had the MIC values 1250 mg/L. As indicated in Table 3, among the extracts DFM showed moderate antimicrobial activity against *E. coli* (MIC: 625 mg/L) and *C. tropicalis* (MIC: 312.5 mg/L). Concerning the antifungal activity of DFM and FFJ, *T. rubrum* was found resistant to both extracts of all the tested concentrations.

Conforming to the literature survey, there are not many studies that covered the antimicrobial activity of *S. ebulus* fruits. In one of the studies among them, antimicrobial activities of chloroform, acetone, hexane, water, and methanol extracts obtained from *S. ebulus* fruits were tested against *Staphylococcus aureus, Bacillus subtilis, Pseudomanas aeruginosa, Escherichia coli, Salmonella typhimurium, Candida guillermondii,* and *Candida albicans* by using well diffusion method. The result showed that the extracts did not show activity against these strains compared to gentamicin and nystatin (Ginovyan & Trchounian, 2017). In another study, *S. ebulus* fruit extract was examined against *Staphylococcus aureus, S. epidermidis, E. coli, Klebsiella pneumoniae, P. aeruginosa, Proteus mirabilis,* and *C. albicans* to determine the antimicrobial activity by using the microbroth dilutions method. According to the result of the study, the extract had shown moderate antifungal activity against *C. albicans* and no activity against the other strains (Meriç, Bitiş, Birteksöz-Tan, Turan, & Akbuga, 2014). In the other study, antibacterial and antifungal activity of *S. ebulus* fruit extract were analyzed against *S. aureus, Bacillus cereus, B. subtilis, E. coli, Enterococcus faecalis, Pseudomonas fluorescens, Botrytis cinerea, Phytophthora infestans*, and *Rhizochtoniasolani* using the disc and the well diffusion methods. It was reported that the well diffusion method was better than the disc diffusion method to obtain the best results. In line with the results, it was observed that the extract had dose-dependent activity against *S. aureus, B. subtilis, E. fecalis,* and *P. fluores*, and it had resistance against *B. subtilis* and *E. coli* strains (Rodino et al., 2015). It seems that the antimicrobial activity results of the current study are in line with the results from literature.

Evaluation of the antioxidant activity

DPPH, a free radical cleaning method, was used in this study to measure antioxidant capacity. It is a colorimetric method that measures the conversion of the color of the radical solution from purple to yellow as a result of hydrogen or electron transfer (Braham et al., 2020; Do et al., 2014). This method has high sensitivity because it can detect antioxidant components in low concentrations. Besides this, it can analyze many samples simultaneously as a first step scanning strategy (Meza, Rojas, Cely-Veloza, Guerrero-Perilla, & Coy-Barrera, 2020). Many compounds are used as antioxidant standards. Ascorbic acid is most commonly used as the standard due to its strong scavenging activity (Al-Rifai, 2018). Table 4 shows the DPPH radical scavenging activity results of *S. ebulus* extracts compared to ascorbic acid.

Table 3.The MIC results (mg/L) of *Sambucus ebulus* fruits extracts.

	Microorganisms									
	***P. aeruginosa* ATCC 27853**	***E. coli* ATCC 25922**	***K. pneumoniae* ATCC 4352**	***P. mirabilis* ATCC 14153**	***E. faecalis* ATCC 29212**	***S. epidermidis* ATCC 12228**	***S. aureus* ATCC 29213**	***C. albicans* ATCC 10231**	***C. parapsilosis* ATCC 22019**	***C. tropicalis* ATCC 750**
FFJ	-	-	-	1250	-	-	1250	-	-	625
DFM	-	625	-	1250	-	-	1250	-	-	312.5
Reference antimicrobials	CAZ: 2.4	CFX: 4.9	CFX: 4.9	CFX: 2.4	AMK: 128	CFX: 9.8	CFX: 1.2	CL: 4.9	AMPHO: 0.5	AMPHO: 1

DFM: Dried fruit methanol extract; FFJ: Fresh fruit juice; CAZ: cefazidime; CFX: cefuroxime; AMK: amikacin; CL: clotrimazole; AMPHO: Amphotericin B.

Table 4. Antioxidant activities of *Sambucus ebulus* extracts.

	IC_{50} (µg/mL)	EC_{50} (mg/mg DPPH)	ARP Values	AEAC Values
DFM [a]	5.941±0.236	0.151	6.637	163075
FFJ [a,b]	24.784±0.873	0.629	1.591	39090
Ascorbic acid	9.688±1.025	0.246	4.070	-

IC_{50} values expressed are means ±S.D. of four measurements. The values having different superscript (Small alphabet and special character) letters within a column were significantly different ($p \leq 0.05$).
DFM: Dried fruit methanol extract, FFJ: Fresh fruit juice, EC_{50}: Effective concentration, ARP: Antiradical power, AEAC:Ascorbic acid equivalent antioxidant capacity.

Antioxidant parameters with IC_{50}, effective concentration (EC_{50}), antiradical power (ARP), and ascorbic acid equivalent antioxidant capacity (AEAC) values were calculated by drawing a logarithm graph with % radical scavenging capacity against the sample concentration. The AEAC value was calculated according to the formula 'AEAC=($IC_{50(AA)}$/$IC_{50(sample)}$) $\times 10^5$' after calculating the IC_{50} value. As can be seen from the formula, the AEAC value has no units. If low IC_{50} – EC_{50} and high ARP – AEAC values are the case, an antioxidant is reported to be stronger (Kedare & Singh, 2011). According to antioxidant parameters, the order of antioxidant potency for *S. ebulus* extract was as follow: DFM (IC_{50}: 5.941±0.236 µg/mL)> FFJ (IC_{50}: 7.893±0.939 µg/mL)> ascorbic acid (IC_{50}: 9.6880±1.02490 µg/mL) (Table 4). When the antioxidant activity of *S. ebulus* fruits was investigated by a DPPH experiment, different IC_{50} values were found in the literature. The study was conducted by Ebrahimzadeh et al. (2009) reported that the aqueous and methanol extracts of *S. ebulus* fruits collected from Mazandaran forest, Iran showed DPPH radical scavenging activity with IC_{50} value of 202.50 ± 1.38 µg/mL and 723.62 ± 3.36 µg/mL, respectively (M. A. Ebrahimzadeh, Ehsanifar, & Eslami, 2009). Another study, that was managed by Rodino et al. (2015), showed that the ethanol extract (70%) of *S. ebulus* fruits collected from Romania exhibited DPPH free radical scavenging potent with EC_{50} value of 68.45 ± 0.441µg/mL. The other study results indicated that aqueous and methanol extracts of *S. ebulus* fruits collected from Serbia demonstrated DPPH scavenging activity with IC_{50} value of 128.23 ± 0.65 µg/mL and 82.15 ± 0.33 µg/mL, respectively (Topuzović, Stanković, Jakovljević, & Bojović, 2016). The study carried on by Meric et al. (2014) expressed that the methanol extract of *S. ebulus* fruits collected from Istanbul, Turkey showed DPPH radical scavenging activity with IC_{50} value of 8.895 ± 1.391 mg/mL (Meriç et al., 2014).

An overview of the discussion

The reason for the identified differences between the results of the current study and the other studies might be correlated with using different plant materials collected from various areas and methods used for the extraction of the *S. ebulus* fruits. Furthermore, according to the present study results, it seems that *S. ebulus* extracts have more potential antioxidant activity than ascorbic acid used as a standard compound. In a study conducted by Glassman et al. (2003), a topical formulation containing urea and an antioxidant agent was developed to use in nail fungus (onychomycosis) treatment. The results of the study showed that a combination of the antifungal agent and antioxidant compound increases the efficacy of nail fungus treatment. The invention was patented by Glassman et al. in 2003. According to the research, a nail is continuously exposed to photooxidative damage and oxidative environment, including air pollutants, ultraviolet radiation, chemical oxidants, and aerobic microorganisms. The antioxidant compound protects the permeability and stability of the cell membrane of the nail (Glassman, Bhagwat, & Glassman, 2004). The current study results support this information and showed that due to having strong antioxidant potential, *S. ebulus* fruits might be beneficial for the treatment of nail fungus infections.

The fruits of *S. ebulus* have traditionally been used to treat nail fungus (onychomycosis) by local folk in Kahramanmaras, Turkey. Therefore, the current study focused on the identification of the potential of *S. ebulus* fruits for the treatment of nail fungus caused by *Trichophyton rubrum*. However, the antifungal activity results indicated that *S. ebulus* fruit extracts did not show activity against *T. rubrum*. On the other hand, antioxidant activity results demonstrated that the extracts have strong antioxidant potential. According to literature, antioxidant compounds contribute to the topical treatment of nail fungus (onychomycosis). As a conclusion, although *S. ebulus* fruits did not show antifungal activity against *T. rubrum*, it might help to treat nail fungus (onychomycosis) with its antioxidant properties and might be useful when used in the antifungal topical formulations for its antioxidant features. In addition to this, the antibacterial activity results for tested organisms are parallel to the results of studies in this particular so far. In the other part, the study showed that a few compounds, namely acacetin, chrysin, eupatilin, hederagenin, isosakuranetin, myricitrin, and rhamnocitrin, quantified by LC-MS/MS defined to *S. ebulus* fruits for the first time. We strongly encourage that pharmacological activity studies should be inspired by the traditional usage of medicinal plants.

CONCLUSION

S. ebulus samples were found to have a potent antioxidant effect on the phytochemical components of the extract. These findings support the use of *S. ebulus* in traditional medicine in the treatment of dermatophytes infections such as onychomycosis. In conclusion, the effectiveness of *S. ebulus* extracts in the field of human health and phytopathology should be complemented by further studies.

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REFERENCES

- Ahmadiani, A., Fereidoni, M., Semnanian, S., Kamalinejad, M., & Saremi, S. (1998). Antinociceptive and anti-inflammatory effects of *Sambucus ebulus* rhizome extract in rats. *Journal of Ethnopharmacology, 61*(3), 229-235.
- Al-Rifai, A. (2018). Identification and evaluation of in-vitro antioxidant phenolic compounds from the *Calendula tripterocarpa* Rupr. *South African Journal of Botany, 116*, 238-244.
- Andreicuţ, A.-D., Pârvu, A. E., Moţ, A. C., Parvu, M., Fischer-Fodor, E., Feldrihan, V., . . . Irimie, A. (2018). Anti-inflammatory and antioxidant effects of *Mahonia aquifolium* leaves and bark extracts. *Farmacia, 66*(1), 49.

- Atay, I., Kirmizibekmez, H., Gören, A. C., & Yeşilada, E. (2015). Secondary metabolites from *Sambucus ebulus*. *Turkish Journal of Chemistry, 39*(1), 34-41.
- Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils–a review. *Food and Chemical Toxicology, 46*(2), 446-475.
- Balea, Ş. S., Pârvu, A. E., Pop, N., Marín, F. Z., & Pârvu, M. (2018). Polyphenolic compounds, antioxidant, and cardioprotective effects of pomace extracts from *Feteasc*ă *Neagr*ă *Cultivar*. *Oxidative Medicine and Cellular Longevity, 2018*.
- Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature, 181*(4617), 1199-1200.
- Braham, F., Carvalho, D., Almeida, C., Zaidi, F., Magalhães, J., Guido, L., & Gonçalves, M. (2020). Online HPLC-DPPH screening method for evaluation of radical scavenging phenols extracted from *Moringa oleifera* leaves. *South African Journal of Botany, 129*, 146-154.
- Brand-Williams, W., Cuvelier, M.-E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food science and Technology, 28*(1), 25-30.
- Cvetanović, A. (2020). *Sambucus ebulus* L., antioxidants and potential in disease. In *Pathology* (pp. 321-333): Elsevier.
- Cvetanovic, A., Djurovic, S., Maskovic, P., Radojkovic, M., Svarc-Gajic, J., & Zekovic, Z. (2016). Polyphenolic profile of *sambucus ebulus* root, leaf and fruit extracts. *Zbornik Radova, 21*(24), 631-637.
- Cvetanović, A., Zeković, Z., Švarc-Gajić, J., Razić, S., Damjanović, A., Zengin, G., . . . Moreira, M. (2018). A new source for developing multi-functional products: biological and chemical perspectives on subcritical water extracts of *Sambucus ebulus* L. *Journal of Chemical Technology & Biotechnology, 93*(4), 1097-1104.
- Demirci, S., & Özhatay, N. (2012). An ethnobotanical study in Kahramanmaraş (Turkey); wild plants used for medicinal purpose in Andirin, Kahramanmaraş. *Turkish Journal of Pharmaceutical Sciences 9*(1), 75-92.
- Do, Q. D., Angkawijaya, A. E., Tran-Nguyen, P. L., Huynh, L. H., Soetaredjo, F. E., Ismadji, S., & Ju, Y.-H. (2014). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of Limnophila aromatica. *Journal of Food and Drug Analysis, 22*(3), 296-302.
- Duhard, É. (2014). Les paronychies. *La Presse Médicale, 43*(11), 1216-1222.
- Ebrahimzadeh, M., Mahmoudi, M., Saiednia, S., Pourmorad, F., & Salimi, E. (2006). Anti-inflammatory and anti-nociceptive properties of fractionated extracts in different parts of *Sambucus ebulus*. *Journal of Mazandaran University of Medical Sciences, 16*(54), 35-47.
- Ebrahimzadeh, M., Mahmoudi, M., & Salimi, E. (2006). Antiinflammatory activity of *Sambucus ebulus* hexane extracts. *Fitoterapia, 77*(2), 146-148.
- Ebrahimzadeh, M. A., Ehsanifar, S., & Eslami, B. (2009). *Sambucus ebulus* elburensis fruits: A good source for antioxidants. *Pharmacognosy Magazine, 5*(19), 213.
- Ginovyan, M., & Trchounian, A. (2017). Screening of some plant materials used in Armenian traditional medicine for their antimicrobial activity. *Proceedings of the YSU B: Chemical and Biological Sciences, 51*(1), 44-53.
- Glassman, B. P., Bhagwat, D., & Glassman, D. (2004). Method of treating onychomycosis with urea and an antioxidant. In: Google Patents.
- Granato, D., Shahidi, F., Wrolstad, R., Kilmartin, P., Melton, L. D., Hidalgo, F. J., . . . Ismail, A. B. (2018). Antioxidant activity, total phenolics and flavonoids contents: Should we ban in vitro screening methods? *Food Chemistry, 264*, 471-475.
- Gupta, A. K., Versteeg, S. G., & Shear, N. H. (2017). Onychomycosis in the 21st century: an update on diagnosis, epidemiology, and treatment. *Journal of Cutaneous Medicine and Surgery, 21*(6), 525-539.
- Gülçin, I., Bursal, E., Şehitoğlu, M. H., Bilsel, M., & Gören, A. C. (2010). Polyphenol contents and antioxidant activity of lyophilized aqueous extract of propolis from Erzurum, Turkey. *Food and Chemical Toxicology, 48*(8-9), 2227-2238.
- Han, H., Yilmaz, H., & Gulcin, I. (2018). Antioxidant activity of flaxseed (*Linum usitatissimum* L.) shell and analysis of its polyphenol contents by LC-MS/MS. *Records of Natural Products, 12*(4), 397-402.
- Hashemi, Z., Ebrahimzadeh, M. A., & Khalili, M. (2019). Sun protection factor, total phenol, flavonoid contents and antioxidant activity of medicinal plants from Iran. *Tropical Journal of Pharmaceutical Research, 18*, 1443-1448.
- Ivanova, D., Tasinov, O., & Kiselova-Kaneva, Y. (2014). Improved lipid profile and increased serum antioxidant capacity in healthy volunteers after *Sambucus ebulus* L. fruit infusion consumption. *International Journal of Food Sciences and Nutrition, 65*(6), 740-744.
- Jabbari, M., Daneshfard, B., Emtiazy, M., Khiveh, A., & Hashempur, M. H. (2017). Biological effects and clinical applications of dwarf elder (*Sambucus ebulus* L): A review. *Journal of Evidence-Based Complementary & Alternative Medicine, 22*(4), 996-1001.
- Kaveh, K., Mohamadyan, M., & Ebrahimzadeh, M. A. (2019). Antihypoxic activities of *sambucus ebulus* leaf and fruit and myrtus communis leaf in mice. *Journal of Mazandaran University of Medical Sciences, 29*(176), 61-73.
- Kaya, Y., Haji, E. K., Arvas, Y. E., & Aksoy, H. M. (2019). *Sambucus ebulus L.: Past, present and future.* Paper presented at the AIP Conference Proceedings.
- Kedare, S. B., & Singh, R. (2011). Genesis and development of DPPH method of antioxidant assay. *Journal of Food Science and Technology, 48*(4), 412-422.
- Kültür, Ş. (2007). Medicinal plants used in Kırklareli province (Turkey). *Journal of Ethnopharmacology, 111*(2), 341-364.
- Lipner, S. R., & Scher, R. K. (2019). Onychomycosis: Treatment and prevention of recurrence. *Journal of the American Academy of Dermatology, 80*(4), 853-867.
- Meriç, Z. İ., Bitiş, L., Birteksöz-Tan, S., Turan, S. Ö., & Akbuga, J. (2014). Antioxidant, antimicrobial and anticarcinogenic activities of *Sambucus ebulus* L. flowers, fruits and leaves. *Marmara Pharmaceutical Journal, 18*(1), 22-25. doi:10.12991/mpj.201414122
- Meza, A., Rojas, P., Cely-Veloza, W., Guerrero-Perilla, C., & Coy-Barrera, E. (2020). Variation of isoflavone content and DPPH• scavenging capacity of phytohormone-treated seedlings after in vitro germination of cape broom (Genista monspessulana). *South African Journal of Botany, 130*, 64-74.
- Mikulic-Petkovsek, M., Ivancic, A., Todorovic, B., Veberic, R., & Stampar, F. (2015). Fruit phenolic composition of different elderberry species and hybrids. *Journal of Food Science, 80*(10), C2180-C2190.
- Morais, S., Lima, K., Siqueira, S., Cavalcanti, E., Souza, M., Menezes, J., & Trevisan, M. (2013). Correlação entre as atividades antiradical, antiacetilcolinesterase e teor de fenóis totais de extratos de plantas medicinais de farmácias vivas. *Revista Brasileira de Plantas Medicinais, 15*, 575-582.
- Nogueira, A. C., Morais, S. M. d., Souza, E. B. d., Albuquerque, M. R. J. R., Santos, H. S. d., Cavalcante, C. S. d. P., . . . Fontenelle, R. O. d. S. (2020). Antifungal and antioxidant activities of Vernonia chalybaea Mart. ex DC. essential oil and their major constituent β-caryophyllene. *Brazilian Archives of Biology and Technology, 63*.
- Pârvu, M., Moț, C. A., Pârvu, A. E., Mircea, C., Stoeber, L., Roșca-Casian, O., & Țigu, A. B. (2019). *Allium sativum* extract chemical composition, antioxidant activity and antifungal effect against *Meyerozyma guilliermondii* and *Rhodotorula mucilaginosa* causing onychomycosis. *Molecules, 24*(21), 3958.
- Rodino, S., Butu, A., Petrache, P., Butu, M., Dinu-Pirvu, C.-E., & Cornea, C. P. (2015). Evaluation of the antimicrobial and antioxidant activity of *Sambucus ebulus* extract. *Farmacia, 63*(5), 751-754.

- Salehzadeh, A., Asadpour, L., Naeemi, A. S., & Houshmand, E. (2014). Antimicrobial activity of methanolic extracts of *Sambucus ebulus* and *Urtica dioica* against clinical isolates of methicillin resistant *Staphylococcus aureus*. *African Journal of Traditional, Complementary and Alternative Medicines, 11*(5), 38-40.
- Scopel, M., Nunes, E. C. M., Silva, M. V., Vendruscolo, G. S., Henriques, A. T., & Mentz, L. A. (2007). Caracterização farmacobotânica das espécies de *Sambucus (Caprifoliaceae) utilizadas* como medicinais no Brasil: Parte I. *Sambucus nigra* L. *Revista brasileira de farmacognosia. São Paulo, SP. Vol. 17, n. 2 (Abr./Jun. 2007), p. 249-261*.
- Senica, M., Stampar, F., & Mikulic-Petkovsek, M. (2019). Harmful (cyanogenic glycoside) and beneficial (phenolic) compounds in different *Sambucus* species. *Journal of Berry Research, 9*(3), 395-409.
- Shi, D., Lu, G., Mei, H., De Hoog, G. S., Zheng, H., Liang, G., . . . Liu, W. (2016). Onychomycosis due to *Chaetomium globosum* with yellowish black discoloration and periungual inflammation. *Medical Mycology Case Reports, 13*, 12-16.
- Shokrzadeh, M., & Saravi, S. S. (2010). The chemistry, pharmacology and clinical properties of *Sambucus ebulus*: A review. *Journal of Medicinal Plants Research, 4*(2), 95-103.
- Sinikumpu, S.-P., Huilaja, L., Auvinen, J., Jokelainen, J., Puukka, K., Ruokonen, A., . . . Tasanen, K. (2018). The association between low grade systemic inflammation and skin diseases: a cross-sectional survey in the Northern Finland birth cohort 1966. *Acta Dermato-Venereologica, 98*(1-2), 65-69.
- Süntar, I. P., Akkol, E. K., Yalçın, F. N., Koca, U., Keleş, H., & Yesilada, E. (2010). Wound healing potential of Sambucus ebulus L. leaves and isolation of an active component, quercetin 3-O-glucoside. *Journal of Ethnopharmacology, 129*(1), 106-114.
- Topuzović, M. D., Stanković, M. S., Jakovljević, D. Z., & Bojović, B. M. (2016). Plant part variability of *Sambucus ebulus* L. secondary metabolites content and antioxidant activity. *Agro Food Ind Hi Technol, 27*(2), 60-64.
- Tuzlacı, E., & Tolon, E. (2000). Turkish folk medicinal plants, part III: Şile (Istanbul). *Fitoterapia, 71*(6), 673-685.
- Vankova, D. V., Todorova, M. N., Kisselova-Kaneva, Y. D., & Galunska, B. T. (2019). Development of new and robust LC-MS method for simultaneous quantification of polyphenols from *Sambucus ebulus* fruits. *Journal of Liquid Chromatography & Related Technologies, 42*(13-14), 408-416.
- Yesilada, E. (1997). Evaluation of the anti-inflammatory activity of the Turkish medicinal plant *Sambucus ebulus*. *Chemistry of Natural Compounds, 33*(5), 539-540.
- Yesilada, E., Gürbüz, İ., & Toker, G. (2014). Anti-ulcerogenic activity and isolation of the active principles from *Sambucus ebulus* L. leaves. *Journal of Ethnopharmacology, 153*(2), 478-483.
- Zahmanov, G., Alipieva, K., Denev, P., Todorov, D., Hinkov, A., Shishkov, S., . . . Georgiev, M. I. (2015). Flavonoid glycosides profiling in dwarf elder fruits (*Sambucus ebulus* L.) and evaluation of their antioxidant and anti-herpes simplex activities. *Industrial Crops and Products, 63*, 58-64.

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Original Article

Antioxidant and prooxidant properties of selected herbs and *Citrus bergamia* Risso et Poiteau (bergamot) used for the management of hyperlipidemia

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ABSTRACT

Background and Aims: In recent years, there has been an increased interest in the search for herbs to aid the management of hyperlipidemia. There is currently very little data on the simultaneous evaluation of the antioxidant and prooxidant properties of antihyperlipidemic herbs. This study was designed to evaluate the antioxidant and prooxidant properties of four antihyperlipidemic herbal drugs and also of bergamot.
Methods: Antioxidant property was determined by ferric ions (Fe^{3+}) reducing capacity (IRC), DPPH radical scavenging activity (DPPH IC_{50}) and trolox equivalent antioxidant capacity (TEAC); deoxyribose degradation test was used for prooxidant property.
Results: The highest total phenolic content (TPC) was in the myrtle leaf (ML) (135.35±3.46 mg GAE/g, $p<0.05$) whereas the highest total flavonoid content (TFC) was in green tea (GT) (48.76±0.69 mg QE/g, $p<0.05$) both of which were maintained from a pharmacy. Among the bergamot samples, the highest TPC and TFC values were in filtered fruit juice (BFFJ) as 197.35±6.29 mg GAE/100 mL; and 94.14±1.39 mg QE/100 mL; $p<0.05$, respectively. GT showed the highest antioxidant capacity in IRC and TEAC assays (2.29±0.12 mM TE/g; and 2.32±0.07 mmol TE/mg, $p<0.05$). The lowest DPPH IC_{50} was identified in ML from a pharmacy (6.95±0.08 µg/mL; $p<0.01$). BFFJ had the highest IRC (2.94±0.031 mM TE/10µL), TEAC (5.14±0.084 mmol TE/10 µL) and the lowest DPPH IC_{50} value (10.561±0.17 µL). GT from a pharmacy and 1mg/mL concentration BFLFJ (filtered and lyophilized) were associated with the lowest hydroxyl radical scavenger activity (0.171±0.013 µM MDA equivalent, $p<0.05$ and 0.144±0.015 µM MDA equivalent, $p<0.05$).
Conclusion: BFLFJ and GT got the highest attention due to high TPC, TFC, antioxidant and low prooxidant properties. Our results highlight the necessity of clarifying the value of bergamot and GT in this field with further studies.

Keywords: Antioxidant, Bergamot, *Citrus bergamia*, Medicinal plants, Oxidative stress, Prooxidant

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INTRODUCTION

Cardiovascular (CV) diseases (CVD) are the leading cause of morbidity and mortality in the world (Mach et al., 2019). Hyperlipidemia (HL) is one of the major risk factors for CVD. Statins are effective in reducing CV events and are safe for almost all HL patients (Arca & Pigna, 2011). However, treatment plans do not always work properly, since there may be problems in patient adherence, statin intolerance, and doctors' attitudes to low CV risk patients (Blaha, Nasir, & Blumenthal, 2012; Redberg & Katz, 2012). Thus both patients and scientific societies are searching for natural products (NP) for HL treatment (Arca & Pigna, 2011). There are scientific studies supporting the use of NP alone or associated with other drugs in clinical practice (Cicero, Parini, & Rosticci, 2015). However, there is still an insufficient number of studies demonstrating morbidity and mortality outcomes(Cicero et al., 2017). Due to lack of outcome data, anti-HL NP are still accepted as food supplements or functional foods (Mach et al., 2019). Red yeast rice, bergamot, berberine, dietary fiber, green tea, phytosterols, spirulina and artichoke are the most studied NP for HL (Banach et al., 2018). There are studies for NP to be used alone or in conjunction with anti-HL pharmaceuticals (Patti et al., 2017). NP act through different mechanisms such as decreasing intestinal cholesterol absorption, inhibiting hepatic cholesterol synthesis, and decreasing hepatic low-density lipoprotein cholesterol uptake. In addition to anti-HL effects, NP also improve endothelial function and have anti-inflammatory, antioxidant, and anti-atherosclerotic activities (Cicero et al., 2017).

Phytonutrients are candidate sources of anti-HL actions. However, there is very little data on anti-HL actions of NP in this study. Green tea extract suppressed HL and non-alcoholic fatty liver disease in mice (Kang et al., 2011). Hawthorn extract were found to be capable of decreasing glucose production and triacylglycerol synthesis (Shih, Lin, Lin, & Wu, 2013). Ethanolic extracts of calyces and leaves of *Hibiscus sabdariffa* L. were found to possess significant antioxidant and anti-HL activities (Ochani & D'Mello, 2009). In a review on anti-HL actions of *Citrus bergamia* Risso et Poiteau some studies showed significant decrease in total cholesterol, triglycerides, low-density lipoprotein cholesterol and an increase in high-density lipoprotein cholesterol (Lamiquiz-Moneo et al., 2020).

Oxidant and antioxidant balance is crucially important in maintaining healthy biological systems. This balance seems to be in favor of mild prooxidant to achieve proper signaling through cells for a strong immune reaction (Ho, Karimi Galougahi, Liu, Bhindi, & Figtree, 2013). CVD such as HL leads to an increase in the reactive oxygen species and oxidative stress, thus the vascular endothelial integrity becomes disrupted (Jamwal & Sharma, 2018). Antioxidant properties of anti-HL NP may have important roles against this mechanism. Flavonoids and other phenolic compounds have strong antioxidant properties and the ability to protect cells from reactive radicals (Shayganni, Bahmani, Asgary, & Rafieian-Kopaei, 2016). Although antioxidant activities are well known, exogenous antioxidants also show prooxidant activity at high doses and particularly in the presence of metal ions such as iron and copper (Azam, Hadi, Khan, & Hadi, 2004). Antioxidant and prooxidant effects of NP have been studied in the literature. To the best of our knowledge, there are as yet no data on the simultaneous evaluation of antioxidant and prooxidant capacities of anti-HL NP. Therefore, in the present study, we aimed to screen the antioxidant and prooxidant properties of *Citrus bergamia* juice and bergamot albedo fragment along with selected plant extracts.

MATERIAL AND METHODS

Methodology

Plant material

Various pharmacy and herbal market commercial samples of hawthorn (*Crataegus* L. spp.) flower-leaf (CFL), hibiscus (*Hibiscus sabdariffa*) flower (HF), green tea (*Camellia sinensis* L.) (GT) and myrtle (*Myrtus communis* L.) leaf (ML) were analyzed in our study. Fruit juice and albedo fragments of two different commercial samples of bergamot fruit harvested in Antalya, Turkey, (named as *Citrus bergamia* rissofemminello and Native A41) were also tested. Codes used for the extracts prepared from plant material and for bergamot albedo and fruit juice samples are listed in Table 1.

Preparation of extracts

Methanol was used to prepare the extracts from dried CFL, HF, GT, ML and albedo fragment of bergamot fruit. First, the plant material was ground to powder to obtain fine particles. 10 g of powdered plant material was extracted with 100 mL methanol in an ultrasonic bath for 30 minutes at 30°C. The extract was

Table 1. Extracts prepared from herbal drug specimens and bergamot fruit.

Herbal drug specimens	**From pharmacy**	**From herbal market**
Hawthorn flower-leaf	CFL-1	CFL-2
Hibiscus sabdariffa flower	HF-1	HF-2
Green tea (loose)	GT-1	GT-2
Myrtle leaf	ML-1	ML-2
Bergamot fruit	***Citrus bergamia* rissofemminello**	**Native A41**
Bergamot albedo fragment	BA-1	BA-2
Bergamot Filtered &Lyophilized Fruit Juice	BFLFJ-1	BFLFJ-2
Bergamot Filtered Fruit Juice	BFFJ-1	BFFJ -2

CFL, hawthorn (*Crataegus* L. spp.) flower-leaf; HF, hibiscus (*Hibiscus sabdariffa* L.) flower; GT, green tea (*Camellia sinensis* L.); ML, myrtle (*Myrtus communis* L.) leaf; BA, bergamot albedo fragment; BFLFJ, Bergamot Filtered & Lyophilized Fruit Juice; BFFJ, Bergamot Filtered Fruit Juice.

filtered. This procedure was repeated three times. The filtrates were pooled and then concentrated using a rotary evaporator. The extracts were aliquoted and stored at -20°C until use.

Preparation of bergamot fruit juice

Bergamot fruit juice (BFJ) was obtained by mechanical pressure. The freshly squeezed juices were filtered. Half of the BFFJ was lyophilized then stored at -20°C until use. The other half of the BFFJ was directly stored at -20°C in small aliquots (25 mL).

Determination of total phenolic content (TPC)

TPC was determined by colorimetric Folin–Ciocalteau method (McDonald, Prenzler, Antolovich, & Robards, 2001). Briefly, 500 µL of crude extract (0.1 mg/mL) was mixed thoroughly with 5 ml of %10 Folin–Ciocalteau reagent (1:10 with distilled water) and incubated for 30 minutes; then 4 mL of 1M Na_2CO_3 was added. The mixture was allowed to stand for 60 min in the dark, and absorbance was measured at 760 nm. Gallic acid which was prepared in different concentrations (25, 50, 100, 150, 250 and 250 mg/mL) was used as standard. The TPC was calculated from the gallic acid calibration curve, and the results were expressed as mg of gallic acid equivalent (GAE)±Standard Deviation (SD) per g extract and mg of GAE±SD per 100 mL BFJ.

Determination of total flavonoid content (TFC)

TFC was determined by the colorimetric aluminum chloride method(Chang, Yang, Wen, & Chern, 2002). In brief, 1.5 mL methanol, 0.1 mL of 10% $AlCl_3$ and 0.1 mL of 1M CH_3CO_2Ksolutions were added in the same order to 500 µL of samples (1 mg/mL). Distilled water was added into the tube and the solution was made up to 5 mL. The tubes were incubated for 30 minutes at room temperature and absorbance was measured at 415 nm. TFC of extracts were calculated from the quercetin standard curve (12.5, 25, 50, 75 and 100 µg/mL) and the results were expressed as mg of Quercetin Equivalent (QE)±SD per g extract and mg of QE±SD per 100 mL BFJ.

Determination of antioxidant properties

The antioxidant properties were determined by three methods.

Ferric ions (Fe^{3+}) reducing capacity (IRC)

IRC was determined by a colorimetric method (Oyaizu, 1986). In order to perform IRC assay, 0.4 mL of the sample solution (150 µg/mL and 300 µg/mL) was mixed with 0.4 mL of 1% $[K_3Fe(CN)_6]$. The mixture was incubated at 50˚C for 20 minutes. 0.4 mL TCA (10%) was added to the mixture and centrifuged at 3000xg for 10 minutes. 0.5 mL of the supernatant was mixed with 0.5 mL $FeCl_3$ 1% and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing capacity. The results were expressed as mM Trolox Equivalent (TE) per g extract±SD and mM TE±SD per 10 ul BFJ.

Trolox equivalent antioxidant capacity (TEAC)

TEAC was measured by decolorization of the ABTS radical cation ($ABTS^{+}$)(Re et al., 1999). 7.4 mM ABTS stock solution and 2.45 mM $K_2S_2O_8$ solutions were mixed within the ratio of 2:1 to produce 7 mM $ABTS^{+}$ radical cation. This radical solution was left in the dark at room temperature for 16 hours to reach stable absorbance at 734 nm. $ABTS^{+}$ solution was diluted with 5 mM PBS (pH.7.4) until it had an absorbance of 0.700±0.02 at 734 nm. Then, 1 mL of radical solution was added to the well and the absorbance was measured at 734 nm using 5 mM PBS (pH:7.4) as blank. The absorbance value was recorded as $A_{ABTS^{+}}$.1 mL of radical solution was mixed with 10 µL test solution (0.1 mg/mL) and the absorbance was recorded at 6 minutes after the initial mixing. Percentage of inhibition was calculated as follows:

Inhibition% = $[(A_{ABTS^{\cdot+}} - A_{6.min}) \times / A_{ABTS^{\cdot+}}] \times 100$ where $A_{ABTS^{\cdot+}}$ is the absorbance of the $A_{ABTS^{\cdot+}}$ at 734 nm (0.70±0.02) and $A_{6.min}$ is the absorbance after the addition of the sample to the $A_{ABTS^{\cdot+}}$.

Trolox was used as an antioxidant standard (0, 0.25, 0.5, 1, 2 and 2.5 mM). The absorbances of the samples were compared to that of the trolox standard curve. The results were expressed as mmol TE/mg extract±SD and mmol TE/10 µL BFJ±SD.

DPPH radical scavenging activity (DPPH IC50)

Free radical scavenging activity was performed according to DPPH method (K.-J. Wang, Zhang, & Yang, 2006). The samples were reacted with the stable DPPH radical in a methanol solution. Samples or standard antioxidant (ascorbic acid) solution were prepared in methanol. In a 96-well plate, 100 µL of DPPH (200 µM) was mixed with 100 µL of different concentrations of samples or standard. Plates were covered and incubated at room temperature for 30 minutes in the dark. The absorbance of the residual DPPH solution was determined at 517 nm. The inhibition percentage of the samples was calculated as follows:

Inhibition% = $[(A_B - A_A) / A_B \times 100$ where A_B is the absorbance of DPPH radical and A_A is the absorbance of the sample or standard. The results were calculated as average Inhibition $Concentration_{50}$ (IC_{50})±SD and expressed as µg/mL±SD for the extracts and µL±SD for the BFJ.

Determination of prooxidant properties

Deoxyribose degradation test

Determination of prooxidant properties was carried out by deoxyribose degradation test(Mathew & Abraham, 2006). Briefly, 100 µL 2-deoxy- D-ribose (3.36 mM), 100 µL H_2O_2 (1 mM), 100µL $FeCl_3$ (1 mM), 100 µL EDTA (1 mM), 100 µL ascorbic acid (0.1 mM) were mixed with 100 µL sample solution (0.1 mg/mL or 1 mg/mL) in a test tube. The final volume was adjusted to 1 mL with potassium phosphate buffer solution (20 mM, pH 7.4). Tubes were incubated for 1 hour at 37°C. The extent of deoxyribose degradation was measured by the TBA method (Ohkawa, Ohishi, & Yagi, 1979). 1 mL TBA (1 % w/v) and 1 mL TCA (2.8% w/v) were added to the reaction mixture and heated in a water bath at 100 ˚C for 30 minutes. The absorbances of the reaction mixtures were measured spectrophotometrically at 532 nm. Chromogenic TBA reactive product (TBARP) concentrations were calculated from the corresponding absorbance values using MDA standard curve prepared at different concentrations of MDA (0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1 µM). The results were expressed as µM MDA equivalent TBARP.

Statistical analysis

The experiments were performed in triplicate. The results were expressed as mean±SD. The statistical analysis of the results was carried out using GraphPad Prism 5 software (San Diego, CA) and statistical comparisons were made using analysis of variance (ANOVA). A value of $p < 0.05$ was considered as statistically significant.

RESULTS

TPC and TFC

Total phenol and flavonoid contents of the methanol extracts of the herbal drug specimens and bergamot samples are shown in Table 2 and 3, respectively. The highest TPC was detected in ML-1 (135.35±3.46 mg GAE/g extract, $p<0.05$) whereas the lowest was in HF-2 (42.30±1.05 mg GAE/g extract, $p< 0.05$). The highest TFC was measured in GT-1 (48.76±0.69 mg QE/g extract, $p<0.05$) whereas the lowest was in HF-2 (17.09±0.62 mg QE/g extract).

TPC of BFLFJ-1 was as 130.35±8.91 mg GAE/g extract ($p< 0.05$) and TFC of BFLFJ-2 was as 65.93±4.31 mg QE/g extract, ($p<0.05$). BFLFJ samples had the highest TPC and TFC capacity among non-liquid bergamot samples. BFFJ-1 values of TPC

Table 2. TPC, TFC, antioxidant and prooxidant properties of herbal drug specimens.

Herbal Drug Specimens	TPC (mg GAE/g extract)	TFC (mg QE/g extract)	IRC (mM TE/g extract)	TEAC (mmol TE/mg extract)	DPPH IC_{50} (µg/mL)	TBARS	
						0.1 mg/mL extract	1 mg/mL extract
CFL-1	116.22±3.25	41.140±1.39	0.77±0.02	1.66±0.09	27.32±0.18	0.754±0.013	0.261±0.024
CFL-2	83.61±1.32	36.381±2.27	0.65±0.03	1.55±0.12	49.80±0.29	0.811±0.022	0.303±0.011
HF-1	47.09±2.11	17.570±1.01	0.87±0.06	0.73±0.02	41.38±0.49	0.837±0.026	0.594±0.033
HF-2	42.30±1.05	17.090±0.62	0.68±0.03	0.59±0.01	48.13±0.81	0.823±0.022	0.551±0.027
GT-1	115.78±2.74	48.760±0.69 *	2.12±0.10	2.3±0.07*	9.72±0.67 **	0.203±0.007 *	0.171±0.013*
GT-2	114.48±3.49	43.760±1.42	2.29±0.12 *	1.76±0.04	25.19±0.23	0.284±0.009	0.211±0.008
ML-1	135.35±3.46 *	46.620±0.97	1.81±0.05	2.01±0.08	6.95±0.08*	0.803±0.015	0.285±0.016
ML-2	110.56±4.17	39.950±1.98	1.29±0.08	1.69±0.15	8.57±0.43	0.871±0.019	0.337±0.100

Values expressed are means±SD of three parallel measurements. * denotes significant differences ($p<0.05$); BA, bergamot albedo fragment; BFLFJ, Bergamot Filtered & Lyophilized Fruit Juice; BFFJ, Bergamot Filtered Fruit Juice; CFL, hawthorn (*Crataegus* L. spp.) flower-leaf; DPPH IC_{50}, DPPH radical scavenging activity; HF, hibiscus (*Hibiscus sabdariffa* L.) flower; GAE, Gallic Acid Equivalent; GT, green tea (*Camellia sinensis* L.); ML, myrtle (*Myrtus communis* L.) leaf; IRC, Ferric ions (Fe^{3+}) reducing capacity; QE, Quercetin Equivalent; TEAC, Trolox equivalent antioxidant capacity; TE, Trolox Equivalent; TFC, total flavonoid content; TPC, total phenolic content.

Table 3. TPC, TFC, antioxidant and prooxidant properties of bergamot albedo and fruit juice.

Bergamot Albedo and Fruit Juice	TPC (mg GAE/g extract)	TFC (mg QE/g extract)	IRC (mM TE/g extract)	TEAC (mmol TE / mg extract)	DPPH IC_{50} (µg/mL)	TBARS (µM MDA equivalent)	
						0.1 mg/mL extract	1 mg/mL extract
BA-1	39.26±3.70	20.19±0.37	2.11±0.04	1.3±0.047	25.19±0.37	0.709±0.033	0.567±0.029
BA-2	48.83±3.64	39.65±0.74	2.04±0.08	1.24±0.039	26.65±0.74	0.26±0.027	0.598±0.038
BFLFJ-1	130.35±8.91*	50.68±3.38	2.73±0.12*	1.86±0.086*	26.65±0.96	0.254±0.025*	0.151±0.011
BFLFJ-2	80.62±6.54	65.93±4.31*	1.98±0.18	1.19±0.067	14.18±0.59*	0.220±0.016	0.144±0.015*
	TPC (mg GAE/100 mL BFJ)	TFC (mg QE/100 mL BFJ)	IRC (mM TE/10 µL BFJ)	TEAC (mmol TE/10 µL BFJ)	IC_{50} (µL)	TBARS (µM MDA equivalent)	
						10 µL	100 µL
BFFJ-1	197.35±6.29 *	71.14±1.04	2.94±0.031*	5.14±0.084*	12.735±0.08	0.251±0.026	0.073±0.006
BFFJ-2	185.61±8.14	94.14±1.39*	2.69±0.047	4.29±0.091	10.561±0.1*	0.287±0.017	0.091±0.003

Values expressed are means±SD of three parallel measurements. * denotes significant differences ($p<0.05$); BA, bergamot albedo fragment; BFLFJ, Bergamot Filtered & Lyophilized Fruit Juice; BFFJ, Bergamot Filtered Fruit Juice; DPPH IC_{50}, DPPH radical scavenging activity; GAE, Gallic Acid Equivalent; IRC, Ferric ions (Fe^{3+}) reducing capacity; QE, Quercetin Equivalent; TEAC, Trolox equivalent antioxidant capacity; TE, Trolox Equivalent; TFC, total flavonoid content; TPC, total phenolic content.

were found to be higher than BFFJ-2 (197.35±6.29 mg GAE/100 mL BFJ; 185.61±8.14 mg GAE/100 mL BFJ, p<0.05 respectively). BFFJ-2 had higher TFC than BFFJ-1 (94.14±1.39 mg QE/100 mL BFJ, 71.14±1.04 mg QE/100 mL BFJ, p<0.05 respectively). Since the other bergamot samples were in non-liquid form, TPC and TFC of BFFJ-1 and BFFJ-2 were not compared and no further analysis was done.

Antioxidant properties

GT-2 had the highest IRC among the methanol extracts of herbal drug specimens (2.29±0.12 mM TE/g extract *p*<0.05). The highest TEAC values were measured in GT-1 (2.32±0.07 mmol TE/mg extract, p<0.05). The lowest DPPH IC_{50} was identified in ML-1 (6.95±0.08 µg/mL; p<0.01). The second lowest IC_{50} was detected in GT-1 (9.72±0.67 µg/mL; p<0.05). Antioxidant properties of herbal drug specimens are shown in Table-2.

The highest IRC among BA and BFLFJ samples was identified in BFLFJ-1 (2.73±0.12 mM TE/g extract, *p*<0.05). The highest TEAC among BA and BFLFJ samples was measured in BFLFJ-1 (1.86±0.086 mmol TE/mg extract, p<0.05). The lowest DPPH IC_{50} values were detected in BFLFJ-2 (14.18±0.59 µg/mL, p<0.05) among BA and BFLFJ. The IRC of BFFJ-1 was 2.94±0.031 mM TE/10µL (*p*<0.05); TEAC value of BFFJ-1 was 5.14±0.084 mmol TE/10µL, (p<0.05), and the DPPH IC_{50} value of BFFJ-2 was 10.561±0.17 µL, (p<0.05) among the BFFJ samples. The antioxidant capacity of BFFJ was not compared with other bergamot samples since they were in non-liquid form and therefore any further analysis was not performed. The antioxidant properties of bergamot albedo and fruit juice samples are presented in Table-3.

Prooxidant properties

Among all the non-liquid samples, GT-1 and BFLFJ-2 with 1mg/mL concentration were associated with the lowest TBARP (0.171±0.013 µM MDA equivalent, p<0.05; 0.144±0.015 µM MDA equivalent, p<0.05 respectively). The prooxidant capacity of BFFJ-1 was 0.073±0.006 µM MDA equivalent however, since the other bergamot samples were in non-liquid form no further analysis was done. Prooxidant activity of herbal drug specimens and bergamot samples are shown in Table 2 and Table 3, respectively.

DISCUSSION

TPC and TFC

Phenolic compounds are the most abundant antioxidants in the human diet. They have a considerable structural diversity. Flavonoids and other phenolic compounds are well-known plant secondary metabolites for their antioxidant, antibacterial, anti-cancer, cardioprotective, anti-HL, immune system promoting and anti-inflammatory effects (Tungmunnithum, Thongboonyou, Pholboon, & Yangsabai, 2018). In our study, the highest statistically significant TPC was detected in ML purchased from a pharmacy. Studies have shown that ML must be considered as good sources of phenolic compounds(Amensour, Sendra, Abrini, Pérez-Alvarez, & Fernández-López, 2010; Benchikh, Amira, & Benabdallah, 2018). Benchikh et al. found TPC as 149.25±3.11 mg GAE/g of dry extract resembling our result as 135.35±3.46 mg GAE/g of extract in ML (Benchikh et al., 2018).

In our study, the highest statistically significant TFC was measured in GT purchased from a pharmacy. According to Graham et al. GT had high amounts of flavonoids (Graham, 1992). Wang et al. reported that flavonoid was believed to be responsible for antioxidant, anticarcinogenic and anti-atherosclerotic activities (H. Wang & Helliwell, 2001).

Bergamot is a common Italian citrus fruit, cultivated almost exclusively to produce essential oils; the juice is considered a waste product (Pernice et al., 2009). However, other parts of bergamot have also drawn attention recently due to their polyphenolic, mainly flavonoid, content (Mannucci et al., 2017). Flavonoids from citrus fruits have many health benefits including anticancer, antiviral, and anti-inflammatory properties , as well as effects on capillary fragility, inhibition activity on human platelet aggregation, and prevention of diet-induced HL (Picerno et al., 2011).

In our study, among all the non-liquid samples, the highest TPC and TFC were identified in BFLFJ samples. BFFJ also had high TPC and TFC; however, these samples were not comparable due to liquid/non-liquid forms. Previous studies on bergamot juice were mostly conducted on its liquid form, to the best of our knowledge. Ercisli et al. showed similar results to our study in which the TPC of Turkish bergamot cultivated in Mersin was measured as "30.37±2.15 mg GAE/100 mL"(Sezai Ercisli et al., 2015). However, Yıldız Turgut et al. demonstrated a much lower TPC of Native A41 BJ than our study which was "30.37±2.15 mg GAE/100 mL" (Yıldız Turgut, D; Seçmen,Tuba; Tanır, 2018). TPC and TFC obtained by Pozzo et al. from lyophilized juices of "femminello" cultivars of *C. bergamia*, were also lower than the results of our study (Da Pozzo et al., 2018).

Antioxidant properties

In our study, GT-2 had the highest statistically significant IRC among the tested drug specimens. IRC is a good indicator for potential antioxidant activity and this is mainly based on reductones. One of the mechanisms of the antioxidant action of reductones is based on the breaking of free radical chain by donating a hydrogen to neutralize free radical(Jayaprakasha, Singh, & Sakariah, 2001). Simamora et al. found similar high IRC for methanol extract of GT(Simamora, Steven, Santoso, Rumiati, & Timotius, 2018). This study also suggested that GT extracts may act as electron donors which react with free radicals, converting them to more stable products, thus enabling a terminate radical chain reaction.

In the current study, the highest statistically significant TEAC was measured in GT-1 among herbal drug specimens. Zhao et al. showed TEAC as 1.89±0.31 mmol TE/g extract in aqueous extract of GT (Zhao et al., 2019). However, De la Luz Cádiz-Gurrea et al. demonstrated a high TEAC with methanol extract of GT, 9.66±1.27 mmol TE/g extract in accordance with our results (de la Luz Cádiz-Gurrea, Fernández-Arroyo, & Segura-Carretero, 2014).

The lowest statistically significant DPPH IC_{50} value was identified in ML; GT was the second lowest among the herbal drug specimens. Free radical scavenging ability is an important antioxidant capacity. Oxidative stress, caused by reactive oxygen or free radicals, has been shown to be associated with the pro-

gression of many diseases including cancer, heart disease, and depression, among others (Kovacic & Jacintho, 2001) Benchikh et al. had found significantly low levels of DPPH IC_{50} values of aqueous and methanol extracts of ML, thus supporting our data (Benchikh et al., 2018). Another supporting data for powerful DPPH radical scavenging capacity of GT was demonstrated by Simamora et al. in different extracts of GT (Simamora et al., 2018).

In this study, BFLFJ-1 had the highest statistically significant IRC, TEAC and BFLFJ-2 had the lowest DPPH IC_{50} value among the non-liquid bergamot samples. Furthermore, among all the non-liquid samples, the highest antioxidant capacity was identified in BFLFJ samples. BFFJ also displayed remarkable antioxidant capacities, however these samples were not comparable since BFFJ was in liquid form. Most of the previous studies were conducted on BFFJ liquid form; Turgut et al. demonstrated a higher DPPH IC_{50} value of Native A41 BJ (Turgut, Demet Yıldız; Seçmen, Tuba; Tanır, 2018). The antioxidant potential of BJ was examined in the DPPH and ferric reducing power assays, the BJ showed a noticeable antioxidant effect in hypercholesterolemic diet-induced renal damage *in-vitro* models (Trovato et al., 2009). DPPH values of different cultivars of *C. bergamia* were found to be significantly associated with the TPC of BJ, thus supporting our data (Vincenzo, Sicari. Pellicanò, 2016). Pernice et al. also suggested that adding BJ to apricot and apple juices significantly increased the antioxidant capacity (Pernice et al., 2009).

Prooxidant properties

Although medicinal plant effects in prevention and treatment of disorders have been widely attributed to their antioxidant activities, there is increasing evidence pointing to their prooxidant hazardous effects, too. Polyphenols in medicinal plants can act as either antioxidants or prooxidants, depending on conditions such as the presence of oxygen or transition metalsand the concentration of the extract (Nasri & Rafieian-Kopaei, 2014). Therefore, prooxidant capacity was evaluated in our study via TBARS. GT-1 had the lowest statistically significant TBARS. There is an increasing amount of evidence suggesting epigallocatechin-3-gallate (EGCG), the main polyphenolic constituent in GT, has prooxidative properties (Elbling et al., 2005). High concentration of EGCG is suggested to make spontaneous H_2O_2 induced oxidative cell damage in *in vitro* models. Joubert et al. demonstrated *in vitro* prooxidant activity of potent antioxidant dietary supplement extracts via TBARS (Joubert, Winterton, Britz, & Gelderblom, 2005). In addition to having potent free radical scavenging activities and TEAC, GT-1 also have low prooxidant property. Our findings suggest that GT purchased from a pharmacy can be used as a safe source of antioxidant.

There is very little data on the prooxidant property of citrus plants. Simić et al. demonstrated the relationship between antioxidative and prooxidative activities and oxidation potentials among grapes, citrus, apple(Simić, Manojlović, Šegan, & Todorović, 2007). This study demonstrated the need for simultaneous evaluation of antioxidant and prooxidant capacities. Our data on prooxidant capacity showed that among the herbal drug specimens and the bergamot samples, GT purchased from a pharmacy and BFLFJ with 1mg/mL concentration were associated with the lowest prooxidant capacity.

CONCLUSION

Flavonoids and other phenolic compounds have recently been getting the attention of HL researchers. Beyond anti-HL actions, these phytochemicals also exhibit antioxidant activities which may further enhance anti-HL effects. However, recent evidence has shown that exogenous antioxidants also show prooxidant activity. Thus, selecting the least hazardous anti-HL NP with a balanced antioxidant and prooxidant capacity becomes important. Our study suggests that BFLFJ and GT may be used as safe antioxidant sources since they have high TPC, TFC, antioxidant and low prooxidant capacities. This study highlights the necessity of clarifying the value of bergamot and GT in this field with further studies.

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REFERENCES

- Amensour, M., Sendra, E., Abrini, J., Pérez-Alvarez, J. A., & Fernández-López, J. (2010). Antioxidant activity and total phenolic compounds of myrtle extracts Actividad antioxidante y contenido de compuestos fenólicos totales en extractos de myrtus. *CyTA - Journal of Food*, *8*(2), 95–101. https://doi.org/10.1080/19476330903161335
- Arca, M., & Pigna, G. (2011). Treating statin-intolerant patients. *Diabetes, Metabolic Syndrome and Obesity : Targets and Therapy*, *4*, 155–166. https://doi.org/10.2147/DMSO.S11244
- Azam, S., Hadi, N., Khan, N. , & Hadi, S. (2004). Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. *Toxicology in Vitro*, *18*(5), 555–561. https://doi.org/10.1016/j.tiv.2003.12.012
- Banach, M., Patti, A. M., Giglio, R. V., Cicero, A. F. G., Atanasov, A. G., Bajraktari, G., … Rizzo, M. (2018). The role of nutraceuticals in statin intolerant patients. *Journal of the American College of Cardiology*, *72*(1), 96–118. https://doi.org/10.1016/j.jacc.2018.04.040
- Benchikh, F., Amira, S., & Benabdallah, H. (2018). The evaluation of antioxidant capacity of different fractions of *Myrtus communis* L. Leaves. *Annual Research & Review in Biology*, *22*(5), 1–14. https://doi.org/10.9734/ARRB/2018/39217
- Blaha, M. J., Nasir, K., & Blumenthal, R. S. (2012). Statin therapy for healthy men identified as "increased risk". *JAMA*, *307*(14), 1489–1490. https://doi.org/10.1001/jama.2012.425
- Chang, C.-C., Yang, M.-H., Wen, H.-M., & Chern, J.-C. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis*, *10*(3), 178–182.

- Cicero, A. F. G., Colletti, A., Bajraktari, G., Descamps, O., Djuric, D. M., Ezhov, M., … Banach, M. (2017). Lipid lowering nutraceuticals in clinical practice: position paper from an International Lipid Expert Panel. *Archives of Medical Science*, *5*, 965–1005. https://doi.org/10.5114/aoms.2017.69326
- Cicero, A. F. G., Parini, A., & Rosticci, M. (2015). Nutraceuticals and cholesterol-lowering action. *IJC Metabolic & Endocrine*, *6*, 1–4. https://doi.org/10.1016/j.ijcme.2014.10.009
- Da Pozzo, E., De Leo, M., Faraone, I., Milella, L., Cavallini, C., Piragine, E., … Martini, C. (2018). Antioxidant and antisenescence effects of bergamot juice. *Oxidative Medicine and Cellular Longevity*, *2018*, 1–14. https://doi.org/10.1155/2018/9395804
- De la Luz Cádiz-Gurrea, M., Fernández-Arroyo, S., & Segura-Carretero, A. (2014). Pine bark and green Tea concentrated extracts: antioxidant activity and comprehensive characterization of bioactive compounds by HPLC–ESI-QTOF-MS. *International Journal of Molecular Sciences*, *15*(11), 20382–20402. https://doi.org/10.3390/ijms151120382
- Elbling, L., Weiss, R.-M., Teufelhofer, O., Uhl, M., Knasmueller, S., Schulte-Hermann, R., … Micksche, M. (2005). Green tea extract and (–)-epigallocatechin-3-gallate, the major tea catechin, exert oxidant but lack antioxidant activities. *The FASEB Journal*, *19*(7), 807–809. https://doi.org/10.1096/fj.04-2915fje
- Graham, H. N. (1992). Green tea composition, consumption, and polyphenol chemistry. *Preventive Medicine*, *21*(3), 334–350. https://doi.org/10.1016/0091-7435(92)90041-F
- Ho, E., Karimi Galougahi, K., Liu, C.-C., Bhindi, R., & Figtree, G. A. (2013). Biological markers of oxidative stress: Applications to cardiovascular research and practice. *Redox Biology*, *1*(1), 483–491. https://doi.org/10.1016/j.redox.2013.07.006
- Jamwal, S., & Sharma, S. (2018). Vascular endothelium dysfunction: a conservative target in metabolic disorders. *Inflammation Research*, *67*(5), 391–405. https://doi.org/10.1007/s00011-018-1129-8
- Jayaprakasha, G. K., Singh, R. P., & Sakariah, K. K. (2001). Antioxidant activity of grape seed (Vitis vinifera) extracts on peroxidation models in vitro. *Food Chemistry*, *73*(3), 285–290. https://doi.org/10.1016/S0308-8146(00)00298-3
- Joubert, E., Winterton, P., Britz, T. J., & Gelderblom, W. C. A. (2005). Antioxidant and pro-oxidant activities of aqueous extracts and crude polyphenolic fractions of rooibos (*Aspalathus linearis*). *Journal of Agricultural and Food Chemistry*, *53*(26), 10260–10267. https://doi.org/10.1021/jf051355a
- Kang, J. S., Lee, W. K., Yoon, W. K., Kim, N., Park, S.-K., Park, H. K., … Kim, H. M. (2011). A Combination of grape extract, green tea extract and <scp>l</scp> -Carnitine improves high-fat diet-induced obesity, hyperlipidemia and non-alcoholic fatty liver disease in mice. *Phytotherapy Research*, *25*(12), 1789–1795. https://doi.org/10.1002/ptr.3476
- Kovacic, P., & Jacintho, J. (2001). Mechanisms of carcinogenesis focus on oxidative stress and electron transfer. *Current Medicinal Chemistry*, *8*(7), 773–796. https://doi.org/10.2174/0929867013373084
- Lamiquiz-Moneo, I., Giné-González, J., Alisente, S., Bea, A. M., Pérez-Calahorra, S., Marco-Benedí, V., … Mateo-Gallego, R. (2020). Effect of bergamot on lipid profile in humans: A systematic review. *Critical Reviews in Food Science and Nutrition*, *60*(18), 3133–3143. https://doi.org/10.1080/10408398.2019.1677554
- Mach, F., Baigent, C., Catapano, A. L., Koskinas, K. C., Casula, M., Badimon, L., … ESC Scientific Document Group. (2019). 2019 ESC/EAS Guidelines for the management of dyslipidaemias: lipid modification to reduce cardiovascular risk. *European Heart Journal*. https://doi.org/10.1093/eurheartj/ehz455
- Mannucci, C., Navarra, M., Calapai, F., Squeri, R., Gangemi, S., & Calapai, G. (2017). Clinical pharmacology of *Citrus bergamia* : A systematic review. *Phytotherapy Research*, *31*(1), 27–39. https://doi.org/10.1002/ptr.5734
- Mathew, S., & Abraham, T. E. (2006). In vitro antioxidant activity and scavenging effects of *Cinnamomum verum* leaf extract assayed by different methodologies. *Food and Chemical Toxicology*, *44*(2), 198–206. https://doi.org/10.1016/j.fct.2005.06.013
- McDonald, S., Prenzler, P. D., Antolovich, M., & Robards, K. (2001). Phenolic content and antioxidant activity of olive extracts. *Food Chemistry*, *73*(1), 73–84. https://doi.org/10.1016/S0308-8146(00)00288-0
- Nasri, H., & Rafieian-Kopaei, M. (2014). Medicinal plants And antioxidants: why they are not always beneficial? *Iranian Journal of Public Health*, *43*(2), 255–257. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/26060753
- Ochani, P. C., & D'Mello, P. (2009). Antioxidant and antihyperlipidemic activity of *Hibiscus sabdariffa* Linn. leaves and calyces extracts in rats. *Indian Journal of Experimental Biology*, *47*(4), 276–282. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19382724
- Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, *95*(2), 351–358. https://doi.org/10.1016/0003-2697(79)90738-3
- Oyaizu, M. (1986). Studies on products of browning reaction. Antioxidative activities of products of browning reaction prepared from glucosamine. *The Japanese Journal of Nutrition and Dietetics*, *44*(6), 307–315. https://doi.org/10.5264/eiyogakuzashi.44.307
- Patti, A. M., Toth, P. P., Giglio, R. V., Banach, M., Noto, M., Nikolic, D., … Rizzo, M. (2017). Nutraceuticals as an important part of combination therapy in dyslipidaemia. *Current Pharmaceutical Design*, *23*(17), 2496–2503. https://doi.org/10.2174/1381612823666170317145851
- Pernice, R., Borriello, G., Ferracane, R., Borrelli, R. C., Cennamo, F., & Ritieni, A. (2009). Bergamot: A source of natural antioxidants for functionalized fruit juices. *Food Chemistry*, *112*(3), 545–550. https://doi.org/10.1016/j.foodchem.2008.06.004
- Picerno, P., Sansone, F., Mencherini, T., Prota, L., Aquino, R. P., Rastrelli, L., & Lauro, M. R. (2011). *Citrus bergamia* juice: phytochemical and technological studies. *Natural Product Communications*, *6*(7), 1934578X1100600. https://doi.org/10.1177/1934578X1100600707
- Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*(9–10), 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3
- Redberg, R. F., & Katz, M. H. (2012). Healthy men should not take statins. *JAMA*, *307*(14), 1491–1492. https://doi.org/10.1001/jama.2012.423
- Ercisli,S., Uzun A.,, Ugurtan Yilmaz. K., , Sengul M.,, Yildiz, H.,, & Uysal O.. (2015). Antioxidant activity and total phenolic content of bergamot (*Citrus bergamia* Risso) fruits. *Acta Horticulturae*, (1065), 1471–1474. https://doi.org/10.17660/ActaHortic.2015.1065.186
- Shayganni, E., Bahmani, M., Asgary, S., & Rafieian-Kopaei, M. (2016). Inflammaging and cardiovascular disease: Management by medicinal plants. *Phytomedicine*, *23*(11), 1119–1126. https://doi.org/10.1016/j.phymed.2015.11.004
- Shih, C.-C., Lin, C.-H., Lin, Y.-J., & Wu, J.-B. (2013). Validation of the antidiabetic and hypolipidemic effects of hawthorn by assessment of gluconeogenesis and lipogenesis related genes and AMP-activated protein kinase phosphorylation. *Evidence-Based Complementary and Alternative Medicine*, *2013*, 1–12. https://doi.org/10.1155/2013/597067
- Simamora, A., Steven, M., Santoso, A. W., Rumiati, F., & Timotius, K. H. (2018). Antioxidant activity of methanol extract from several

indonesian green teas. *YARSI Medical Journal*, *26*(1), 018. https://doi.org/10.33476/jky.v26i1.460

- Simić, A., Manojlović, D., Šegan, D., & Todorović, M. (2007). Electrochemical behavior and antioxidant and prooxidant activity of natural phenolics. *Molecules*, *12*(10), 2327–2340. https://doi.org/10.3390/12102327
- Trovato, A., Taviano, M. F., Pergolizzi, S., Campolo, L., De Pasquale, R., & Miceli, N. (2009). *Citrus bergamia* risso & poiteau juice protects against renal injury of diet-induced hypercholesterolemia in rats. *Phytotherapy Research*, n/a-n/a. https://doi.org/10.1002/ptr.2971
- Tungmunnithum, D., Thongboonyou, A., Pholboon, A., & Yangsabai, A. (2018). Flavonoids and other phenolic compounds from medicinal plants for pharmaceutical and medical aspects: an overview. *Medicines*, *5*(3), 93. https://doi.org/10.3390/medicines5030093
- Yıldız Turgut, D., Seçmen,Tuba; Tanır, A. (2018). A comparative study of the antioxidant properties of different citrus juices. In *International Agricultural, Biological & Life Science Conference, Edirne, Turkey* (pp. 179–185).
- Vincenzo, Sicari. Pellicanò, T. M. (2016). Phytochemical properties and antioxidant potential from Citrus bergamia, Risso (bergamot) juice extracted from three differen cultivars. *Journal of Applied Botany and Food Quality*, *89*, 171–175.
- Wang, H., & Helliwell, K. (2001). Determination of flavonols in green and black tea leaves and green tea infusions by high-performance liquid chromatography. *Food Research International*, *34*(2–3), 223–227. https://doi.org/10.1016/S0963-9969(00)00156-3
- Wang, K.-J., Zhang, Y.-J., & Yang, C.-R. (2006). New phenolic constituents from *Balanophora polyandra* with radical-scavenging activity. *Chemistry & Biodiversity*, *3*(12), 1317–1324. https://doi.org/10.1002/cbdv.200690135
- Zhao, C.-N., Tang, G.-Y., Cao, S.-Y., Xu, X.-Y., Gan, R.-Y., Liu, Q., … Li, H.-B. (2019). Phenolic profiles and antioxidant activities of 30 Tea infusions from green, black, oolong, white, yellow and dark teas. *Antioxidants*, *8*(7), 215. https://doi.org/10.3390/antiox8070215

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Original Article

Investigation of antioxidant properties, essential oil, and fatty acid composition of *Onobrychis armena* Boiss. & Huet

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ABSTRACT

Background and Aims: In this study, *Onobrcyhis armena* Boiss. & Huet was screened for its antioxidant potential, fatty acids and volatile compounds.
Methods: Antioxidant activities of different extracts (ethyl acetate, methanol and water) were measured using the phosphomolybdenum assay, free radical scavenging assay, β-carotene/linoleic acid method, and ferric and cupric reducing power assay. Total phenolic and flavonoid contents were also calculated spectrophotometrically.
Results: GC analysis revealed that the oil was dominated by palmitic (22.67%) and linoleic (15.09%) acids. Unsaturated acids levels were higher than saturated fatty acids. The essential oil was analyzed by GC-MS system and twenty-two volatile compounds were identified. The identified major components were n-hexadecanoic acid, 9-12 octadecanoic acid, tetradecanoic acid and hexahydro farnesyl acetone.
Conclusion: The results of this study show that *O. armena* can be used as an easily accessible source of natural antioxidants and unsaturated fatty acids in food and pharmaceutical industries.

Keywords: *Onobrychis*, Fatty acids, Essential oil, Phenolic compounds, GC-MS

INTRODUCTION

Free radicals are atoms or groups of atoms having an unpaired electron in their last orbital. Generally, they are known as reactive oxygen species consisting of hydroxyl radical, hydrogen peroxide, peroxynitrite, nitric oxide, peroxyl radical, singlet oxygen and superoxide anion. Oxidative stress is caused by the discrepancy between the production of free radicals in the body and the attempt to render their bad effects harmless with antioxidants. These render free radicals harmless by complementing their unpaired end electrons with extra electrons or by breaking them down (Halliwell & Gutteridge, 1984).

Antioxidants play a significant role in the prevention of diseases with their ability to repair or cleanse the damage caused by free radicals (Alonso, Guillen, Barroso, Puertas, & Garcia, 2002). Epidemiological studies have revealed an inverse relationship between dietary antioxidant-rich foods, degenerative processes, and death (Hertog, Feskens, Hollman, Katan, & Kromhout, 1993; Pietta, 2000). Natural chemicals obtained from plants, especially phenolic compounds with high antioxidant activity, have enabled them

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to be used in the pharmaceutical and food industry. In addition to their antioxidative properties, herbal ingredients are valuable for their antiviral, antimicrobial and anti-inflammatory activities (Rice-Evans, Miller, & Paganga, 1997).

Essential oils and chemical components obtained from medicinal plants are used as deodorizing and flavoring additives in the cosmetics and food industry. Also, essential oils play an important role in the protection of plants against insects in nature (Bakkali, Averbeck, Averbeck, & Waomar, 2008).

Plant-based products used against oxidative damage and diseases, such as various foods, dietary supplements, and pharmaceuticals, have attracted worldwide attention in recent years (Huang, Ou, & Prior, 2005).

In recent years, besides natural antioxidants or plant-based foods containing antioxidants, synthetic antioxidants such as Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT), PG (propyl gallate) and TBHQ (tertiary butyl hydroquinone) have been used to prevent oxidative damage in the body, as well as to prevent lipid oxidation in foods. However, some studies have shown that synthetic antioxidants can harm human health (Valentao et al., 2002).

Fabaceae comprise the second largest plant family after the Asteraceae family with more than 18,000 species common worldwide (Zengin et al., 2015). The Fabaceae plant family is medicinally, economically, and culturally important (Erbil, Duzguner, Durmuskahya, & Alan, 2015).

Onobrychis is a member of the Fabaceae family of which 162 species have been identified in the world. In Turkiye there are a total of 52 Onobrychis genera,27 of which are endemic. These have a rich phenolic content (such as *p*-hydroxybenzoic acid, ferulic acid, rutin, benzoic acid, caffeic acid, *p*-coumaric acid, quercetin) (Karakoca, Asan-Ozusaglam, Cakmak, & Teksen, 2015), and therefore these have a significant antioxidant (Karamian & Asadbegy, 2016) and antimicrobial activity (Usta, Yildirim, & Turker, 2014).

In this study, antioxidant properties, fatty acids and essential oil composition of the aerial parts of *Onobrychis armena* Boiss. & Huet plant collected from Konya (Turkiye) were studied. For this purpose, different extracts (ethyl acetate, methanol and water) of *Onobrychis armena* were prepared and its antioxidant activities were measured using the phosphomolybdenum experiment, free radical scavenging test, *β*-carotene/linoleic acid method, and ferric and copper reducing power test. Also, the total phenolic and flavonoid contents were determined to understand the usefulness of this plant as a foodstuff as well as in medicine.

MATERIAL AND METHODS

Plant materials

Onobrychis armena was collected during the flowering period from the area between Yükselen and Kestel villages in Konya province (Turkiye). The plants were identified by Prof. Dr. Murad Aydin Sanda from the Division of Botany within the Department of Biology, Science Faculty, Selcuk University (Konya). The voucher specimens were deposited in the KNYA herbarium at the Department of Biology, Selcuk University.

Chemicals

Potassium ferricyanide, Folin–Ciocalteu's reagent, BHT, BHA and methanol were purchased from Merck (Germany); 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene/linoleic acid and Tween 40 were purchased from Sigma–Aldrich GmbH (Germany). All other chemicals and solvents were analytical grade.

Preparation of the extracts

The collected plant samples were dried in the shade, then they were ground thoroughly in the mill. 15 g of powdered samples were weighed and extracted separately with ethyl acetate and methanol for 6-8 hours in the soxhlet apparatus. After this time, the mixture obtained was filtered with Whatman paper. Then the solvents were completely evaporated at 40 ^{0}C in a rotary evaporator. The obtained dry extracts were stored at +4 ^{0}C until analysis. 15 g of dry herbal material was boiled in 250 mL of water for 30 minutes to obtain a water extract. The water extract was frozen after filtering and lyophilized to completely remove the water. The lyophilized dry herbal drug was stored at +4 ^{0}C until analysis. The extraction yields were 2.4%, 10.8% and 14.2% for ethyl acetate, methanol, and water, respectively.

Total phenolic (folin-ciocalteu method) substance determination

A concentration of plant extracts was prepared consisting of 2 mg/mL, and 200 µl of each concentration was taken into separate test tubes. Then 1.5 mL of water and 100 µL of Folin-Ciocalteu reagent were added to each tube. After this, 500 µL of 2% Na_2CO_3 solution was added to each tube. After the mixtures were left in the dark for 2 hours at room temperature, their absorbance was measured at 765 nm. Spectrophotometric measurements in all antioxidant capacity determination tests were carried out using the Shimadzu UV-1800 spectrophotometer. The same procedures were repeated for the standard gallic acid. The phenolic content of the plants was given as gallic acid equivalent (mg GAE/g) (Slinkard & Singleton, 1977).

Total flavonoid substance determination

The total flavonoid content in plant extracts was determined spectrophotometrically. Accordingly, 1 mL of the methanolic solution of 2% $AlCl_3$ was taken and mixed with the same volume and 2 mg/mL concentration of plant extract. After waiting for 10 minutes, the absorbance of the mixture against blank was determined at 415 nm. The same procedures were done for the standard flavonoid routine, and the calibration curve for the routine was drawn. As a result, the total flavonoid substance contents of the extracts were given as routine equivalent (mg RE/g) (Arvouet-Grand et al., 1994).

Determination of total antioxidant capacity

The basis of the method is the reduction of Mo (VI) to Mo (V) and the formation of green colored phosphate/Mo (V) complex in acidic medium. Following this method, the first step was to prepare solutions of plant extracts with a concentration of 2 mg/mL.

The reagent solution was prepared by mixing the prepared solutions in a cylinder. 0.3 mL of herbal solutions at a concentration of

1 mg/mL was taken into a tube and 3 mL of the reagent solution was added. Tubes were mixed vigorously and stored at 95 ^{0}C.

The incubation lasted for 90 minutes,at the end of which the absorbance of the solutions was read at 695 nm. The same procedures were followed for ascorbic acid, which is used as a standard antioxidant. Antioxidant activity was calculated as ascorbic acid equivalent (mg AAE/g) (Prieto, Pineda, & Aguilar, 1999).

β-carotene/Linoleic acid emulsion system

In this method, the emulsion solution was first prepared. For this, 1 mg of β-carotene was dissolved in 2 mL of chloroform. 50 µL linoleic acid and 200 mg Tween 40 were added to this mixture. The mixture was thoroughly mixed. Chloroform was thoroughly evaporated at 40 ^{0}C in a rotary evaporator. 200 mL of pure water was added on the remaining part. Thus, the emulsion solution was prepared.

350 µl of herbal drugs and standard substances with a concentration of 2 mg/mL were taken and 2.5 mL of emulsion solution was added to them. As soon as the emulsion solution was added, the absorbances were read at 490 nm. The tubes were then incubated at 50 ^{0}C for 120 minutes. In addition, the control solution was prepared by adding 350 µL of methanol instead of the plant material and then adding 2.5 mL of emulsion solution. The absorbance of the control solution was also read as soon as the emulsion solution was added and was likewise incubated at 50 ^{0}C for 120 minutes (Sokmen et al., 2004). The reduction percentage was given as shown below.

$$I\ (\%)=(A_0-A_1)/A_0x100$$

In this formula, A_0 is the absorbance of the control and A_1 is the absorbance of the extract/standard.

DPPH free-radical scavenging assay

Free radical removal activities of plant samples were determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH). 0.4 mM DPPH solution was subjected to dilution with ethanol by controlling the absorbance at 517 nm. Subsequently, 40 µL of different concentrations of plant samples were placed in microplates. Then, 120 µL of ethanol and 40 µL of DPPH solution were added to be incubated for 30 minutes in a dark condition. The absorbances were read at 517 nm. The absorbance results of the herbal extracts were examined against the control. Free radical removal activity was used as shown below and the percentage of the inhibition values was calculated from these absorbance values (Sarikurkcu et al., 2009).

$$I\ (\%)=(A_0-A_1)/A_0x100$$

In this formula, A_0 is the absorbance of the control and A_1 is the absorbance of the extract/standard.

Reducing power activity [Iron (III) to iron (II) reduction]

In this method, concentrations of 0.2 to 2 mg/mL of herbal extracts were used. 2.5 mL of herbal solutions of different concentrations were taken. After that 0.2M pH:6.6 2.5 mL phosphate buffer and 2.5 mL 1% potassium ferricyanide were added. The tubes were left to incubate at 50 °C for 20 minutes. 10% Trichloroaceticacid (TCA) was added to the tubes after incubation. After mixing the tubes, 2.5 mL were transferred from their upper part to another tube. 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$ solution were added into this tube. Absorbances of the solutions were read at 700 nm (Oyaizu, 1986).

CUPRAC assay

The cupric ion reducing activity (CUPRAC) was determined according to the method of Apak et al. (2006). Sample solution (0.5 mL) was added to premixed reaction mixture containing $CuCl_2$ (1 mL, 10 mM), neocuproine (1 mL, 7.5 mM) and NH_4Ac buffer (1 mL, 1M, pH:7.0). Similarly, a blank was prepared by adding a sample solution (0.5 ml) to the premixed reaction mixture (3 mL) without $CuCl_2$. Then, the sample and blank absorbances were read at 450 nm after a 30 min incubation at room temperature. The absorbance of the blank was subtracted from that of the sample. CUPRAC activity was expressed as Trolox equivalent (TE/g extract).

Determination of fatty acid composition of plants
oil extraction from plants

The ground and powdered 10 g of plant material was first extracted with petroleum ether for 6-8 hours in the soxhlet apparatus. At the end of the extraction, the residue after the solvent was evaporated in the evaporator was used in fatty acid analysis.

Preparation of methyl esters of fatty acids

0.1-0.2 g of the oil samples were transferred to the flasks. 4 mL of 2% NaOH solution was added to the oil samples and boiled for 10 minutes for saponification to occur. After saponification was completed, 5 mL of 14% BF_3-methanol complex was added and boiled for 5 minutes. Then, 2 mL of n-heptane was added to the mixture and left to boil for one minute. After boiling was complete, 4 mL of saturated NaCl solution was added. After mixing thoroughly, the balloons were transferred to separation funnels for phase separation and left for 5-10 minutes. At the end of this period, the lower aqueous part was discarded, and the upper yellow phase was transferred to vials and stored at -20 ^{0}C until analyzed (IUPAC, 1979).

Gas chromatography-mass spectrometry

The samples to be analyzed for essential oil were ground and subjected to water distillation with the Clevenger apparatus. The extracts obtained were made in HP Agilent 7890A Gas Chromatography using Agilent 5975C MS detector and HP Innowax column. Wiley and Nist libraries were used for the identification of essential oil components.

Helium was used as carrier gas in the analyzes and the flow rate was set at 1.2 mL/min. The initial temperature of the column was determined to be 60 ^{0}C and held at this temperature for 10 minutes. Later, by increasing 4 ^{0}C per minute, 220 ^{0}C was reached. After being kept at this temperature for 10 minutes, it reached 240 ^{0}C by increasing 1 ^{0}C per minute. Finally, it was held at this temperature for 30 minutes. Thus, the total analysis time was determined as 110 minutes. The temperature of the injector block was set at 240 ^{0}C. Mass spectrums were recorded at 70 eV.

RESULTS AND DISCUSSION

Total phenolic and flavonoid content

The total phenolic and flavonoid contents of the ethyl acetate, methanol and water extracts of *Onobrychis armena* are given in Table 1. Among the extracts, the phenolic content was mostly observed in the methanol extract. 62.199±0.001 mg GAE/g phenolic substance was found in the methanol extract. Methanol extract is followed by water (34.689±1.136 mg GAE/g) and ethyl acetate (18.323±0.852 mg GAE/g) in terms of content, respectively. Godevac et al. (2008), in their study investigating the antioxidant properties of nine Fabaceae members, determined the phenolic content in *O. scardica* methanol extract to be higher (115.23 mg GAE/g) than *O. armena*. However, *Astragalus glycyphyllos* L. (44.6 mg GAE/g) and *Coronilla emerus* L. (38 mg GAE/g) extracts had lower phenolic content than *O. armena*. The total phenolic contents determined in our study agree with the values given for some species of the Fabaceae family (Orhan et al., 2009; Orhan et al., 2011).

Flavonoids constitute the largest and most important group of phenolic compounds. Flavonoid contents of *O. armena* were calculated as routine equivalents. The richest extract in terms of flavonoid content is methanol extract with 32.589±0.465 mg RE/g content. Methanol extract is followed by water (20.046±0.141 mg RE/g) and ethyl acetate (16.846±0.061 mg RE/g) in terms of content, respectively. Hayet et al. (2008) determined the flavonoid contents of ethyl acetate and methanol extracts as 53.81 and 41.58 mg CAE/g, respectively, in their study on the biological activities of *Retama raetam* (Forssk.) Webb, a member of Fabaceae.

Total antioxidant capacity (Phosphomolybdate test)

The phosphomolybdate test is based on the reduction of the antioxidant compounds Mo (VI) to Mo (V) in an acidic environment and spectrophotometric measurement of the phosphate/Mo (V) complex formed.

The results of the method are given using a standard antioxidant and usually ascorbic acid. According to the results of this test, methanol extract has the highest efficiency (103.118±0.795 mg AAE/g). This extract is followed by ethyl acetate (38.933±0.596 mg AAE/g) and water (35.562±1.390 mg AAE/g) extracts, respectively (Table 1).

DPPH free radical removal method

The change in free radical scavenging activity of *O. armena* extracts depending on the concentration is presented in Table 2. These results show the methanolic extract to be the highest radical scavenging efficiency among the extracts. Although the water and methanol extracts show relatively similar activity, the activity of the ethyl acetate extract is very low. On the other hand, the free radical scavenging efficiency of BHA and BHT, which are synthetic antioxidants, is considerably higher than the extracts despite their low concentrations.

DPPH scavenging activity of *Vicia sativa* ssp. *nigra* was determined as 8.7% and 15.4% at a concentration of 0.5 mg/ml and 1 mg/mL, respectively (Orhan et al., 2009). The activity of the methanolic extract at a concentration of 1 mg/mL of *O. armena* used in our study is higher compared with the water extract. Of the other concentrations studied, the activities of water and methanol extracts were relatively close to each other.

Ferric reducing power

Reducing power or potential is one of the most important indicators of antioxidant capacity. This potential antioxidant activity indicates the electron donating ability of the compounds or extracts investigated. Therefore, high reducing power means high antioxidant activity. For this purpose, the ferric and copper reduction powers of *O. armena* extracts were investigated. The ferric reducing power is based on the investigation of the ability of antioxidant molecules to convert Fe^{+3}-Fe^{+2} and the resulting Prussian Blue color spectrophotometric determination. The higher the absorbance, the higher the reducing power. The ferric reducing power results are shown in Table 3, depending on the concentrations of the studied *O. armena* extracts (Table 3). Among the extracts, the best reducing power belongs to

Table 1. Total phenolic, flavonoid contents and total antioxidant capacity of *O. armena* extracts.

	Ethyl acetate	Methanol	Water
Total Phenolic (mg GAEs/g*)	18.323±0.852	62.199±0.001	34.689±1.136
Total Flavonoid (mg REs/g*)	16.846±0.061	32.589±0.465	20.046±0.141
Total Antioxidant (mg AAEs/g*)	38.933±0.596	103.118±0.795	35.562±1.390

*GAEs-gallic acid equivalents, *REs/g-rutin equivalents, *AAEs-Ascorbic acid equivalents

Table 2. DPPH free radical % scavenging activities of *O. armena* extracts and synthetic antioxidant.

	0.125 mg/mL	0.25 mg/mL	0.5 mg/mL	1 mg/mL
Ethyl acetate	6.540±0.001	11.958±1.368	16.796±0.274	26.529±2.764
Methanol	10.700±0.958	23.084±0.629	44.021±0.520	80.611±0.930
Water	14.222±1.450	23.491±1.806	44.621±2.080	76.645±0.137
BHA	95.627±0.328	-	-	-
BHT	62.481±0.301	-	-	-

the methanolic extract and the ferric reduction power at a concentration of 1 mg/mL is 1.457±0.047. However, although synthetic antioxidants are at 10 times more dilute concentration, they have a stronger reducing potential than all the extracts. The concentration of 1 mg/ml of *Vicia sativa* ssp. nigra, a member of Fabaceae, was determined as 0.230±0.1 at 700 nm (Orhan et al., 2009). This value is quite low compared to *O. armena*. Likewise, in a study investigating the antioxidant and DNA protective effects of *Bauhinia variegata* L., a Fabaceae member, the highest reducing power was reported in the methanol extract (Sharma, Bhardwaj, Kumar, & Kaur, 2001). Similarly, methanol extract of *O. armena* also exhibited stronger activity compared to water. Another study on the nutritional characteristics of Fabaceae members shows that the ferric reduction power of seeds and fruit extracts is quite weak compared to *O. armena* (Chanda, Dudhatra, & Kaneria 2010).

CUPRAC test

As with ferric reducing power, the copper reducing power of extracts increases with concentration. The concentration-dependent copper reduction capabilities of *O. armena* extracts are shown in Table 4. Similar to the ferric reduction power, the highest copper reduction capacity was found in the methanol extract of *O. armena*. Similarly, many studies have reported a strong correlation between ferric and copper reduction powers. The extracts studied and synthetic antioxidants can be listed in terms of copper-reducing efficiency as follows: BHA> BHT>Methanol> Water> Ethyl acetate. Various other studies also state that methanol extracts have stronger copper reducing power compared to water extracts (Zahin, Aqil, & Ahmad, 2010).

β-carotene/Linoleic acid test system

The percentages of the inhibition values of *O. armena* extracts and synthetic antioxidants in β-carotene/linoleic acid test system are given in Table 5. Unlike other methods, the highest inhibition rate (84.437%) was observed in ethyl acetate extract in this method. Methanol extract showed 58.557% inhibition, while the water extract showed 33.649% inhibition. The results observed in this test system may be due to the higher presence of other antioxidant compounds with antioxidant activity other than phenolic compounds that prevent linoleic acid oxidation in the ethyl acetate extract. Not only do phenolic compounds play an active role in the method, but also other molecules with antioxidant properties. Similar situations have been reported for this method in various studies (Mishra, Yosouf, & Singh, 2009; Baghiani et al., 2012). Methanolic extract of *Sesbania sesban*, a member of the Fabaceae family, like *O. armena*, inhibited linoleic acid oxidation by 49.80%. Although this value seems low compared to *O. armena*, considering that the *S. sesban* extract has a concentration of 1 mg/mL, it is not correct to make a direct comparison. In a study on 15 *Lathryus* species, inhibition values in the β-carotene/linoleic acid test vary between 28.47% and 57.83% (Pastor-Cavada, Juan, Pastor, Alaiz, & Vioque, 2009).

Results of fatty acid analysis

As a result of the gas chromatographic analysis of the oil of *Onobrychis armena* Boiss. & Huet, it was determined that the fatty acid composition was formed by 25 different fatty acids. Carbon numbers of these fatty acids vary between 8 and 22.

Table 3. Ferric reducing power of *O. armena* extracts and synthetic antioxidants.

	0.2 mg/mL	0.5 mg/mL	1 mg/mL	2 mg/mL
Ethyl acetate	-	0.062±0.008	0.209±0.037	0.441±0.023
Methanol	0.072±0.025	0.307±0.017	0.710±0.023	1.457±0.047
Water	0.036±0.001	0.229±0.032	0.458±0.010	0.843±0.001
BHA	2.685±0.028	-	-	-
BHT	1.810±0.028	-	-	-

Table 4. Copper reducing power of *O. armena* extracts and synthetic antioxidants.

	0.2 mg/mL	0.5 mg/mL	1 mg/mL	2 mg/mL
Ethyl acetate	0.115±0.021	0.328±0.01	0.629±0.022	1.374±0.004
Methanol	0.395±0.094	0.820±0.069	1.557±0.020	3.045±0.007
Water	0.170±0.008	0.453±0.025	0.942±0.005	1.731±0.020
BHA	3.678±0.081	-	-	-
BHT	3.435±0.046	-	-	-

Table 5. Percentage (%) of *O. armena* extracts and synthetic antioxidants to inhibit Linoleic acid oxidation.

	Inhibition (%)
Ethyl acetate	84.437±4.657
Methanol	58.557±0.540
Water	33.649±0.618
BHT	94.395±0.052
BHA	95.735±0.052

When the fatty acid composition was examined, it was found that the major fatty acid was C 16:0, palmitic acid (Table 6). Palmitic acid is followed by C 18:2 ω6 with 15.09%, linoleic acid and C 20:3 ω6 with 12.03% (cis-8-11-14 eicosatrienoic acid). Similarly, palmitic (34.92%) and linoleic acid (25.91%) were determined as the fatty acids with the highest percentage in the fatty acid composition of *Teramnus labialis* belonging to the Fabaceae family such as *Onobrychis* (Wiswanathan, Thangadurai, Vendan, & Ramesh, 1999). Thangadurai et al. (2001), in their study investigating the nutritional properties of *Galactia longifolia* (Jacq.) Benth., determined that the fatty acid composition consists of five fatty acids and that palmitic acid has the highest percentage of these fatty acids. In their study on the fatty acid compositions of some Fabaceae members,Bagci et al. (2004) determined the fatty acid compositions of four species belonging to the genus Onobrcyhis showing them to be linoleic, oleic and palmitic acids, respectively. In the same study, the percentage of linoleic acid in *Onobrychis major* (Boiss.) Hand.-Mazz. increased up to 51.8%.

It was observed that the monounsaturated fatty acids (MUFA) content of *O. armena* was mostly composed of C 18:1 C9 oleic acid (4.47%) in the fatty acid composition. Oleic acid accounts for about 50% of the total monounsaturated fatty acids content. This fatty acid is followed by C 16:1 ω7 palmitoleic acid (1.51%), C 15:1 ω5 pentadecanoic acid (1.37%) and C 14:1 ω5, myristoleic acid (1.22%), respectively. Others of the monounsaturated fatty acids are at very low levels. Similarly, in many studies on fatty acid compositions of Fabaceae members, oleic acid has been reported to be the fatty acid that contributes the greatest to MUFA content (Mao et al., 2012; Uzun, Arslan, Karhan, & Toker, 2007).

When looking at the saturated (SFA), mono (MUFA) and polyunsaturated (PUFA) fatty acid contents of the studied *O. armena* oil, the polyunsaturated fatty acids are higher than saturated and monounsaturated fatty acids. In *O. armena*, PUFA, SFA, and MUFA contents were determined as 56.62%, 33.84% and 9.54%, respectively (Table 7). This situation has been similarly reported in *Onobrychis* and many Fabaceae members. The polyunsaturated fatty acids (PUFA) content of *O. armena* is mostly C 18:2 ω6 and C 20:3 ω6. Linoleic acids cannot be synthesized by humans and are considered "essential fatty acids" since they must be taken from the diet. The percentage of essential fatty acids in *O. armena* is 26.96%. This high level of essential fatty acids suggests that *O. armena* oil may be considered as an important source of essential fatty acids. In addition, the positive effects of polyunsaturated fatty acids on health and the high levels of polyunsaturated fatty acids in *O. armena* oil increase the importance of this oil on health.

Various values such as ω3/ω6, atherogenic index (AI) and thrombogenic index (TI) have recently been used more frequently in evaluating the nutritional quality of fat. A high ratio of ω3/ω6 and low AI and TI values is desirable in the nutritional quality of the oil. The 3/ω6 ratio, AI and TI values of *O. armena* oil used in the study were determined as follows: 0.65, 0.47 and 0.42. It has been reported that the AI value of various vegetable oils, for example cocoa butter, is between 13-20, and this value is around 7 for palm oil. From this , it might be concluded that the indicated indexes increase due to the increase in saturated fatty acid content, but on the contrary, the increase in unsaturated fatty acids contributes to the decrease of these values. Therefore, the high unsaturated fatty acid content of *O. armena* oil is the most important indicator that this oil might also be nutritionally valuable.

Results of essential oil composition

During the flowering period of *Onobrcyis armena*, the essential oils of the above-ground parts of this plant were obtained by water distillation method, and the compositions of these essential oils were investigated by GC-MS. Analysis results of these plants are given in Table 8. A total of 22 different essential oil components were determined in the examined *O. armena* essential oil. The components of the essential oil were identified at a rate of 96.957%. N-hexadecanoic acid was determined as the major component of essential oil. This component makes up 56.609% of the total essential oil content. In the same way, n-hexadecanoic acid was determined as the major

Table 6. Fatty acids of *O. armena*.

Carbon Number	Common and Systematic Name
C8:0	Caprylic acid (Octanoic acid)
C10:0	Capric acid (Decanoic acid)
C11:0	Andesilic acid (Andecanoic acid)
C12:0	Lauric acid (Dodecanoic acid)
C13:0	Tridesilic acid (Tridecanoic acid)
C14:0	Myristic acid (Tetradecanoic acid)
C14:1ω5	Myristoleic acid (cis-9-Tetradecanoic acid)
C15:0	Pentadecylic acid (Pentadecanoic acid)
C15:1ω5	Pentadecanoic acid (cis-10-Pentadecanoic acid)
C16:0	Palmitic acid (Hexadecanoic acid)
C16:1ω7	Palmitoleic acid (cis-9-Hexadecanoic acid)
C17:0	Margaric acid (Heptadecanoic acid)
C17:1ω8	Margaroleic acid (cis 10-Heptadecanoic acid)
C18:0	Stearic acid (Octadecanoic acid)
C18:1ω9	Oleic acid (cis-9-Octadecanoic acid)
C18:1ω7	cis-vaccenic acid (cis-11-Octadecanoic acid)
C18:2ω6	Linoleic acid (cis-9-12-Octadecadienoic acid)
C18:3ω6	γ-Linolenic acid (cis-6-9-12-Octadecatrienoic acid)
C18:3ω3	Linolenic acid (α-linoleic acid.ALA) (cis-9-12-15-Octadecatrienoic acid)
C20:0	Arachidic acid (Eicosanoic acid)
C20:1ω9	Gadoleic acid (cis -11 Eicosenoic acid)
C20:3ω3	cis-11,14,17- Eicosatrienoic acid
C20:3ω6	cis-8-11-14 Eicosatrienoic acid
C22:1ω9	Erucic acid (cis-13 Docosanoic acid)
C22:6ω3	Docosahexaenoic acid (DHA) (cis-4,7,10,13,16,19-Docosahexaenoic)

Table 7. *O. armena* fatty acid composition (%).

Carbon Number	*O.armena*	Carbon Number	*O.armena*
C8:0	0.05±0.01*	C18:2ω6	15.09±0.01
C10:0	0.10±0.01	C18:3ω6	11.81±0.07
C11:0	0.04±0.01	C18:3ω3	11.87±0.01
C12:0	0.45±0.01	C20:3ω6	12.03±0.03
C13:0	0.84±0.01	C20:3ω3	0.11±0.01
C14:0	1.97±0.01	C22:6ω3	5.71±0.35
C15:0	1.09±0.01	**ΣPUFA****	**56.62±0.26**
C16:0	22.67±0.07	**ΣUFA****	**66.16±0.01**
C17:0	0.64±0.03	**ΣEFA****	**26.96±0.01**
C18:0	5.87±0.01	**PUFA/SFA**	**1.67±0.01**
C20:0	0.12±0.02	**ω3**	**17.69±0.35**
ΣSFA*	**33.84±0.01**	**ω6**	**27.17±0.02**
C14:1ω5	1.22±0.01	**ω3/ω6**	**0.65±0.01**
C15:1ω5	1.37±0.20	**ω6/ω3**	**1.54±0.03**
C16:1ω7	1.51±0.10	**AI****	**0.47±0.01**
C17:1ω8	0.33±0.01	**TI****	**0.42±0.01**
C18:1ω9	4.47±0.01		
C18:1ω11	0.46±0.01		
C20:1ω9	0.04±0.01		
C22:1ω9	0.14±0.05		
ΣMUFA*	**9.54±0.27**		

*Arithmetic mean ± standard deviation
**SFA:Saturated fatty acids, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids, UFA: Unsaturated fatty acids, EFA: Essential fatty acids, AI: Atherogenic index, TI: Thrombogenic index.

Table 8. *O. armena* essential oil components (%).

	RTa	Component Name	%
1	27.500	Tetradecane	0.517
2	28.910	Farnesan	0.127
3	44.371	β-Ionone	0.262
4	45.363	2-propenoicacid, pentadecy-lester	0.097
5	47.718	Heneicosane	0.484
6	48.575	Hexahydrofarnesylacetone	5.222
7	48.903	Spathulenol	0.923
8	50.939	Spiro[4,5]dec-6-en-8-one,1,7-dimethyl-4-1(1-methylethyl)	0.156
9	52.468	Docosane	0.955
10	57.097	9-octadecanoicacid	0.289
11	58.400	Dodecanoicacid	3.153
12	58.600	Tetratetracontane	0.880
13	58.891	9-15octadecanoicacid	0.569
14	63.481	Phytol	1.538
15	67.274	Heneicosane,11-(1-ethylpro-pyl)	0.951
16	67.541	Tetradecanoicacid	6.783
17	75.026	2-ısobutyl-1,3,2-oxazaborolane	0.549
18	77.780	Nonocosane	2.888
19	78.717	n-Hexadecanoicacid	56.609
20	80.652	15-Tetracosenoicacid	3.567
21	90.053	Hexadecane	1.453
22	99.995	9-12-Octadecanoicacid	8.985
		Total defined component	**96.957**

RT: RetentionTime

component in *Samanea saman* (Jacq.) Merr. essential oil (Oguwande, Walker, Setzer, & Essien, 2006). Studies have reported that the essential oil composition varies depending on certain factors such as climatic conditions, season, and altitude (Daferera, Ziogas, & Polissiou, 2000; Grosso et al., 2007).

Major components after n-hexadecanoic acid in the essential oil composition of the studied *O. armena* can be listed as follows: 9-12 Octadecanoic acid (8.985%), tetradecanoic acid (6.783%), hexahydro farnesyl acetone (5.222%), 15-tetracosenoic acid (3.567%) and dodecanoic acid (3.153%). In *Retama raetam* essential oil, a member of the Fabaceae family, 12 components were determined quite differently from *O. armena* essential oil and-linalool (50.9%) was reported as the major component. Differently, 1,8-cineol (39.8%) was determined as the major component in the composition of the essential oil of *Cassia alata*, which belongs to the same family (Bhaksu & Raju, 2009). Kicel et al. (2010) identified hexahydro farnesyl acetone in the essential oil composition of *Trifolium repens* at a rate of 6.2%, close to our study. Phytol, which is relatively low as 1.538% in *O. armena*, is the major component of the essential oil of *Prosopis farcta* leaves, a member of the same family.

CONCLUSION

In this study, the *in vitro* antioxidant properties of ethyl acetate, methanol and water extracts of *Onobrychis armena* Boiss. & Huet were investigated using five different chemical test systems including total antioxidant, free radical capture, β-carotene/linoleic acid, copper and ferric reduction powers. In addition, total phenolic and flavonoid content was also determined. The antioxidant activity of methanol extract of *O. armena* was found to be stronger than other extracts in all test systems (except the β-carotene/linoleic acid test). This shows that, as in many other studies, the antioxidant activity changes depending on the solvent used. When the results of these test systems and the total phenolic and flavonoid content are evaluated, the antioxidant activity of *O. armena* extracts can be shown as follows: Methanol>Water>Ethyl acetate.

The fatty acid composition of *O. armena* was investigated using GC and it was found that the main part of the fatty acid composition was composed of unsaturated fatty acids (66.16%), which are of great importance for health. The major fatty acids

in the oil composition are palmitic acid (22.67%) and linoleic acid (15.09%). The composition of the essential oil obtained by hydrodistilation from *O. armena* was investigated by GC-MS and a total of 22 components were identified, the major component being n-hexadecanoic acid (56.609%).

In recent years, the search for new natural raw material resources has been increasing day by day, especially in the field of food and pharmacology. In this study, antioxidant properties and polyunsaturated fatty acids of *Onobrychis armena* were investigated. According to the determined results, it can be concluded that *Onobrychis armena* can be used as a natural raw material source in the field of food and pharmacology.

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REFERENCES

- Apak, R., Guclu, K., Ozyurek, M., Karademir, S.E. & Ercag, E. (2006). The cupric ion reducing antioxidant capacity and polyphenolic content of some herbal teas. *International Journal Food Sciences Nutrition, 57*, 292–304.
- Alonso, A.M., Guillen, D.A., Barroso, C.G., Puertas, B. & Garcia, A. (2002). Determination of antioxidant activity of wine by products and its correlation with polyphenolic content.*Journal of Agriculture and Food Chemistry, 50*, 5832–5836.
- Arvouet-Grand, A., Vennat, B., Pourrat, A. & Legret, P. (1994). Standardisation d'un extrait de propolis et identification des principaux constituants. *Journal de Pharmacie de Belgique, 49*, 462–468.
- Bagci, E., Bruehl, L., Özçelik, H., Aitzetmuller, K., Vural, M. & Sahin, A. (2004). Study of the fatty acid and tocochromanol patterns of some Turkish Fabaceae (Leguminosae) Plants I. *Grases y Aceites, 55*, 378–384.
- Baghiani, A., Djarmouni Boumerfeg, S., Trabsa, H., Charef, N., Khennouf, S. & Arrar L. (2012). Xanthine oxidase inhibition and antioxidant effects of peaganum harmala seed extracts, *European Journal of Medicinal Plants, 2*(1), 42–56.
- Bakkali, F., Averbeck, S., Averbeck, D. & Waomar, M. (2008). Biological effects of essential oils-A review, *Food and Chemical Toxicology,46*(2), 446–475.
- Bhaksu, L.M. & Raju, R.R.V. (2009). Chemical composition and in vitro antimicrobial activity of essential oil of Rhynchosia heynei, an endemic medicinal plant from Eastern Ghats of India. *Pharmaceutical Biology, 47*(11), 1067–1070.
- Chanda, S., Dudhatra, S. & Kaneria, M. (2010). Antioxidative and antibacterial effects of seeds and fruit rind of nutraceutical plants belonging to the Fabaceae family. *Food & Function, 1*, 308–315.
- Daferera, D.J., Ziogas, B.N. & Polissiou, M.G. (2000). GC–MS analysis of essential oils from Greek aromatic plants and their fungitoxicity on Penicillum digitatum. *Journal of Agricultural and Food Chemistry, 48*, 2576–2581.
- Erbil, N., Duzguner, V., Durmuskahya, C. & Alan, Y. (2015). Antimicrobial and antioxidant effects of some Turkish fodder plants belongs to Fabaceae family (*Vicia villosa, Trifolium ochroleucum* and *Onobrychis altissima*). *Oriental Journal of Chemistry, 31*, 53–58.
- Godevac, D., Zdunic, G., Savikin, K., Vajs, V. & Menkovic, N. (2008). Antioxidant activity of nine Fabaceae species growing in Serbia and Montenegro. *Fitoterapia, 79*, 185–187.
- Grosso, A.C., Costa, M.M., Ganco, L., Pereira, A.L., Teixeira, G., Lavado, J.M.G., Figueiredo, A.C., Barosso, J.B. & Pedro, L.G. (2007). Essential oil composition of *Pterospartum tridentatum* grown in Portugal. *Food Chemistry, 102*, 1083–1088.
- Halliwell, B. & Gutteridge, J. (1984). Oxygen toxicity oxygen radicals, transitionmetals and disease. *Biochemical Journal, 219*, 1–14.
- Hayet, E., Maha, M., Samia, A., Mata, M., Gros, P., Raida, H., Ali, M.M., Mohammed, A.S., Gutmann, L., Mighri, Z. & Mahjoub, A. (2008). Antimicrobial, antioxidant, and antiviral activities of *Retama raetam* (Forssk.) Webb flowers growing in Tunisia. *World Journal of Microbiology and Biotechnology, 24*, 2933–2940.
- Hertog, M. G. L., Feskens, E. J. M., Hollman, P. C. H., Katan, M. B. & Kromhout, D. (1993). Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. *The Lancet, 342*, 1007–1011.
- Huang, D., Ou, B. & Prior, R.L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry, 53*, 1841–1856.
- IUPAC, (1979). Standards methods for analysis of oils, fats and derivatives, (Paquot, C. Ed.), 6th Edn, *Oxford Pergamon Press,* pp. 59–66.
- Karakoca, K., Asan-Ozusaglam, M., Cakmak, Y. S. & Teksen, M. (2015). Phenolic compounds, biological and antioxidant activities of *Onobrychis armena* Boiss. & Huet flower and root extracts. *Chiang Mai University Journal of Natural of Sciences, 42*, 376–392.
- Karamian, R. & Asadbegy, M. (2016). Antioxidant activity, total phenolic and flavonoid contents of three Onobrychis species from Iran. *Journal of Pharmaceutical Sciences ,22*, 112–119.
- Kicel, A., Wolbis, M., Kalemba, D. & Wajs, A. (2010). Identification of volatile constituents in flowers and leaves of Trifolium repens L. *Journal of Essential Oil Research, 22*(6), 624–627.
- Mao, Z., Fu, H. & Wan, C. (2012). Effect of temperature on fatty acid content in Vicia sativa. *Journal of Consumer Protection and Food Safety, 7*, 133–135.
- Mishra, J., Yosouf, A. & Singh, R.D. (2009). Phytochemical investigation and in-vitro antioxidant potential of leaves of Murraya koenigii. *International Journal of Integrative Biology, 7*(3), 171–174.
- Oguwande, I.A., Walker, TM., Setzer, WN. & Essien, E. (2006). Volatile constituents from *Samanae saman* (Jacq.) Merr. Fabaceae. *African Journal of Biotechnology, 5*(20), 1890–1893.
- Oyaizu, M. (1986). Studies on products of browning reactions: antioxidative activities of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition, 44,* 307-315.
- Orhan, I., Kartal, M., Abu-Asaker, M., Şenol, F.S., Yilmaz, G. & Şener, B. (2009). Free radical scavenging properties and phenoli characterization of some edible plants. *Food Chemistry, 114*, 276–281.
- Orhan, I., Tosun, F., Tamer, U., Duran, A., Alan, B. & Kok, A.F. (2011). Quantification of genistein and daidzein in two endemic Genista species and their antioxidant activity. *Journal of the Serbian Chemical Society, 76*(1), 35–42.
- Pastor-Cavado, E., Juan, R., Pastor, J.E., Alaiz, M. & Vioque, Javier (2009). Antioxidant activity of seed polyphenols in fifteen wild Lathyrus species from South Spain. *LWT.42*, 705–709.
- Pietta, P.G. (2000). Flavonoids as antioxidants(review). *Journal of Natural Products,63*, 1035–1042.
- Prieto, P., Pineda, M. & Aguilar, M. (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphor molybdenum complex: Specific application to the determination of vitamin E. *Analytical Biochemistry, 269*, 337–341.
- Rice-Evans, C.A., Miller, N.J. & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine, 20*, 933–956.

- Sarikurkcu, C., Arisoy, K., Tepe, B., Cakir, A., Abali, G. & Mete, E. (2009). Studies on the antioxidant activity of essential oil and different solvent extracts of Vitex agnus castus L. fruits from Turkey. *Food and Chemical Toxicology, 47*, 2479–2483.
- Sharma, N., Bhardwaj, R., Kumar, S. & Kaur, S. (2011). Evaluation of Bauhinia variegata L. bark fractions for in vitro antioxidant potential and protective effect aganist H_2O_2-induced oxidative damage to pBR322 DNA. *African Journal of Pharmacy and Pharmacology, 5*, 1494–1500.
- Singleton, V.L. & Rossi, J.A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungsticacid reagents. *American Journal of Enology and Viticulture, 16*, 144–158.
- Slinkard, K. & Singleton, V.L. (1977). Total phenol analyses: Automation and comparison with manual methods. *American Journal of Enology and Viticulture, 28*, 49–55.
- Sokmen, A., Gulluce, M., Akpulat, H.A., Daferera, D., Tepe, B., Polissiou, M., Sokmen, M. & Sahin, F. (2004). The in vitro antimicrobial and antioxidant activities of the essential oils and methanol extracts of endemic Thymus spathulifolius. *Food Control, 15*, 627–634.
- Thangadurai, D., Viswanathan, B. & Ramesh, N. (2001). Nutritional potential of biochemical components in Galactia longifolia Benth. (Fabaceae). *Food/Nahrung, 45*, 97–100.
- Usta, C., Yildirim, A. B. & Turker, A.U. (2014). Antibacterial and antitumour activities of some plants grown in Turkey. *Biotechnology & Biotechnological Equipment, 28*, 306–315.
- Uzun, B., Arslan, C., Karhan, M. & Toker, C. (2007). Fat and Fatty acids of White lupin (Lupinus albus L.) in comparison to sesame (Sesamum indicum L.). *Food Chemistry, 102*, 45–49.
- Valentao, P., Fernandes, E., Carvalho, F., Andrade, P. B., Scabra, R. M. & Bastos, M. L. 2002. Antioxidative properties of cardoon (Cynara cardunculus L.) infusion against superoxide radical, hydroxyl radical and hypochlorus acid. *Journal of Agricultural and Food Chemistry, 50*, 4989–4993.
- Wiswanathan, M.B., Thangadurai, D., Vendan, K.T. & Ramesh, N. (1999). Chemical analysis and nutritional assessment of Teramnus labialis (L.) Spreng. (Fabaceae). *Plant Foods for Human Nutrition, 54*, 345–352.
- Zahin, M., Aqil, F. & Ahmad, I. (2010). Broad spectrum antimutagenic activity of antioxidant active fraction of Punica granatum L. peel extracts. *Mutation Research, 703*, 99–107.
- Zengin, G., Guler, G.O., Aktumsek, A., Ceylan, R., Picot, C.M.N. & Mahomoodally, M.F. (2015). Enzyme Inhibitory Properties, Antioxidant Activities, and Phytochemical Profile of Three Medicinal Plants from Turkey. *Advances in Pharmacological Sciences,* 1–8.

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Original Article

Prevention of antibiotic resistance created by experimental evolutionary microbiology in *Staphylococcus aureus* and *Escherichia coli* with herbal substances

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ABSTRACT

Background and Aims: Recently, one of the biggest problems of the world is a bacterial antimicrobial resistance, that is developing against most of the existing antibiotics. In addition to conducting studies that continue to discover new antimicrobial agents for combating multidrug resistant bacteria, steps should be taken for the protection of existing antibiotics. With this in mind, many modern and classical strategies have been developed, and among them, using essential oils or extracts obtained from plants, which may be a practical and effective alternative.
Methods: We used the experimental evolutionary microbiology method to determine the effects of herbal substances, such as cinnamaldehyde from cinnamon, epigallocatechin gallate from greentea, curcumin from turmeric, punicalagin from pomegranate, and clove oil from clove, on the prevention or delay of antimicrobial resistance. In this study, *Staphylococcus aureus* and *Escherichia coli* standard and clinical strains were gradually exposed to increasing sub-inhibitory concentrations of meropenem and ciprofloxacin with or without the presence of herbal substances.
Results: Resistance was developed in the *E. coli* and *S. aureus* control groups which were exposed only to ciprofloxacin, but, when herbal substances were included to the test, there was no resistance development. When the control groups were exposed only to meropenem, there was only an increase in the minimum inhibitory concentrations (MIC), but they did not become resistant, and we observed similar MIC values when we added the herbal substances to the test.
Conclusion: These results showed that herbal substances might contribute to lowering MIC values of antibiotics and may help prevent the development of resistance in the studied bacteria.

Keywords: Antibiotic resistance, Escherichia coli, Evolutionary microbiology, Herbal substance, Staphylococcus aureus

INTRODUCTION

Bacterial resistance to antibiotics is steadily becoming a bigger problem, and it has reached a critical level both in our country (Türkiye) and around the world. There are major problems, such as increasing morbidity and mortality rates in the treatment of infections, especially the nosocomial infections caused by the multi drug resistant strains (Koksal, Ak, Kucukbasmaci & Samasti, 2009; Kunz & Brook, 2010). According to the World Health Organization (WHO), the most frequently reported multi drug resistant bacteria include *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterococcus* and *Salmonella* sp. (WHO, 2021). There are several resistance mechanisms which happen

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in the development of resistance, such as limiting the uptake of drugs, modification of drug targets, inactivation of drugs with enzymes, and active efflux pumps. Some situations, such as the irrational or misuse of antibiotics or using bacteriostatic antibiotics for immunosuppressive patients, facilitate the selection or development of antibiotic resistance in bacteria (Aslam et al., 2018).

There are many ongoing studies to combat antibiotic resistance. While some studies prioritize the discovery of new and effective antibiotics, some of them focus on the re-use of existing antibiotics. The experimental evolution studies are one prominent concept for re-use strategies (Allen, Popat, Diggle & Brown, 2014). Experimental evolution includes evolutionary dynamics through controlled field studies and/or laboratory experiments. In this technique, small organisms, such as bacteria that exhibit rapid growth, are used to examine changes that would normally take a long time in large living organisms. Evolution can be observed under laboratory conditions, with natural selection of individuals and/or populations under new environmental conditions. In experimental evolutionary studies, the adaptation can be observed in two ways: when organisms undergo a new and beneficial mutation, or when a trait predominates in a population that is observed in a very small number of individuals (Long, Liti, Luptak & Tenaillon, 2015).

Although there are studies that continue to examine new methods that might be used for treating resistant microorganisms, recently, the prevention of the emergence of antibiotic resistance has seemed like the most effective and safest way forward. Therefore, there are many ongoing studies about the use of various natural herbal substances that have been used in the community for many years, either alone or in combination with antibiotics (Yap, Yiap, Ping & Lim, 2014). In this study, we aimed to observe the stages of resistance development against meropenem and ciprofloxacin in *S. aureus* and *E. coli* using the experimental evolutionary microbiology method. In order to slow down or stop this resistance, we tested the effects of some herbal antimicrobial substances, including cinnamaldehyde, punicalagin, epigallocatechin gallate, curcumin, and clove oil combined with the antibiotics against *S. aureus* and *E. coli* standard and clinical strains.

MATERIAL AND METHODS

Microorganisms

Clinical strains of *E. coli* (E1) and *S. aureus* (S1), which were isolated from different patients in Clinical Microbiology Laboratories at Group Florence Nightingale Hospitals in Türkiye, were used in the study. These isolates were identified with the VITEK 2 system and confirmed by routine biochemical tests. We also used *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 (Rockville, MD, USA) standard strains in the study.

Antimicrobial Substances

Two antibiotics, meropenem (activity 986 mg/L) and ciprofloxacin (activity 90.6%), were provided by Astra Zeneca Turkiye and five herbal substances, cinnamaldehyde (activity ≥95%), punicalagin (activity ≥98%), clove oil, curcumin (activity ≥65%), and epigallocatechin gallate (activity ≥95%), were provided by Sigma - Aldrich. The stock solutions of ciprofloxacin and herbal substances, except curcumin, were prepared at a concentration of 5120 mg/L and stored at -80°C for up to 6 months. Meropenem and curcumin were prepared daily in each experiment. Cinnamaldehyde and punicalagin were dissolved in DMSO, epigallocatechin gallate in distilled water, clove oil in Tween 80, and curcumin in ethanol.

Media

Cation-adjusted Mueller-Hinton broth (CAMHB, Difco Laboratories) was used for determining the minimum inhibitory concentration (MIC) and Checkerboard experiments, and Tryptic Soy Agar (TSA, Difco Laboratories) was used for bacterial cultures and colony counts.

Experimental Evolution Method

In accordance with the evolutionary microbiology dynamics, each day, bacteria were exposed to increasing doses of antibiotics, starting from low/sub-MIC concentrations. In this process, the development of resistance in bacteria which survived after exposure to antibiotics, was examined by determining the rising MIC values. For each experiment, two-fold meropenem and ciprofloxacin solutions were added to the TSA medium, starting from 1/20 x MIC up to the concentration which was not allow any bacterial regrowth. We also prepared the sub-MIC concentrations of herbal substances (1/4 x MIC) and added them to the antibiotic including TSA plates. After the each passage of standard and clinical isolates, bacterial colonies that continued to grow in the presence of antimicrobials were collected and stored in -80°C for MIC determinations (McDonald, 2019). Experiments were performed twice.

Determination of Minimum inhibitory concentration (MIC)

MICs of antibiotics and herbal substances were determined by the microbroth dilution technique as described by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2003; EUCAST, 2021). Serial two-fold dilutions of antibiotics and herbal substances were prepared in CAMHB in 96-well, U bottom microtiter plates. Each well was inoculated with overnight bacterial cultures that gave a final concentration of 5×10^5 cfu/mL. The plates were covered and placed in plastic bags to prevent evaporation and incubated at 37°C for 18-20 h. The MIC was defined as the lowest concentration of antimicrobials, producing complete inhibition of visible growth. Experiments were performed twice.

Determination of Fractional Inhibition Concentration (FIC) index

We conducted this experiment to determine whether the antibiotic and herbal substance combinations had any interaction besides the evolutionary microbiology results. The effects of combinations were tested using the microbroth checkerboard technique (Pillai, Moellering & Eliopoulos, 2005). For this purpose, different concentrations of meropenem or ciprofloxacin between 1/8 x MIC - 2 x MIC in the vertical plane and herbal substances in the horizontal plane were mixed and tested against *E. coli* and *S. aureus* strains. Each microplate well containing the mixture of antimicrobials was inoculated with overnight bacterial cultures to give a final concentration of approximately 5×10^5 cfu/mL. After incubation at 37°C for 18-20 h, the FIC values of each antimicrobial

were calculated as combined inhibitory concentration divided by the single concentration, and the FIC index was defined as sum of the FIC values. The combination value was derived from the highest dilution of antibiotic combination permitting no visible growth. Synergy was defined as a FIC index as ≤ 0.5, additive as > 0.5-4, and antagonism as > 4.0 (Odds, 2003). Experiments were performed twice.

RESULTS

Susceptibility

The MIC values of antibiotics and herbal substances against *E. coli* and *S. aureus* strains are shown in Table 1.

Experimental Evolution Results

The changes in the MIC values of meropenem and ciprofloxacin with or without herbal substances against *E. coli* and *S. aureus* are shown in Figures 1 and 2, respectively.

According to these experimental evolution results, when bacteria were exposed to increasing doses of antibiotics, we observed the resistance development for ciprofloxacin, and the higher MIC values for meropenem. After the inclusion of herbal substances to the study, although there was a slight increase in MIC values, the resistance development was not observed. (Table 2, and 3).

Combination studies

When we tested the combined effects of antibiotics and herbal substances, the synergistic interaction was observed against *S. aureus* with meropenem-clove oil, ciprofloxacin-clove oil, and ciprofloxacin-cinnamaldehyde combinations by the checkerboard method (Table 2).

Table 1. MIC values of meropenem, ciprofloxacin, and herbal substances against *S. aureus* and *E. coli* strains (mg/L).

Bacteria	Mer	Cip	Clo	Cur	Cin	Epi	Pun
***S. aureus* (Clinical strain)**	0.032-0.25	0.125-0.5	3.47	40.6	0.125	60.8	0.12
***S. aureus* (ATCC 25923)**	2	0.25	3.47	20.3	0.25	60.8	0.12
***E. coli* (Clinical strain)**	0.003-0.032	0.003-0.5	3.47	325	0.5	121.6	0.12
***E. coli* (ATCC 25922)**	0.016-0.03	0.008	3.47	325	0.25	121.6	0.12

Mer: Meropenem, Cip: Ciprofloxacin, Clo: Clove Oil, Cur: Curcumin, Cin: Cinnamaldehyde, Epi: Epigallocatechingallate, Pun: Punicalagin

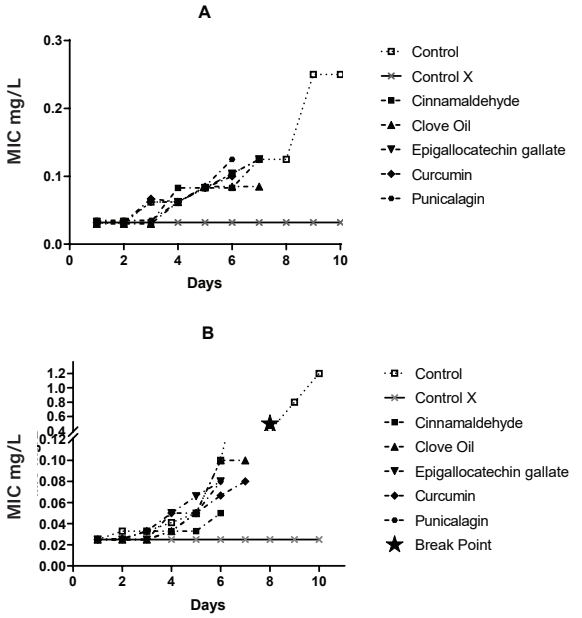

Figure 1. The changing MIC values of A: meropenem and B: ciprofloxacin with herbal substances against E. coli clinical strain. The X axis represents days that bacteria continue to grow; Y axis represents the MIC values after bacteria exposure to the antimicrobials. Break Point: EUCAST resistant breakpoint for E. coli (Meropenem: >8, Ciprofloxacin: >0.5). Control: Bacteria passage with only meropenem or ciprofloxacin. Control X: The bacteria passage without any antimicrobial substance.

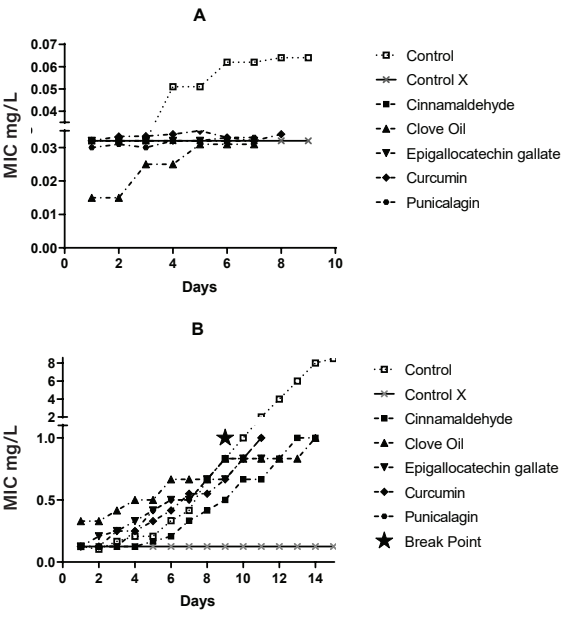

Figure 2. The changing MIC values of A: meropenem and B: ciprofloxacin with herbal substances against S. aureus clinical strain. The X axis represents days that bacteria continue to grow; Y axis represents the MIC values after bacteria exposure to the antimicrobials. Break Point: EUCAST resistant breakpoint for E. coli (Meropenem: >4, Ciprofloxacin: >1). Control: Bacteria passage with only meropenem or ciprofloxacin. Control X: The bacteria passage without any antimicrobial substance.

Table 2. FIC indexes of meropenem and ciprofloxacin combinations with herbal substances against *S. aureus* and *E. coli*.

	Staphylococcus aureus		*Escherichia coli*	
Herbal substances	**Meropenem**	**Ciprofloxacin**	**Meropenem**	**Ciprofloxacin**
Cinnamaldehyde	0.625	**0.5**	0.625	0.75
Curcumin	0.625	0.625	0.625	0.625
Epigallocatechin gallate	0.625	0.625	0.625	0.625
Clove Oil	**0.375**	**0.375**	0.75	0.75
Punicalagin	0.625	0.625	0.625	0.625

According to the FIC index, ≤0.5 as synergistic, 0.5 - 4.0 as additive, and ≥ 4.0 as antagonist

Table 3. The influence of herbal substances on antibiotic activities against *E. coli* and *S. aureus* in evolutionary microbiology assays.

	Escherichia coli		*Escherichia coli*		*Staphylococcus aureus*		*Staphylococcus aureus*	
	Resistance development		**Increase in MIC values**		**Resistance development**		**Increase in MIC values**	
Herbal substances	**Mer**	**Cip**	**Mer**	**Cip**	**Mer**	**Cip**	**Mer**	**Cip**
None	-	**+**	+	**+**	-	**+**	+	+
Cin	-	**-**	-	**-**	-	**-**	-	+
Cur	-	-	-	-	-	-	-	+
Ep	-	-	-	-	-	-	-	+
Clo	**-**	**-**	**-**	**-**	**-**	**-**	-	+
Pun	-	-	-	-	-	-	-	+

Mer: Meropenem, Cip: Ciprofloxacin, Clo: Clove Oil, Cur: Curcumin, Cin: Cinnamaldehyde, Epi: Epigallocatechin gallate, Pun: Punicalagin.

DISCUSSION

In recent years, in parallel with the increasing number of antibiotic resistant microorganisms, the importance of infectious diseases caused by resistant pathogens has increased at an alarming rates. Due to the inadequacy of existing antibiotics for infection control, finding alternative treatment strategies has become very crucial. One novel therapeutic strategy involves using the herbal antimicrobial substances either alone or in combination with antibiotics. Since ancient times, plants have been the main resource that people administer for pain relief and treatment of many kind of diseases. A great deal of evidence suggests that the use of herbs for medicinal purposes dates back thousands of years, and today, the active ingredients of more than 50% of current drugs originate from plants. Considering the various studies about the treatment effects of herbs, used in traditional medicine, it could be said that traditional medicine guides modern medicine (Pan et al., 2013).

In order to slow down or stop the development of resistance and restore the effects of some antibiotics, of which clinical use has become very limited, we investigated the effects of some herbal substances. For this purpose, we studied the resistance development in clinical *S. aureus* and *E. coli* strains against meropenem or ciprofloxacin, and determined the effects of cinnamaldehyde, curcumin, epigallocatechin gallate, clove oil, and punicalagin in this process.

The selected herbal substances have been used by people for thousands of years and their antimicrobial activities have been demonstrated in various studies. Among them, cinnamaldehyde shows its antimicrobial activities by damaging the cell membrane of bacteria, inhibiting ATPases, or damaging cell division, motility, and biofilm formation (Vasconcelos, Croda & Simionatto, 2018). Punicalagin, obtained from pomegranate peel, causes morphological damages on the cell membranes and prevents biofilm formation (Xu et al., 2017). Green tea catechins inhibit the virulence factors and intracellular enzymes of bacteria, damage the cell membrane and cell wall, and cause oxidative stress, DNA damage, and iron chelation (Renzetti, Betts, Fukumoto & Rutherford, 2020). Curcumin, obtained from turmeric, has an antibacterial effect by inhibiting the virulence factors and biofilm formation mechanisms of bacteria, and also by preventing the adhesion to host tissues (Zheng et al., 2020). Clove oil, obtained from the clove plant, affects the movement of bacteria and their adhesion forces. According to in vitro studies such as cytotoxicity on fibroblasts and other cells, these substances are considered as safe (Hu, Zhou & Wei, 2018). Additionally, the presence of these substances in food such as cinnamon, pomegranate, green tea, turmeric, and cloves, which are frequently consumed in daily life, provides the correlation with those studies.

As shown in Figure 1 and Table 3, when *E. coli* was exposed to increasingly larger ciprofloxacin doses, we observed the rising MIC values, resistance development, and surviving bacteria even reaching 0.8 mg/L concentration. On the other hand, when *E. coli* was exposed to increasingly larger concentrations of meropenem, although we observed an increase in MIC values, the resistance breakpoints were not exceeded, and all bacteria died at a concentration of 0.25 mg/L at the end of ten days. There were no changes in the MICs of the *E. coli* control group, which were passage without being exposed any antimicrobial agent, and the MIC values were 0.025 and 0.032 mg/L for ciprofloxacin and meropenem, respectively. Similarly, Fröhlich and friends showed that the sub-MICs of ceftazidime could affect the development of resistance in *E. coli* by evolutionary microbiology, and these results indicates that the exposure to β-lactams at very low concentrations drives the evolution of β-lactamases. On the other hand, Mantage and friends indicated that, when *E. coli* was exposed to low concentrations of rifampicin, there was no development of resistance, and instead their population adapted to the antimicrobial environment via evolving small colony variants. These results indicated that some lifestyle changes have the potential to affect the ultimate fate of populations through mutations and altering genetic make up (Matange, Hegde & Bodkhe, 2019; Fröhlich et al., 2021).

When we included the herbal substances in the experiment, although there was a slight increase in MIC values, we did not observe any resistance for ciprofloxacin or meropenem in *E. coli*. Also, the MIC values were always below the MICs without herbal substances. However, these results might be explained by the meropenem resistance gene that is effective in *E. coli* (Hong et al., 2005; Hitzenbichler et al., 2018); the changes in the MIC values might also results from the deterioration in colony morphology due to the long-term passages. These results suggested that during the antibiotic treatment, consuming the studied herbal substances, could be effective to prevent or delay the development of antibiotic resistance.

As shown in Figure 2 and Table 3, when *S. aureus* was exposed to increasingly larger ciprofloxacin doses, similar to *E. coli*, we observed the rising MIC values, resistance development, and surviving bacteria even reaching 8 mg/L concentration. When *S. aureus* was exposed to increasing concentrations of meropenem, we didn't observe any increase in MIC values and the resistance breakpoints were not exceeded, and also all bacteria died at a concentration of 0.064 mg/L, at the end of nine days. There were no changes in the MICs of the *S. aureus* control group which were passage without any antimicrobial agent, and the MIC values were 0.125 and 0.032 mg/L for ciprofloxacin and meropenem, respectively. Similarly, Johnson and Levin showed that, sub-MIC concentrations of ciprofloxacin and gentamicin resulted in increased surviving bacterial fraction (level of persistence), and they postulated that persistence is an inevitable consequence of a metabolic disruption, similar to mutation in cell replication (Johnson & Levin, 2013).

When we included the herbal substances in the experiment, although there was a slight increase in MIC values, we did not observe any resistance for ciprofloxacin in *S. aureus*. For meropenem, no resistance development was observed, and also the MIC values were not increased, after we added the herbal substances, except cinnamaldehyde. These results indicated that there are different mechanisms required for the development of meropenem resistance in *S. aureus* (Lemaire, Van Bambeke, Mingeot-Leclercq, Glupczynski & Tulkens, 2007; Hassanzadeh et al., 2017). These results also suggested that consuming herbs during the antibiotic course could be effective for preventing the development of antibiotic resistance.

In this study, considering the possible other interactions between studied antibiotics and herbal substances, we perform the microdilution checkerboard assay, which is the most popular and prevalent in-vitro combination test. According to our results, synergy was observed with meropenem-clove oil, ciprofloxacin-clove oil, and ciprofloxacin-cinnamaldehyde combinations against *S. aureus*. When these results are considered together, in evolutionary microbiology studies performed with clove oil, the MIC values of meropenem and ciprofloxacin against *S. aureus* were decreased, and found lower than the antibiotic free control group, as expected.

Considering the differences in *E. coli* and *S. aureus* results, the morphological differences between Gram positive and Gram negative bacteria seems to be an important factor, in addition to the possibilities related to the action mechanisms, mentioned above. Meropenem, is a beta lactam antibiotic that acts on a peptidoglycan layer of the cell wall, while ciprofloxacin belongs to the fluoroquinolone group, which targets the DNA gyrase enzymes of bacteria. The development of resistance against ciprofloxacin is generally acquired spontaneous chromosomal mutations in genes, while beta lactams generally disrupted by beta lactamases enzymes. As a result of mutations, changes in DNA gyrase enzymes or decreases in membrane permeability may occur in bacteria (Hooper, 2001). We thought that the serial passage procedures in the evolutionary microbiology may hasten, and support the mutations that allows the fluoroquinolone resistance in bacteria. On the other hand, the studied herbal substances generally act against the bacterial cell wall or cytoplasmic membrane, and we thought that the prevention of ciprofloxacin resistance by herbal substances might be related to those combinations of different targets.

CONCLUSION

In this study, it was shown that, very low levels of antimicrobials can direct the development of antimicrobial resistance. We also showed that the combination of sub-MIC herbal substances and antibiotics prevents the development of resistance or reduces MICs against *E. coli* and *S. aureus*. According to these results, we thought that the use of herbs such as cinnamon, cloves, turmeric, green tea, and pomegranate, together with antibiotics, might be beneficial in avoiding or delaying resistance, and this should be investigated against different types of bacteria.

Ethics: This study was approved by Istanbul University, Faculty of Medicine, Clinical research ethics committee (07.03.2017-91678).

Peer-review: Externally peer-reviewed.

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

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

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REFERENCES

- Allen, R. C., Popat, R., Diggle, S. P., & Brown, S. P. (2014). Targeting virulence: Can we make evolution-proof drugs? *Nature reviews Microbiology*, *12*(4), 300–308. https://doi.org/10.1038/nrmicro3232
- Aslam, B., Wang, W., Arshad, M. I., Khurshid, M., Muzammil, S., Rasool, M. H., Nisar, M. A., Alvi, R. F., Aslam, M. A., Qamar, M. U., Salamat, M., & Baloch, Z. (2018). Antibiotic resistance: A rundown of a global crisis. *Infection and Drug Resistance*, *11*, 1645–1658. https://doi:10.2147/IDR.S173867
- European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society for Clinical Microbiology and Infectious Diseases (ESCMID). (2003). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clinical Microbiology and Infection, 9*(8), 1-7. https://doi.org/10.1046/j.1469-0691.2003.00790.x
- European Committee on Antimicrobial Susceptibility Testing. (2021). *Breakpoint tables for interpretation of MICs and zone diameters. Version 11.0, 2021*. Retrieved from: http://www.eucast.org
- Fröhlich, C., Gama, J. A., Harms, K., Hirvonen, V., Lund, B. A., van der Kamp, M. W., Johnsen, P. J., Samuelsen, Ø., &Leiros, H. S. (2021). Cryptic β-Lactamase Evolution Is Driven by Low β-Lactam Concentrations. *mSphere*, *6*(2), e00108-21. https://doi.org/10.1128/mSphere.00108-21
- Hassanzadeh, S., Mashhadi, R., Yousefi, M., Askari, E., Saniei, M., &Pourmand, M. R. (2017). Frequency of efflux pump genes mediating ciprofloxacin and antiseptic resistance in methicillin-resistant *Staphylococcus aureus* isolates. *Microbial Pathogenesis*, *111*, 71–74. https://doi.org/10.1016/j.micpath.2017.08.026
- Hitzenbichler, F., Simon, M., Holzmann, T., Iberer, M., Zimmermann, M., Salzberger, B., & Hanses, F. (2018). Antibiotic resistance in *E. coli* isolates from patients with urinary tract infections presenting to the emergency department. *Infection*, *46*(3), 325–331. https://doi.org/10.1007/s15010-018-1117-5
- Hong, T., Moland, E. S., Abdalhamid, B., Hanson, N. D., Wang, J., Sloan, C., Fabian, D., Farajallah, A., Levine, J., & Thomson, K. S. (2005). *Escherichia coli*: development of carbapenem resistance during therapy. *Clinical Infectious Diseases*, *40*(10), e84–e86. https://doi.org/10.1086/429822
- Hooper D. C. (2001). Emerging mechanisms of fluoroquinolone resistance. *Emerging Infectious Diseases*, 7(2), 337–341. https://doi.org/10.3201/eid0702.010239
- Hu, Q., Zhou, M., & Wei, S. (2018). Progress on the Antimicrobial Activity Research of Clove Oil and Eugenol in the Food Antisepsis Field. *Journal of food science*, *83*(6), 1476–1483. https://doi.org/10.1111/1750-3841.14180
- Johnson, P. J., & Levin, B. R. (2013). Pharmaco-dynamics, population dynamics, and the evolution of persistence in *Staphylococcus aureus*. *PLOS Genetics*, *9*(1), e1003123. https://doi.org/10.1371/journal.pgen.1003123
- Koksal, F., Ak, K., Kucukbasmaci, O., &Samasti, M. (2009). Prevalence and antimicrobial resistance patterns of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolated from blood cultures in an Istanbul University Hospital. *Chemotherapy*, 55(4), 293–297. https://doi.org/10.1159/000224657
- Kunz, A. N., & Brook, I. (2010). Emerging resistant Gram-negative aerobic bacilli in hospital-acquired infections. *Chemotherapy*, *56*(6), 492–500. https://doi.org/10.1159/000321018
- Lemaire, S., Van Bambeke, F., Mingeot-Leclercq, M. P., Glupczynski, Y., &Tulkens, P. M. (2007). Role of acidic pH in the susceptibility of intraphagocytic methicillin-resistant *Staphylococcus aureus* strains to meropenem and cloxacillin. *Antimicrobial Agents and Chemotherapy*, *51*(5), 1627–1632. https://doi.org/10.1128/AAC.01192-06
- Long, A., Liti, G., Luptak, A., & Tenaillon, O. (2015). Elucidating the molecular architecture of adaptation via evolve and resequence experiments. *Nature Reviews Genetics*, *16*(10), 567–582. https://doi.org/10.1038/nrg3937
- Matange N, Hegde S &Bodkhe S. (2019). Adaptation through lifestyle switching sculpts the fitness landscape of evolving populations: implications for the selection of drug-resistant bacteria at low drug pressures. *Genetics*, *211*, 1029–1044. https://doi.org/10.1534/genetics.119.301834
- McDonald M. J. (2019). Microbial Experimental Evolution - a proving ground for evolutionary theory and a tool for discovery. *EMBO Reports*, *20*(8), e46992. https://doi.org/10.15252/embr.201846992
- Odds F. C. (2003). Synergy, antagonism, and what the chequerboard puts between them. *The Journal of Antimicrobial Chemotherapy*, *52*(1), 1. https://doi.org/10.1093/jac/dkg301
- Vasconcelos, N. G., Croda, J., &Simionatto, S. (2018). Antibacterial mechanisms of cinnamon and its constituents: A review. *Microbial pathogenesis*, *120*, 198–203. https://doi.org/10.1016/j.micpath.2018.04.036
- Pan S. Y., Zhou S. F., Gao S. H., Yu Z. L., Zhang S. F., Tang M. K., Sun J. N., Ma D. L., Han Y. F., Fong W. F.,& Ko K. M. (2013). New Perspectives on how to discover drugs from herbal medicines: CAM's outstanding contribution to modern therapeutics. *Evidence-Based Complementary and Alternative Medicine*, *2013*(1),1–25. https://doi.org/10.1155/2013/627375
- Pillai, S.K., Moellering, R.C.,& Eliopoulos, G.M. (2005) Antimicrobial combinations. In V. Lorian (Ed.), *Antibiotics in Laboratory Medicine*(5th ed.) (pp. 365–440). Philadelphia, PA: The Lippincott Williams & Wilkins Co.
- Renzetti, A., Betts, J. W., Fukumoto, K., & Rutherford, R. N., (2020). Antibacterial green tea catechins from a molecular perspective: mechanisms of action and structure-activity relationships. *Food & function*, *11*(11), 9370–9396. https://doi.org/10.1039/d0fo02054k
- World Health Organization (2021). *Antimicrobial resistance*. Retrieved from: https://www.who.int/en/news-room/fact-sheets/detail/antimicrobial-resistance
- Xu, Y., Shi, C., Wu, Q., Zheng, Z., Liu, P., Li, G., Peng, X.,& Xia, X. (2017). Antimicrobial Activity of Punicalagin Against Staphylococcus aureus and Its Effect on Biofilm Formation. *Foodborne pathogens and disease*, *14*(5), 282–287. https://doi.org/10.1089/fpd.2016.2226
- Yap, P. S., Yiap, B. C., Ping, H. C., & Lim, S. H. (2014). Essential oils, a new horizon in combating bacterial antibiotic resistance. *The Open Microbiology Journal*, *8*, 6–14. https://doi.org/10.2174/1874285801408010006
- Zheng, D., Huang, C., Huang, H., Zhao, Y., Khan, M., Zhao, H., & Huang, L. (2020). Antibacterial Mechanism of Curcumin: A Review. *Chemistry & Biodiversity*, *17*(8), e2000171. https://doi.org/10.1002/cbdv.202000171

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Original Article

Mineral and heavy metal concentration of nutritionally and therapeutically valued wild plants: Insights into health effects

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ABSTRACT

Background and Aims: The purpose of the study was to determine the concentrations of minerals and heavy metals in nutritive and therapeutically valued wild plants *Allium orientale* Boiss., *Eremurus spectabilis* M. Bieb., *Anchusa officinalis* L. and *Arum elongatum* Steven.

Methods: The presence and quantity of 23 minerals and heavy metals were analyzed by inductively coupled plasma mass spectrometry (ICP-MS).

Results: The most common minerals were P, Mg, K, Ca, Fe and Al. Moderately abundant elements were Na, Sr, Zn, Cu, Mn, B and Ni. Toxic heavy metals such as Sn, Li, Co, Se, Sb, Hg, Cd, As and Pb were present at very low concentrations or were not detected. *A. officinalis* was observed to be rich in K (7496.435 mg/kg) and Ca (2947.378 mg/kg). On the other hand, Fe concentrations were high in *A. orientale* (1022.068 mg/kg) and *A. elongatum* (699.932 mg/kg). The Mg concentration in *A. orientale* (731.012 mg/kg) was almost double that in the other three plants. Al was found in high concentrations in *A. orientale* (889.368 mg/kg) and *A. elongatum* (651.570 mg/kg). Cr concentration of *A. orientale, A. officinalis* and *A. elongatum* exceeded both EPA limits and standard concentrations in plants.

Conclusion: The study reveals the elemental profile, heavy metal content and possible effects on human health of four wild plants that are frequently used in alternative medicine and nutrition. Most of the elements are not at detrimental levels. Additionally, the results can be useful for the food and pharmaceutical industries and to guide nutritional and comparative studies.

Keywords: Edible wild plant, Mineral, Heavy Metal, Nutrition, Medicinal plant

INTRODUCTION

Minerals are the basic nutritional elements involved in the balancing of body fluids, the functioning of the nervous system, homeostasis, the functioning of enzymes and hormones, bones, teeth, muscles, blood formation and many other functions. Even at threshold levels, most minerals contribute significantly to normal growth and play important roles in biochemical functions and enzyme systems (Bhat, Kiran, Arun, & Karim, 2010). Element uptake of plants from the soil is selective, but as the level of essential elements in the soil increases, the uptake of heavy metals by plant tissues increases, and these heavy metals are indirectly involved in the food chain. C, H, O, N, P, K, S, Ca, Mg, Fe, Zn, Mn, Cu, B, Cl and Mo are essential elements for plants, and the elements Co, Al, Na, Si, Ni and V are considered necessary for some specific plants and some specific processes (Okcu, Tozlu, Kumlay, & Pehluvan, 2009).

Although more than sixty elements are considered heavy metals, the most well-known ones are Hg, Mn, Fe, Co, Ni, Cu, Zn, Cd, As, Cr, Pb, Ag, Sn and Se. However, Fe, Mn, Cu, Ni and Zn heavy metals are also known as essential heavy metals for the body.

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For this reason, their effects on the body are closely related to their concentrations in the edible parts of the plants. While decreasing the amounts of these essential elements can cause significant symptoms in the body, it can also cause toxicity in people who accumulate them at excessive levels (Okcu et al., 2009). Ba, Ag, Cu, Cr, Ni, Hg, Pb, Cd, Se and As are known to be the most toxic heavy metals for the human body, The main reasons for their toxic effects on the human body are mainly related to disorders of the intracellular metabolic processes, such as DNA, RNA, ATP damage, organelle and cell membrane damage, biomolecular degradation, enzyme inactivation, mitochondrial damage, apoptosis, organ failure, autoimmune diseases, and neurological disorders (Jaishankar, Tseten, Anbalagan, Mathew, & Beeregowda, 2014).

Even though research on wild edible plants primarily focuses on the medicinal and ethnobotanical properties of plants, studies on gastronomy tourism, nutritional values, and culinary uses have gained momentum recently. Knowing the benefits and harms of wild plants by obtaining information about their nutritional and therapeutic properties provides benefits for human health. Recently, a lot of research has been done to determine the mineral and heavy metal content of nutritional and medicinal plants (Aberoumand & Deokule, 2009; Bedassa, Abebaw, & Desalegn, 2017; Bhat et al., 2010; Ceylan & Alıc, 2015; Maharia, Dutta, Acharya, & Reddy, 2010; Muhammad, Shah, & Khan, 2011). As known for centuries, wild plants have been used as traditional medicines for the treatments such as hemorrhoids, diabetes, peptic ulcer, skin diseases, burns, kidney diseases, ringworm, digestive system diseases, colds, flu, cough, depression, fear, stress, nervous disorder and bronchitis. They are also thought to have important effects such as kidney stone reducer, milk increaser, menstrual remover, cholesterol lowering, snake and scorpion antidote and pain reliever.

The distributions of the four edible wild plants that make up the study material are as follows. *Allium orientale* (Eastern onion), is native to the East Mediterranean, Egypt, Levant, Libya, Cyprus, Sinai and Turkiye and is a species in the Alliaceae family. *Eremurus spectabilis* (Foxtail Lilly) member of the Liliaceae family is native to Turkiye, Iran, Lebanon, Iraq, Ukraine, Syria, North Caucasus, Palestine, southern Russia, Transcaucasia and Turkmenistan. *Anchusa officinalis* (Alkanet) is a species belonging to the family Boraginaceae whose native range extends from Europe to the Caucasus. *Arum elongatum* (Cuckoo pint) is a species in the Araceae family and distributed in Turkiye, Bulgaria, Ukraine, Greece, Palestine, Crimea, southern Russia, the North Caucasus and Transcaucasia (Karahan et al., 2020; Kardaş, 2019). The main objective of the present study is to determine the concentration of minerals and heavy metals in the therapeutic and nutritional wild plants *A. orientale, E. spectabilis, A. officinalis* and *A. elongatum*. On the other hand, the study describes the elementary properties of the species, their conscious consumption as well as their possible positive or negative effects on health.

MATERIAL AND METHODS

Sample collection and preparation

Plant materials were harvested in fresh, edible form during the first two weeks of growth. The species are available in edible form for about 3 weeks in the wild. *A. orientale* was harvested on April 30, 2020, in the village of Balveren, Şırnak (altitude 1342 m, coordinates: 37° 29′ 30″ N and 42° 32′ 57″ E). *E. spectabilis* was harvested on May 10, 2020, to the west of Mount Namaz (altitude 1760 m, coordinates: 37° 32′ 19″ N and 42° 29′ 30″ E). *A. officinalis* was harvested on March 29, 2020, and *A. elongatum* on March 24, 2020, from Şırnak-Cizre road (Kumçatı) (altitude 522 m, coordinates: 37° 27′ 57″ N and 42° 17′ 17″ E). The plants were taken to the Laboratory of Biology and Chemistry of Şırnak University and photographed with a high-resolution camera (Nikon Coolpix P900 camera and 83×Zoom-NIKKOR ED Glass Lens). The plants were marked and identified by the plant physiology and taxonomy experts of the biology department of Dicle University and according to the Flora of Turkiye and Plant Databases. Preserved specimens were labelled according to herbarium techniques. After the identification, some of the species were dried and some were cleaned and stored in a deep freezer at -20 °C in poly-ethylene bags. Edible tissues (leaves) of the species were studied totally. Detailed information on the species, such as family and species names, Turkish and local names, traditional treatment and nutritional uses are given in Table 1.

Experimental devices, materials and chemicals

The analysis was done by the method of determination of trace elements (As, Cd, Hg, Pb) and other elements by inductively coupled plasma-mass spectrometry (ICP-MS: AGILENT/7800), after pressure digestion including the microwave heating technique. ICP-MS is an analytical technique device that can be used to measure elements at trace levels in organic foods. The device is used to determine 23 elements such as P, Na, Mg, K, Ca, Zn, Sn, Mn, Li, Fe, Cu, Cr, Co, B, Al, Sr, Se, Sb, Ni, Hg, As, Cd and Pb. Microwave, Teflon tube, plastic flask, 0.45 µm membrane filter, a vacuum manifold, syringe, plastic spatula, ceramic knife, automatic pipettes and scalpel were used as materials. Ultrapure water, nitric acid (65%, Suprapur), standard solutions of (1000 mg/L) of the elements, tune solution, argon (99.99% purity) and Helium (99.99% purity) were used as chemicals.

Processing of samples, standard solutions and concentration analysis

First, 15.4 mL of HNO_3 (Nitric acid-2%) and 6.75 mL of HCl (Hydrochloric acid-0.5%) were taken and transferred into a 500 mL volumetric flask. Then the flask was filled to 500 mL with ultra-pure water. For 500 ppb internal standard solutions, a 12.5 mL internal standard was taken. It was filled to the volume line with solutions of HNO_3 (2%) and HCl (0.5%) at 250 mL. The edible part (leaves) (0.5 g ± 1 mg) of the species was placed in Teflon tubes and the sample was accurately weighed. Ten mL of HNO_3 (65%) solution was added. The tightly closed Teflon tubes were placed in a microwave as indicated in the program and the burning process was started. After the combustion process was completed, the tubes were allowed to cool down to room temperature in the Fume Hood. The obtained solutions were checked to be transparent and completely burned. Then the solution was transferred to the appropriate volume of a plastic flask according to the dilution factor to be applied and completed with ultrapure water to the sample and increasing to the total volume to 100 mL. Then it was injected into the

Table 1. General characteristics of *A. orientale, E. spectabilis, A. officinalis* and *A. elongatum* and their therapeutic and nutritional usages.

Family Species	Turkish name Local name	Studied part (edible part)	Therapeutic and nutritional usages
(Alliaceae) *Allium orientale* Boiss.	Doğu soğanı, Soryaz	Young leaves	The bulbs and leaves are eaten raw or cooked. Flowers and leaves are used as a garnish in salads. Particularly the leaves are added to cheese and several traditional dishes. The therapeutic effects are antimicrobial, antioxidant, anticancer and antiseptic. The plant increases robustness, reduces cholesterol, reduces arteriosclerosis, is used as a tonic for the digestive system and strengthens the circulatory system (Karahan et al., 2020; Kardaş, 2019).
(Liliaceae) *Eremurus spectabilis* M. Bieb.	Çiriş otu, Gulik	Young leaves	It is used in many traditional dishes, especially in the omelet. It is consumed for vitamin C, B and antioxidant requirements. The therapeutic effects are antifungal and antimicrobial. It is used for the treatment of hemorrhoids, diabetes, peptic ulcer, skin diseases (eczema, boils, acne), burns, kidney diseases, ringworm, stomach ulcer, prostate and breast cancer, and as a milk and blood enhancer (Karahan et al., 2020; Kardaş, 2019).
(Boraginaceae) *Anchusa officinalis* L.	Sığırdili, Guriz	Young leaves	It is used in several traditional dishes and soups. It is used for the treatment of colds, flu, cough, depression, fear, stress, bronchitis, and as a diuretic and diaphoretic. It reduces the symptoms of rheumatism and stomach aches. It is used for healing open wounds in the intestines, stomach and duodenal ulcers. After boiling, it is wrapped in a cloth and left on the forehead of epilepsy patients for calming and relaxation. It is used as a sedative and antipyretic and also as an antidepressant, and for headache, dizziness and tinnitus (Karahan et al., 2020; Kardaş, 2019).
(Araceae) *Arum elongatum* Steven	Yılanyastığı, Kari	Young Leaves	It is cooked as a vegetable in dishes such as soup, stew, and omelets. It is mixed with mulberry molasses and applied to female breasts for healing wounds and reducing swelling. It is used as an anti-parasitic for the intestines. Its leaf and fruit are used for hemorrhoids, bladder diseases, and as an antidote to snake and scorpion poison. Drinking its boiled juice contributes to body regeneration, breastfeeding and relieves postpartum pain. It increases body resistance. It is eaten for menstruation, menopause and bleeding. It is added to the dowry of newly married brides as a gift to prevent gynecological diseases in southern Anatolia (Karahan et al., 2020; Kardaş, 2019).

ICP-MS. The results were obtained in µg/kg according to the value corresponding to the calibration curve obtained in the device. The device provides an automatic calculation of the element amount in the sample by entering the sample amount and the total volume. Measurement result (mg/kg) = (Device result × Completion volume) / Sample Quantity /1000. Each measurement was performed in triplicate and the results were averaged. Data, including the elemental properties of the species, were represented as the arithmetic mean, ranges and standard deviation values of three replicates of each sample.

RESULT AND DISCUSSION

A. orientale, E. spectabilis, A. officinalis and *A. elongatum* species are significant therapeutic and nutritious plants that are thought to have many positive effects on human health (Table 1) (Kardaş, 2019). Apart from minerals and heavy metals, other possible benefits of these species to human health have been described in many studies (Jakovljević, Vasić, Stanković, Topuzović, & Čomić, 2016; Jaradat & Abualhasan, 2016; Tosun et al., 2012); however, there are no detailed studies about their mineral and heavy metals accumulations. Although mostly the positive aspects of the species are investigated, they can also cause irreversible damages due to their toxic heavy metals levels. Therefore, examining these therapeutic plants from all aspects will help to reach a clear conclusion.

Macro-elements analysis of *A. orientale, E. spectabilis, A. officinalis* and *A. elongatum* showed that; P (462.856 mg/kg), Mg (731.012 mg/kg), K (4743.113 mg/kg), Ca (906.746 mg/kg)

and Fe (1022.068 mg/kg) were major elements in *A. orientale*. P (472.188 mg/kg), Mg (260.743 mg/kg), K (3689.566 mg/kg) and Ca (732.388 mg/kg) were major elements in *E. spectabilis*. P (475.193 mg/kg), Mg (387.009 mg/kg), K (7496.435 mg/kg), Ca (2947.378 mg/kg) and Fe (268.862 mg/kg) were the main elements in *A. officinalis*. P (363.409 mg/kg), Mg (393.178 mg/kg), K (4069.964 mg/kg), Ca (1191.365 mg/kg) and Fe (699.932 mg/kg) were major elements in *A. elongatum* (Table 2). Acceptable and normal limits of some beneficial minerals in plants ranged as follows: 1000-50,000 mg/kg for K, 3000-30,000 mg/kg for Ca, 100-1000 mg/kg for Mg, 50-250 mg/kg for Fe (Kabata-Pendias & Pendias, 2001; Ozyigit et al., 2018), 360-470 mg/kg for P (on average in *E. spectabilis*) (Tosun et al., 2012). In general, the concentrations of the basic minerals P, K, and Mg in the current study were found within the acceptable and normal limits of edible plants. Only the Ca level was below the required values; however, just in *A. officinalis* was the Ca level very close to the sufficient level. Also, in the study on *E. spectabilis* (collected from Erzurum, Turkiye) the average concentration of P, K, Ca and Mg were found to be 430, 4040, 309 and 390 mg/kg, respectively (Tosun et al., 2012). The concentrations of P, K, Ca and Mg in *E. spectabilis* in the present study were 472.188, 3689.566, 732.388 and 260.743 mg/kg, respectively. The concentrations of the P, K, and Mg appear to be roughly close to the mentioned study (Tosun et al., 2012). This indicates that the plant generally accumulates essential elements at genetically similar proportions. However, the Ca level of the current study seems to be almost twice of the previous study (Tosun et al., 2012). This high amount may be related to the chemical composition of the soil or the collection season. Ca is the most abundant essential mineral with many functions such as the repair and construction of bones and teeth, the functioning of muscles and nerve tissues, the functioning and coordination of the brain and nervous system, blood circulation, and the hormonal system (Karahan et al., 2020; Soetan, Olaiya, & Oyewole, 2010). On the other hand, Fe concentrations in *A. orientale* and *A. elongatum* were found higher than the normal limit of an average plant (Table 3). However, the Fe concentra-

Table 2. The average concentration of minerals and heavy metals in the edible tissues (leaves) of *A. orientale*, *E. spectabilis*, *A. officinalis* and *A. elongatum* (mg/kg).

	Plant species			
Mineral and heavy metal	***A. orientale* (Mean*±SD)**	***E. spectabilis* (Mean*±SD)**	***A. officinalis* (Mean*±SD)**	***A. elongatum* (Mean*±SD)**
Phosphor (P)	462.856±48.554	472.188±49.533	475.193±49.848	363.409±38.122
Sodium (Na)	21.975±2.373	4.206±0.454	12.586±1.359	30.379±3.281
Magnesium (Mg)	731.012±71.201	260.743±25.396	387.009±37.695	393.178±38.296
Potassium (K)	4743.113±490.438	3689.566±381.501	7496.435±775.131	4069.964±420.834
Calcium (Ca)	906.746±95.571	732.388±77.194	2947.378±310.654	1191.365±125.570
Zinc (Zn)	7.839±0.810	6.809±0.703	21.757±2.248	9.906±1.023
Tin (Sn)	nd	nd	nd	nd
Manganese (Mn)	26.004±2.234	4.886±0.420	8.552±0.735	14.659±1.259
Lithium (Li)	0.794±0.067	nd	nd	nd
Iron (Fe)	1022.068±89.431	49.720±4.351	268.862±23.525	699.932±61.244
Copper (Cu)	2.252±0.184	1.379±0.113	2.518±0.206	2.066±0.169
Chromium (Cr)	3.433±0.272	nd	1.405±0.111	1.645±0.130
Cobalt (Co)	0.660±0.055	nd	nd	0.384±0.032
Boron (B)	2.741±0.224	2.344±0.191	2.410±0.197	1.980±0.162
Aluminum (Al)	889.368±78.443	33.489±2.954	150.737±13.295	651.570±57.468
Strontium (Sr)	2.308±0.306	1.533±0.203	8.001±1.059	2.831±0.375
Selenium (Se)	0.116±0.019	nd	0.175±0.029	0.085±0.014
Antimony (Sb)	nd	nd	0.010±0.001	nd
Nickel (Ni)	3.851±0.402	0.279±0.029	2.492±0.260	2.290±0.239
Mercury (Hg)	nd	nd	nd	nd
Arsenic (As)	0.124±0.012	0.008±0.001	0.073±0.007	0.087±0.008
Cadmium (Cd)	nd	nd	0.064±0.005	0.013±0.001
Lead (Pb)	0.183±0.016	0.047±0.001	0.202±0.018	0.156±0.014

Results expressed as a concentration (mg/kg) of mineral and heavy metals in the edible plant leaves.
*Mineral and heavy metal concentrations of the plants are an average of three replicates; Means ± SD (standard deviation), nd: not detected.
Some minerals were not detected in the study due to the limited detectable range of the instrument and methods.

tion of *E. spectabilis* was lower than expected. The examined plants may not be rich in Ca, but rich in Fe, therefore *A. orientale* and *A. elongatum* might be recommended to be consumed as Fe sources. As known, Fe is a vitally essential element and due to its ability to exchange electrons, it is of great importance in ATP, DNA, RNA and protein synthesis and in the transport of oxygen by participating in the structure of hemoglobin as well as being necessary for the production and functioning of many enzymes and biomolecules (Roy & Enns, 2000; Soetan et al., 2010). Its deficiency adversely affects reproductive functions, growth and cognitive development and sometimes severe deficiency even causes anemia, mental disorders in children and causes premature births and infant weight loss in early pregnancy (Karahan et al., 2020; Soetan et al., 2010). It is worth noting that, *A. orientale* and *A. elongatum*, which are rich in Fe, are consumed as milk enhancers and strengthening agents for postpartum women in most of the rural part of Anatolia. Besides, *A. orientale* and *A. elongatum* consumed for therapeutic purposes during the menstrual period and postpartum has been considered to be involved in the synthesis of hemoglobin and compensate for blood loss. Additionally, Mg and K have many high-quality outcomes on human health (Karahan et al., 2020; Long & Romani, 2015; Soetan et al., 2010).

The heavy metal accumulation of a plant can be influenced by many factors such as the physiology of the plant, the structure of the soil, the pH of the soil, the chemical environment of the soil, the presence of mines, factories or facilities close to the growing region, the concentration of heavy metals in polluted air and irrigation water, etc. In the present study Mn concentration was determined as 26.004, 4.886, 8.552 and 14.659 mg/kg in *A. orientale, E. spectabilis, A. officinalis* and *A. elongatum*, respectively (Table 2). The level of Mn was below the sufficient concentration of 30-300 mg/kg in an average plant leaf (Table 3). Only the Mn level in *A. orientale* was close to the adequate limit. Mn is vital for glucose metabolism, bone structure, immune and neural functions, activation of many enzymes, the functions in cartilage and connective tissue, lipid and cholesterol metabolism, and antioxidants for free radicals, formation of amino acids and coenzyme activity (Karahan et al., 2020; Santos, Batoreu, Mateus, Marreilha dos Santos, & Aschner, 2014; Soetan et al., 2010).

Zn was detected at the level of 7.839, 6.809, 21.757 and 9.906 mg/kg in *A. orientale, E. spectabilis, A. officinalis* and *A. elongatum,* respectively (Table 2). The amounts did not exceed the sufficient concentration of 27-150 mg/kg in an average plant leaf (Table 3). Zn level in *A. officinalis* was 21.757 mg/kg and the concentration of Zn was quite above the EPA maximum contamination level (5 mg/L) (Table 4). However, it was near the sufficient level of the general plants limit (Table 3). It has been emphasized that the Zn level is higher than the sufficient concentration of a leaf 27-150 mg/kg in many studies. In a study on Zn, *Polygonum hydropiper* L. growing on contaminated soils in a sewage pond had accumulated 1061 mg/kg of Zn in its shoots and *Rumex acetosa* L. growing near a smelter accumulated more than 900 mg/kg of Zn both in its shoots and roots (Wang, Cui, Liu, Dong, & Christie, 2003). In addition, although Zn is an essential element, its high accumulation in the body causes dizziness and fatigue (Table 4) (Dixit et al., 2015). The Cu level of the current study ranged from 1.379 to 2.518 mg/kg (Table 2). According to the EPA maximum contamination limit of drinking water, Cu should be 1.3 mg/L, and according to the sufficient limit of a plant leaf, it should be between 5-30 mg/kg. The Cu concentration was determined as 2.252, 1.379, 2.518 and 2.066 mg/kg in *A. orientale, E. spectabilis, A. officinalis* and *A. elongatum*, respectively (Table 2). The quantities of Cu did not exceed the sufficient concentration of an average plant leaf (Table 3). As is known, high levels of Cu cause discomfort such as brain and kidney damage, liver cirrhosis and chronic anemia, and stomach and intestine irritation (Dixit et al., 2015; Nik Abdul Ghani et al., 2021).

Al concentration was determined as 889.368, 33.489, 150.737 and 651.570 mg/kg in *A. orientale, E. spectabilis, A. officinalis* and *A. elongatum*, respectively. Al concentration in soil is between 10,000 and 300,000 mg/kg (Neenu & Karthika, 2019). Al is not considered an essential nutrient for plants, but small concentrations can sometimes increase plant growth or bring other desired effects (Neenu & Karthika, 2019). There is no clear value regarding the limit of Al in medicinal plants. For example, Al could not be detected in some medicinal plants (Bhat et al., 2010), but moderate concentrations (30.983-368.877 mg/kg) were mentioned in another study (Karahan et al., 2020). Al concentration was high in *A. orientale* 889.368 mg/kg and *A. elongatum* (651.570 mg/kg). These high concentrations indicate that consuming *A. orientale* and *A. elongatum* may lead to drawbacks (Malluche, 2002; Molloy et al., 2007).

In the study of medicinal plants *Euphorbia hirta, Peristrophe bycaliculata, Tinospora cordifolia*, *Abutilon indicum* and *Calotropis procera*, it was observed that the heavy metal levels (particularly Pb, Cd, Cr and Ni) are different in the same medicinal plant collected from different parts of the same city (Barthwal, Nair, & Kakkar, 2008). In another study on onion (*Allium*) tuber and leaf, the level of Cr in the tuber and Fe in the leaf were found at high levels of 2.3 mg/kg and 425.5 mg/kg, respectively (Bedassa et al., 2017). The mineral and heavy metal contents of wild plants in the Kohistan region (Pakistan) were investigated. The concentration of Na, K, Ca, Mg, Fe, Mn, Cr, Ni, Co, Cu, Pb, Zn and Cd were as follows; 19 to 225 mg/kg, 2515 to 12595 mg/kg, 1602 to 24687 mg/kg, 898 to 5487 mg/kg, 187 to 5054 mg/kg, 22 to 857 mg/kg, 6 to 27 mg/kg, 10 to 44 mg/kg, 1 to 15 mg/kg, 4 to 66 mg/kg, 8 to 31 mg/kg, 7 to 328 mg/kg and 0.2 to 2.1 mg/kg, respectively (Muhammad et al., 2011). It was reported that most of the elements were not at a detrimental level. In a report on 17 medicinal plants from different parts of Turkiye, the measured element levels were (in mg/kg) 30.983 - 368.877 for Al, 13.845 - 186.015 for B, 1335.699 - 11213.951 for Ca, 0.016 - 0.653 for Cd, 0.379 - 30.708 for Cr, 23.838 - 90.444 for Cu, 78.960 - 1228.845 for Fe, 1035.948 - 6393.491 for K, 83.193 - 2252.031 for Mg, 12.111 - 362.570 for Mn, 278.464 - 1968.775 for Na, 1.945 - 35.732 for Ni, 0.796 - 17.162 for Pb and 166.910 - 395.252 for Zn (Karahan et al., 2020). In the study, *Vitiveria zizinalis* had the highest concentration of toxic heavy metals, including As (5.31 mg/kg), Cr (0.674 mg/kg), Co (1.02 mg/kg), Hg (0.36 mg/kg), and Ni (0.328 mg/kg), conversely, Al, Ba, Cd, and Mo heavy metals were not detected (Bhat et al., 2010).

Table 3. Average concentrations of trace and heavy elements (mg/kg) in a mature leaf tissue for various plant species. The table is adapted from the literature (Kabata-Pendias & Pendias, 2001).

Trace and heavy Elements	Excessive (toxic) concentration	Sufficient (normal) concentration	Trace and heavy Elements	Excessive (toxic) concentration	Sufficient (normal) concentration
Ag	5-10	0.5	Mn	400-1000	30-300
As	5-20	1-1.7	Mo	10-50	0.2-5
B	50-200	10-100	Ni	10-100	0.1-5
Ba	500	-	Pb	30-300	5-10
Be	10-50	1-7	Se	5-30	0.01-2
Cd	5-30	0.05-0.2	Sn	60	-
Co	15-50	0.02-1	Sb	150	7-50
Cr	5-30	0.1-0.5	Ti	50-200	-
Cu	20-100	5-30	Tl	20	-
F	50-500	5-30	V	5-10	0.2-1.5
Hg	1-3	-	Zn	100-400	27-150
Li	5-50	3	Zr	15	-

Values are not given for very sensitive or highly tolerant plant species. Values are given in mg/kg.

Table 4. Maximum contaminant level of toxic heavy metals for drinking water and potential health effects from long-term exposure above the maximum contaminant level (EPA, Environmental Protection Agency). The table is adapted from the literature (Apori, Atiah, Hanyabui, & Byalebeka, 2020; Dixit et al., 2015; Nik Abdul Ghani, Jami, & Alam, 2021).

Toxic heavy metals	Potential effects on human health for long-term exposure	EPA maximum contamination limit (mg/L)
As	Adversely affects the basic cellular processes oxidative phosphorylation and ATP synthesis	0.01
Cd	Mutagenic, endocrine disruptor, carcinogenic, lung damage, fragile bones, affects calcium regulation in body systems	0.005
Cr	Hair loss, skin irritation, skin sensitization, allergy	0.1
Cu	High levels cause liver cirrhosis and chronic anemia, stomach and intestine irritation, brain and kidney damage	1.3
Hg	Autoimmune diseases, fatigue, drowsiness, hair loss, depression, insomnia, amnesia, restlessness, defect of vision, temper outbursts, brain damage, tremors, lung and kidney failure	0.002
Pb	Overexposure in children and babies causes developmental impairment, decreased intelligence, short-term memory loss, learning and coordination problems, and cardiovascular disease risk	0.015
Se	Dietary exposure of approximately 300 µg per day affects endocrine function, impairment of natural killer cell activity, gastrointestinal disturbances and hepatotoxicity	0.050
Zn	Excessive exposure causes fatigue, dizziness, nausea, vomiting, diarrhea, metal taste, and kidney and stomach damage	5

Maximum contaminant level of toxic heavy metals is prepared according to *Drinking Water Contaminants;* United States Environmental Protection Agency (EPA): Washington, DC, USA, 2009.

On the other hand, some wild herbal plants contain very high amounts of specific heavy metals. As known, *Hypericum perforatum* L. is one of the most important medicinal plants used as an anti-depressive agent (Kim, Streltzer, & Goebert, 1999; Verotta, 2003). Recent studies have shown that *H. perforatum* can accumulate higher concentrations of Cd than other plants grown under the same conditions (Chizzola & Lukas, 2006; Masarovičová, Katarina, Kummerová, & Kmentova, 2004). The species of the present study do not show consistent results when compared with the above studies. Undoubtedly, heavy metal accumulation in a plant is mainly due to internal and external factors. Notably, the harvesting area of *A. orientale,*

E. spectabilis, A. officinalis and *A. elongatum* are mountainous lands far from industrial areas and settlements. The location of the species strengthens the fact that the heavy metal accumulation capacity of the examined species is due to internal factors. Considering all these results, although each species has its heavy metal and macro-element accumulation feature, the chemical structure of the soil and environmental heavy metal contamination can also change the elemental profile of a plant (Sarma, Deka, Deka, & Saikia, 2012).

Li was found at the level of 0.794 mg/kg in *A. orientale*, and Sb was found at the level of 0.010 mg/kg in *A. officinalis*. The excessive concentration of Li for leaf tissue is 5 mg/kg and for Sb, the excessive concentration is 150 mg/kg. Exceeding the concentration limit poses a danger to the body (Table 3). Cr concentration was determined as 3.433 mg/kg in *A. orientale*, 1.404 mg/kg in *A. officinalis* and 1.645 mg/kg in *A. elongatum* (Table 2). These levels were high when compared to both the EPA limit (0.1 mg/L) (Table 4) and an average plant leaf limit (0.1-0.5 mg/kg) (Table 3). Nevertheless, the results of the current study did not exceed the toxic level of an average plant tissue. On the other hand, the amounts of Ni in *A. orientale, E. spectabilis, A. officinalis* and *A. elongatum* were determined as 3.851, 0.279, 2.492 and 2.290 mg/kg, respectively (Table 2). Ni levels do not exceed the danger limit accepted in Table 3. The concentration of Pb varied between 0.047 and 0.202 mg/kg. The concentration of Pb in plant leaves is between 5 and10 mg/kg (Table 3). The highly toxic As ranged from 0.008 to 0.124 mg/kg in the studied species. Cd, another dangerous heavy metal, was not found in *A. orientale* or *E. spectabilis*, however, it was detected at levels of 0.064 and 0.013 mg/kg in *A. officinalis* and *A. elongatum*, respectively. Concentrations of toxic heavy metals Co, B and Se in *A. orientale, E. spectabilis, A. officinalis* and *A. elongatum* plants did not exceed the limit applicable for an average plant tissue (Table 3).

CONCLUSION

As a result, the concentration of the elements and heavy metals, with the exception of Cr and Al, in the plants, were within acceptable limits. However, the high concentration of Al in *A. orientale* and *A. elongatum* indicated that these plants should be consumed carefully. On the other hand, the fact that high levels of Fe in *A. orientale* and high levels of K and Ca in *A. officinalis* show that these plants can be consumed as nutrients in the supply of essential elements for the body. Moreover, the traditional postnatal use of *A. orientale* and *A. elongatum* is probably related to the presence and high concentration of Fe, a precursor in blood synthesis. The concentrations of toxic metals such as As, Cd, Pb, Ni, Sn, Co, Se, Sb, Hg and Cu were determined far from the danger limits. Finally, as the studied plants are frequently sold as vegetables in public markets, any study on the nutritional analysis of these plants will benefit the consumer sector.

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REFERENCES

- Aberoumand, A., & Deokule, S. S. (2009). Determination of elements profile of some wild edible plants. *Food Analytical Methods*, *2*(2), 116–119. https://doi.org/10.1007/s12161-008-9038-z
- Apori, S. O., Atiah, K., Hanyabui, E., & Byalebeka, J. (2020). Moringa Oleifera seeds as a low-cost biosorbent for removing heavy metals from wastewater. *STED Journal*, *2*(1), 45–52. https://doi.org/10.7251/STED2002045O
- Barthwal, J., Nair, S., & Kakkar, P. (2008). Heavy metal accumulation in medicinal plants collected from environmentally different sites. *Biomedical and Environmental Sciences*, *21*(4), 319–324. https://doi.org/10.1016/S0895-3988(08)60049-5
- Bedassa, M., Abebaw, A., & Desalegn, T. (2017). Assessment of selected heavy metals in onion bulb and onion leaf (Allium cepa L.), in selected areas of central rift valley of Oromia region Ethiopia. *Journal of Horticulture*, *4*(4), 217. https://doi.org/10.4172/2376-0354.1000217
- Bhat, R., Kiran, K., Arun, A. B., & Karim, A. A. (2010). Determination of mineral composition and heavy metal content of some nutraceutically valued plant products. *Food Analytical Methods*, *3*(3), 181–187. https://doi.org/10.1007/s12161-009-9107-y
- Ceylan, O., & Alıc, H. (2015). Antibiofilm, antioxidant, antimutagenic activities and phenolic compounds of Allium orientale BOISS. *Brazilian Archives of Biology and Technology*, *58*(6), 935–943. https://doi.org/10.1590/S1516-89132015060309
- Chizzola, R., & Lukas, B. (2006). Variability of the cadmium content in Hypericum species collected in Eastern Austria. *Water, Air, and Soil Pollution*, *170*(1–4), 331–343. https://doi.org/10.1007/s11270-005-9004-y
- Dixit, R., Wasiullah, Malaviya, D., Pandiyan, K., Singh, U., Sahu, A., … Paul, D. (2015). Bioremediation of heavy metals from soil and aquatic environment: An overview of principles and criteria of fundamental processes. *Sustainability*, *7*(2), 2189–2212. https://doi.org/10.3390/su7022189
- Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B. B., & Beeregowda, K. N. (2014). Toxicity, mechanism and health effects of some heavy metals. *Interdisciplinary Toxicology*, *7*(2), 60–72. https://doi.org/10.2478/intox-2014-0009
- Jakovljević, D., Vasić, S., Stanković, M., Topuzović, M., & Čomić, L. (2016). The content of secondary metabolites and in vitro biological activity of Anchusa officinalis L. (Boraginaceae). *Indian Journal of Traditional Knowledge*, *15*(4), 587–593.
- Jaradat, N., & Abualhasan, M. (2016). Comparison of phytoconstituents, total phenol contents and free radical scavenging capacities between four Arum species from Jerusalem and Bethlehem. *Pharmaceutical Sciences*, *22*(2), 120–125. https://doi.org/10.15171/PS.2016.19
- Kabata-Pendias, A., & Pendias, H. (2001). *Trace elements in soils and plants* (3rd ed). Boca Raton, Fla: CRC Press.
- Karahan, F., Ozyigit, I. I., Saracoglu, I. A., Yalcin, I. E., Ozyigit, A. H., & Ilcim, A. (2020). Heavy metal levels and mineral nutrient status in different parts of various medicinal plants collected from Eastern Mediterranean region of Turkey. *Biological Trace Element Research*, *197*(1), 316–329. https://doi.org/10.1007/s12011-019-01974-2
- Kardaş, C. (2019). Muş'ta yabani bitkilerin halk hekimliğinde kullanılması [Usage of savage plants in the folk medicine in Muş]. *Lokman Hekim Journal*, *9*(1), 85–96. https://doi.org/10.31020/mutftd.468848
- Kim, H. L., Streltzer, J., & Goebert, D. (1999). St. John's Wort for depression: A meta-analysis of well-defined clinical trials. *Jour-*

nal of Nervous and Mental Disease, *187*(9), 532–538. https://doi.org/10.1097/00005053-199909000-00002
- Long, S., & Romani, A. M. (2015). Role of cellular magnesium in human diseases. *Austin Journal of Nutrition & Food Sciences, 2*(10), 19.
- Maharia, R. S., Dutta, R. K., Acharya, R., & Reddy, A. V. R. (2010). Heavy metal bioaccumulation in selected medicinal plants collected from Khetri copper mines and comparison with those collected from fertile soil in Haridwar, India. *Journal of Environmental Science and Health, Part B*, *45*(2), 174–181. https://doi.org/10.1080/03601230903472249
- Malluche, H. (2002). Aluminum and bone disease in chronic renal failure. *Nephrology, Dialysis, Transplantation: Official Publication of the European Dialysis and Transplant Association - European Renal Association*, *17 Suppl 2*, 21–24. https://doi.org/10.1093/ndt/17.suppl_2.21
- Masarovičová, E., Katarina, K., Kummerová, M., & Kmentova, E. (2004). The effect of cadmium on root growth and respiration rate of two medicinal plant species. *Biologia*, *59*(13), 211–214.
- Molloy, D. W., Standish, T. I., Nieboer, E., Turnbull, J. D., Smith, S. D., & Dubois, S. (2007). Effects of acute exposure to Aluminum on cognition in humans. *Journal of Toxicology and Environmental Health, Part A*, *70*(23), 2011–2019. https://doi.org/10.1080/15287390701551142
- Muhammad, S., Shah, M. T., & Khan, S. (2011). Heavy metal concentrations in soil and wild plants growing around Pb–Zn sulfide terrain in the Kohistan region, northern Pakistan. *Microchemical Journal*, *99*(1), 67–75. https://doi.org/10.1016/j.microc.2011.03.012
- Neenu, S., & Karthika, K. S. (2019). Aluminium toxicity in soil and plants. *Harit Dhara*, *2*(1), 15–19.
- Nik Abdul Ghani, N. R., Jami, M., & Alam, M. (2021). The role of nanoadsorbents and nanocomposite adsorbents in the removal of heavy metals from wastewater: A review and prospect. *Pollution*, *7*(1), 153–179. https://doi.org/10.22059/poll.2020.307069.859
- Okcu, M., Tozlu, E., Kumlay, A. M., & Pehluvan, M. (2009). Ağır metallerin bitkiler üzerine etkileri [The effects of heavy metals on plants]. *Alinteri Journal of Agriculture Science*, *17*(2), 14–26.
- Ozyigit, I. I., Yalcin, B., Turan, S., Saracoglu, I. A., Karadeniz, S., Yalcin, I. E., & Demir, G. (2018). Investigation of heavy metal level and mineral nutrient status in widely used medicinal plants' leaves in Turkey: Insights into health implications. *Biological Trace Element Research*, *182*(2), 387–406. https://doi.org/10.1007/s12011-017-1070-7
- Roy, C. N., & Enns, C. A. (2000). Iron homeostasis: New tales from the crypt. *Blood*, *96*(13), 4020–4027.
- Santos, D., Batoreu, C., Mateus, L., Marreilha dos Santos, A. P., & Aschner, M. (2014). Manganese in human parenteral nutrition: Considerations for toxicity and biomonitoring. *NeuroToxicology*, *43*, 36–45. https://doi.org/10.1016/j.neuro.2013.10.003
- Sarma, H., Deka, S., Deka, H., & Saikia, R. R. (2012). Accumulation of heavy metals in selected medicinal plants. In D. M. Whitacre (Ed.), *Reviews of Environmental Contamination and Toxicology* (pp. 63–86). New York, NY: Springer New York. https://doi.org/10.1007/978-1-4614-0668-6_4
- Soetan, K. O., Olaiya, C. O., & Oyewole, O. E. (2010). The importance of mineral elements for humans, domestic animals and plants: A review. *African Journal of Food Science*, *4*(45), 200–222.
- Tosun, M., Ercisli, S., Ozer, H., Turan, M., Polat, T., Ozturk, E., … Kilicgun, H. (2012). Chemical composition and antioxidant activity of Foxtail Lily (Eremurus spectabilis). *Acta Scientiarum Polonorum Hortorum Cultus*, *11*(3), 145–153.
- Verotta, L. (2003). Hypericum perforatum, a source of neuroactive lead structures. *Current Topics in Medicinal Chemistry*, *3*(2), 187–201. https://doi.org/10.2174/1568026033392589
- Wang, Q. R., Cui, Y. S., Liu, X. M., Dong, Y. T., & Christie, P. (2003). Soil contamination and plant uptake of heavy metals at polluted sites in China. *Journal of Environmental Science and Health, Part A*, *38*(5), 823–838. https://doi.org/10.1081/ESE-120018594

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Original Article

Microscopic examination and comparison of exine layer of bee pollen and bee bread (Perga)

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ABSTRACT

Background and Aims: Thanks to their high nutritional content and therapeutic effects, bee pollen and bee bread (perga) are used as a food supplement. Studies have shown that bee bread has more bioavailability than bee pollen. This situation has been explained by the fragmentation of the exine layers of pollen in bee bread in some studies. However, there is no clear microscopic study showing that the exine layer is broken. This study investigated for the first time whether the pollen grains in bee bread were fragmented in the exine layers after fermentation, in comparison with the pollen grains in bee pollen samples.
Methods: Bee pollen and bee bread samples were collected from the same hives and pollen slides were prepared for examination with light and SEM microscopes. Both of the pollen slides were compared and microscopic photographs were taken.
Results: No deformation was observed in the exine layers of the pollen grains in bee bread after fermentation.
Conclusion: In many studies, the higher bioavailability of bee bread has been explained by the deformation at exine structure of the pollen grains. But it has not been supported microscopically in detail with both light and SEM microscopes. Our study's conclusion was that no deformation was observed in the exine structures of the pollen in bee bread after fermentation.

Keywords: Bee bread (perga), Bee pollen, Exine layer, Microscopic analysis

INTRODUCTION

Plant pollen provides the basic protein needs of honey bees due to its high nutritional content (Standifer, 1980). Pollen is collected by honey bees and stored as pollen loads on the 3rd pair of legs (Alataş, Yalçın, & Öztürk, 1997; Almeida-Muradian, Pamplona, Coimbra, & Barth, 2005). These pollen loads are defined as "bee pollen" (corbicular pollen, bee-collected pollen) (Fuenmayor et al., 2014; Kňazovická et al., 2019). These pollen loads must be fermented in order to be used as a nutrient by honey bees. Therefore, the pollen is stored in the honeycomb, it is compressed, and saliva secretions of honeybees are added. Then, the honeycomb is covered with bee wax (Nagai, Nagashima, Myoda, & Inoue, 2004). Fermentation takes place thanks to microorganisms (Lactic acid bacteria (LAB), *Bifidobacterium* spp., *Saccharomyces* spp., *Pseudomonas* spp., *Streptococcus* spp., etc.) naturally found in the digestive secretions of the honey bees (Gilliam, Wickerham, Morton, & Martin, 1974; Olofsson & Vásquez, 2008). Fermentation is completed in about two weeks and the pollen stored in the honeycombs is called "bee bread" or "perga" (Herbert & Shimanuki, 1978; Nagai et al., 2004; Silici, 2014). So bee bread is probiotic due to the presence and activities of probiotic microorganisms in it (Kieliszek et al., 2018).

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Bee pollen has high nutritional and polyphenolic content and many therapeutic effects, such as antioxidant, antiallergenic, immunomodulator, anticarcinogen, hepatoprotective (Campos et al., 2008; Morais, Moreira, Feás, & Estevinho, 2011; Markiewicz-Żukowskaet al., 2013; Özkök, 2018). Due to these properties, bee pollen has been used as a food supplement in human nutrition for centuries (Özkök, 2018; Howell & Champie, 1981). However, in recent years studies have shown that the dense and durable wall structure of pollen grains can't be fully digested (only 48% to 59% in the human body) by the digestive enzymes in the living body. In contrast, fermented bee bread was observed to have higher digestibility and bioavailability than bee pollen (Campos, Frigerio, Lopes, & Bogdanov, 2010). This situation has been explained by researchers in different ways. Some researchers suggest that the presence of acidic substances formed after fermentation of bee bread and low pH value break down the exine layer of pollen and in this way the pollen protoplast comes out and its bioavailability increases (Mutsaers, Blitterswijk, Leven, Kerkvliet, & Waerdt, 2005; Zuluaga, Serratob, & Quicazana, 2015). But considering the structure of the exine layer, the possibility of this situation is doubtful.

When the structure of the pollen is examined, each of the pollen grains is surrounded by a sporoderm layer, which consists of two main layers, exine and intine (D'Albore, 1997; Wiermann& Gubatz, 1992). The exine layer structure of the sporopollenin consists of the polymerization of monocarboxylic and carboxylic fatty acids and has a high molecular weight (Heslop-Harrison, 1968). The exine layer, which is very resistant and difficult to purify and digest, provides protection of the pollen, and pollen grains are highly resistant even against strong acids (HNO_3, HF, HCL) and at high temperatures (about 400-500°C). Studies have revealed that the pollen can remain unharmed for many years thanks to its stable exine structure (Özkök, 2018; Martin& Byers,1965; Morgan, Flynn, Sena, & Bull, 2014; Southworth, 1990). The intine layer beneath the exine surrounds the protoplast of the pollen. In addition to being rich in polysaccharides, the intine layer also contains protein compounds in its structure and is not as stable as exine (Wiermann & Gubatz, 1992; Kapp, 1969; Halbritteret al., 2018). The germination tube formed during germination of the pollen originates from the intine layer. The apertures (gaps on the exine layer), formed by the weakening or disappearance of the exine on the pollen surface, allow the outward development of the germination tube (Kapp, 1969). Considering the stable nature of exine, it is suggested that the exine layer cannot be easily degraded after fermentation. When the studies on the subject were examined, it was seen that microscopic examinations regarding the structure of the pollen after fermentation were insufficient. In this study, pollen grains in bee pollen and bee bread samples (obtained from the same hive) were examined through light microscopy and a scanning electron microscope (SEM). By examining the pollen structure after fermentation, this study aimed to clarify the situation in the exine layer.

MATERIAL AND METHODS

Collecting samples

Bee pollen and bee bread were samples collected from the same hive from Bursa city in Turkey. Bee pollen samples were collected with pollen traps attached to the hive every 15 days and mixed to represent an entire year. Bee bread samples were collected with a special bee bread tool from the same hive, from honeycomb that was collected after the honey harvest to represent an entire year. Bee pollen and bee bread samples were kept at +4 °C until microscopic analysis.

Examining the samples with light microscope

Bee pollen samples and bee bread samples were separately homogenized through mixing. Pollen slides were prepared according to the method of Mayda, Özkök, Bayram, Gerçek & Sorkun (2020). Slides were fixed with glycerin gelatin and inverted to hold the pollen grains to the lamels' surface. Pollen slides were ready for microscopic examination after 12 hours, and they were examined and photographed under a Nicon Eclipse E400 light microscope.

Examining the samples with scanning electron microscope (SEM)

Pollen slides of bee pollen and bee bread were prepared for examination with SEM according to the method of Doğan & Erdem (2018),with some modifications. Two grams of each homogenized sample were weighed. 10 ml of distilled water were added and they were macerated, then vortexed until homogenized. After centrifuging at 3500 rpm for 20 minutes, the supernatant was removed. 3 ml of glutaraldehyde and 0.1 M 7.2 pH phosphate buffer were added to each sample for each fixation and the samples were kept at room temperature for one night. After fixation, the tubes were washed 3 times with phosphate buffer and centrifuged 20 minutes each time. Before the last centrifuge, the mixture was filtered through gauze and unwanted particles were removed. Samples were kept in ethanol series of 25%, 50%, 75%, 90% and 100% for 5 minutes each time, and the water in it was centrifuged for 20 minutes each time. After the centrifugation process was completed, the supernatant part was removed. The tubes were inverted on a blotter paper and allowed to filter for 10 minutes. The samples remaining at the bottom of the tube were removed with the help of a glass Pasteur pipette and spread over the stabs previously covered with carbon tape. Stabs were left to dry overnight in the oven at 25°C.

RESULTS AND DISCUSSION

As a result of the examination of bee pollen and bee bread samples under light microscope, no fragmentation was observed in the exine layers of the pollen grains. Pollen grains in bee bread preserved their structural integrity as in bee pollen samples (Figure 1).

In an examination of the bee pollen and bee bread samples with the SEM, no fragmentation was observed in the exine layers of the pollen grains. Pollen grains in the bee bread preserved their structural integrity as in the bee pollen samples (Figure 2).

The nutritional properties of bee pollen and bee bread are similar. It has been observed that protein, lipid content and antioxidant capacity of bee bread may decrease slightly compared to bee pollen (Mayda et al., 2020). But it has also been reported that bee bread contains more vitamin K, reduced sugar and

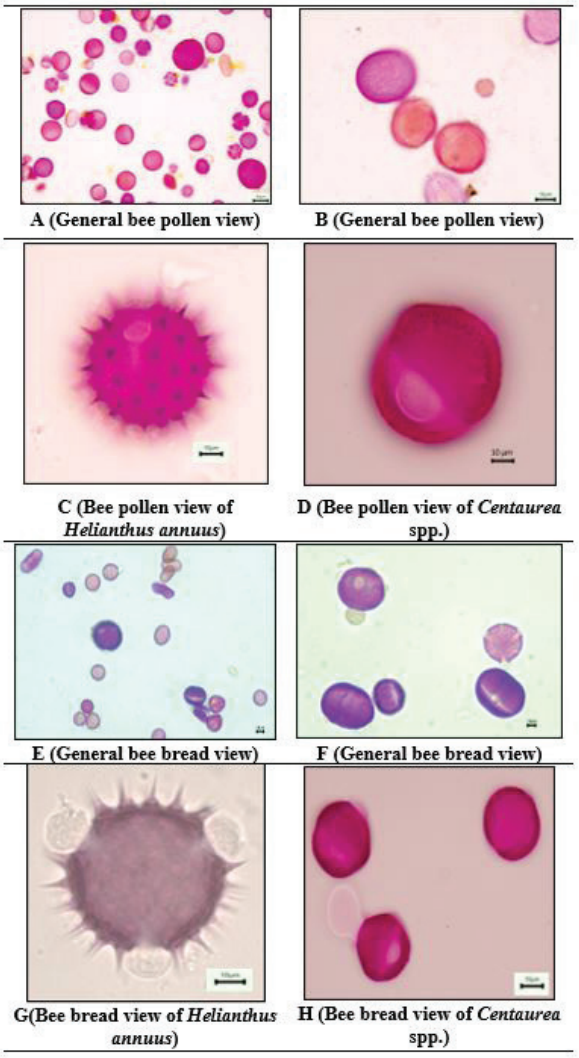

Figure 1. Pollen photos of bee pollen (A-D) and bee bread (E-H from light microscope.

fiber (Ivanišová et al., 2015). Although their nutritional contents are similar, studies show that bee bread has more bioavailability in the human body than bee pollen (Campos et al., 2010; Zuluaga et al., 2015).

Campos et al., (2010) reported that bee pollen can be digested 48%-59% in the living body. Bell et al., (1983) examined the bioavailability of two different commercial bee pollen in mice and observed that both were quite low. In a study conducted by Zuluaga et al. (2015), bee pollen was digested at the rate of 63 g (digested protein) / 100 g (total protein).On the other hand, it was observed that bee bread was digested at the rate of 79 g (digested protein) / 100 g (total protein).

The high bioavailability of bee bread has been explained by various researchers in different ways. It is suggested that the acidic products formed as a result of fermentation in bee bread cause deformation and disintegration in the pollen wall. This deformation occurring in the exine layer allows the emergence of pollen protoplast and thus higher bioavailability of bee bread (Mutsaers et al., 2005; Zuluaga et al., 2015).

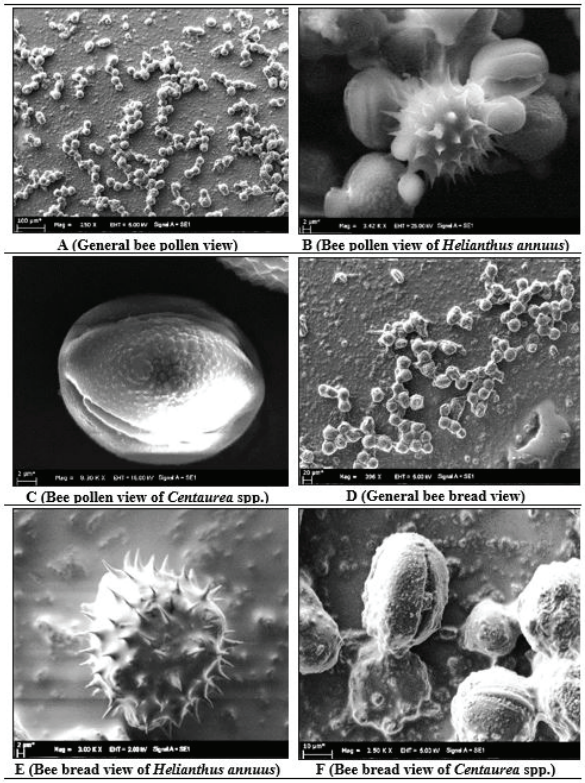

Figure 2. Pollen photos of bee pollen (A-C) and bee bread (D-F) from SEM.

Similarly, Gönül (2016) studied the high bioavailability of the fermented pollen and explained the fragmentation occurring in the exine structure. However, the investigation was carried out under a light microscope did not have sufficient detail to examine the surface of the exine layer.

In this study, pollen grains in bee pollen and bee bread samples collected from the same hives were examined with both light microscopy and Scanning Electron Microscope (SEM). The collection of samples from the same hive is important in terms of obtaining samples with similar botanical origins, because the stability of the wall structure changes according to the botanical origin. Sporoderm is richer and more durable in entomophilic plant pollen in terms of chemical content than anemophilous plant pollen (Campbell, 1991; Twiddle & Bunting, 2010). Hall (1981) reported that pollen of *Taraxacum* spp., which is an entomophilic plant, is more resistant than pollen of *Corylus* spp. and *Quercus* spp., which are anemophilic plants.

As a result of morphological examinations performed under the light microscope, no deformation was observed on the surface of the pollen grains in bee bread. Examinations made with the light microscope were also supported by the SEM. Thus, it was revealed that there was no deformation in the exine layers of the pollen grains in bee bread, as explained in some studies (Dustmann, 2007; Bobiset al., 2017). Pollen grains'structural integrity was preserved as in bee pollen.

The intine, located below the exine and enveloping the protoplast of the pollen, does not have as durable and stable a structure as the exine (Kapp, 1969). Various mechanical and chemi-

cal effects can affect the integrity of the intine. In the acetolysis method developed by Erdtman, intine is eliminated, while the exine structure is preserved in pollen grains treated with acidic substances (Erdtman, 1957). As a result of this study, it was found that the acidic substances occurring as a result of fermentation do not cause deformation of the exine structure. However, acidic substances reaching the intine surface through the openings on the surface of the exine can cause deformations on the intine surface. In this way, it has been suggested that Protoplast can escape from the apertures by disrupting the intine. Similar comments were made by Dustmann (2007) and Bobis et al. (2017). Dustman (2007) explained the high bioavailability of bee bread with the presence of water and sugar from honey. The difference in concentration between sugar and water causes an osmotic shock in the pollen grains. Due to this osmotic shock, the content of pollen is released from the openings (aperture) and bioavailability increases (Komosinska-Vassev, Olczyk, Kaźmierczak, Mencner, & Olczyk, 2015). Similarly, Bobis et al., (2017) explained that sugars and water from honey are absorbed through the aperture of as a result of osmotic pressure and enrich the internal composition of bee bread.

CONCLUSION

In many studies, the high bioavailability of bee bread has been explained by the deformation of the pollen grains in the exine structure, but this has not been supported in detail microscopically. Considering the stable and durable structure of the exine, this seems unlikely. The conclusion of our study is that no deformation was observed in the exine structures of pollen in bee bread after fermentation. However, it is thought that acidic substances formed as a result of acidic activity may enter the apertures on the surface of the exine, causing deformations in the intine structure and the pollen protoplast may come out of these openings. It is also possible that the protoplast content is released as a result of the osmotic pressure of the sugar and water sourced from honey in the bee bread. In addition, the prebiotic property of bee bread may be an effective factor in its easy digestibility. Examining the literature data, we can say that the high bioavailability of bee bread is due to contact with acidic substances and deformation of the intine layer.

As a result of this, pollen content can go out from the apertures. Moreover, it is believed that the fact that bee bread is a probiotic substance increases its absorption and bioavailability in the intestine. Explanations on the bioavailability of bee bread are still insufficient; this question needs to be investigated in further studies.

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REFERENCES

- Alataş, İ., Yalçın, L.İ. & Öztürk, A.İ. (1997). The effect of pollen trapping on the honey bee (*Apis mellifera* L.) colony development and honey field. *Anadolu Journal of Aegean Agricultural Research Institute,7,* 30–42.
- Almeida-Muradian, L., Pamplona, L.C., Coimbra, S. & Barth, O.M. (2005). Chemical composition and botanical evaluation of dried bee pollen pellets. *Journal of Food Composition and Analysis, 18,* 105–111.https://doi.org/10.1016/j.jfca.2003.10.008
- Bell, R.R., Thornber, E.J., Seet, J.L., Groves, M.T., Ho, N.P. & Bell, D.T. (1983). Composition and protein quality of honeybee-collected pollen of *Eucalyptus marginata* and *Eucalyptus calophylla*. *The Journal of Nutrition, 113*, 2479–2484.https://doi.org/10.1093/jn/113.12.2479
- Bobis, O., Dezmirean, D.S., Marghitas, L.A., Bonta, V., Margaoan, R., Pasca, C. … Bandhari, P.S. (2017). Beebread from *Apis mellifera* and *Apis dorsata*, comparative chemical composition and bioactivity. *Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Animal Science and Biotechnologies, 74,* 43–50.http://dx.doi.org/10.15835/buasvmcn-asb:12620
- Campbell, I.D. (1991). Experimental mechanical destruction of pollen grains. *Palynology, 15*(1), 29–33.
- Campos, M.G., Bogdanov, S., de Almeida-Muradian, L.B., Szczesna, T., Mancebo, Y., Frigerio, C. & Ferreira, F. (2008). Pollen composition and standardisation of analytical methods. *Journal of Apicultural Research, 47*(2), 154–161.https://doi.org/10.1080/00218839.2008.11101443
- Campos, M.G.R., Frigerio, C., Lopes, J. & Bogdanov, S. (2010). What is the future of bee-pollen?*Journal of ApiProduct and ApiMedical Science, 2,* 131–144.https://doi.org/10.3896/IBRA.4.02.4.01
- D'Albore, G.R. (1997). Textbook of melissopalynology, Apimondia Congress.Bucharest, Romania.
- Doğan, C. & Erdem, S.Ö. (2018). Pollen morphology of some Arenaria taxa (Caryophyllaceae), *Phytotaxa, 351,* 29–40.
- Dustmann, J. (2007). Fresh pollen offers best therapeutic and nutritional benefits. Paper presented at the 5th German Apitherapy Congress. Passau, Germany.
- Erdtman, G. (1957). Pollen and spore morphology/plant taxonomy. Gymnospermae, Pteridophyta, Bryophyta(illustrations). (An introduction to palynology. II.). Stockholm: Almqvist&Wiksell; New York, USA: The Ronald Press.
- Fuenmayor, C. B., Zuluaga, C. D., Díaz, C. M., Quicazán de, C.M., Cosio, M. & Mannino, S. (2014). Evaluation of the physicochemical and functional properties of Colombian bee pollen. *Revista MVZ Córdoba, 19,* 4003–4014.
- Gilliam, M., Wickerham, L., Morton, H.L. &Martin, R.D. (1974). Yeasts isolated from honey bees, *Apis mellifera*, fed 2, 4-D and antibiotics. *Journal of Invertebrate Pathology, 24,*349–356.https://doi.org/10.1016/0022-2011(74)90013-5
- Gönül, S. (2016). Polen fermentasyonunun fenolik bileşiklerin biyoyararlılığı ve profilleri üzerindeki etkisi [Effect of pollen fermentation on bioavailability and profiles of phenolic compounds]. Master Thesis, Fen Bilimleri Enstitüsü, İstanbul Teknik Üniversitesi, Turkey.
- Halbritter, H., Ulrich, S., Grimsson, F., Weber, M., Zetter, R., Hesse, M. & Frosch-Radivo, A. (2018). Illustrated pollen terminology. Springer International Publishing.
- Hall, S.A. (1981). Deteriorated pollen grains and the interpretation of Quaternary pollen diagrams. *Review of Palaeobotany and Palynology, 32,*193–206.https://doi.org/10.1016/0034-6667(81)90003-8

- Herbert Jr, E.W. & Shimanuki, H.(1978). Chemical composition and nutritive value of bee-collected and bee-stored pollen. *Apidologie, 9,*33–40.
- Heslop-Harrison, J. (1968). Pollen wall development. *Science, 161,* 230–237. doi:10.1126/science.161.3838.230
- Howell, J. & Champie, C. (1981). Market and industrial aspects of bee pollen [USA].*American Bee Journal, 121*(11), 817–818.
- Ivanišová, E., Kačániová, M., Frančáková, H., Petrová, J., Hutková, J., Brovarskyi, V. … Musilová, J.(2015). Bee bread-perspective source of bioactive compounds for future. *Potravinarstvo Slovak Journal of Food Sciences,9,* 592–598.
- Kapp, R.O. (1969). How to know pollen and spores. Pictured Key Nature Series. Wm. C. In: Brown Company Publishers, Dubuque, Iowa, USA.
- Kieliszek, M., Piwowarek, K., Kot, A.M., Błażejak, S., Chlebowska-Śmigiel, A. & Wolska I. (2018). Pollen and bee bread as new health-oriented products: A review. *Trends in Food Science & Technology, 71*, 170–180.
- Kňazovická, V., Mašková, Z., Vlková, E., Švejstil, R., Salmonová, H., Ivanišová, E. … Tokár, M.(2019). Pollen can testing of bee pollen fermentation in model conditions.*Journal of Microbiology, Biotechnology and Food Sciences, 9,*805–811. https://doi: 10.15414/jmbfs.2018.8.2.805-811
- Komosinska-Vassev, K., Olczyk, P., Kaźmierczak, J., Mencner, L. & Olczyk, K.(2015). Bee pollen: chemical composition and therapeutic application.*Evidence-Based Complementary and Alternative Medicine, 2015*,297425.https://doi.org/10.1155/2015/297425
- Markiewicz-Żukowska, R., Naliwajko, S.K., Bartosiuk, E., Moskwa, J., Isidorov, V., Soroczyńska, J. & Borawska, M.H. (2013). Chemical composition and antioxidant activity of beebread, and its influence on the glioblastoma cell line (U87MG).*Journal of Apicultural Science, 57,*147–157.https://doi.org/10.2478/jas-2013-0025
- Martin, P.S.,& Byers, W. (1965). Pollen and archaeology at Wetherill Mesa. Memoirs of Society for American Archaeology. *American Antiquity, 31*(2), 122–135.
- Mayda, N., Özkök, A.,Bayram, N.E., Gerçek, Y.C. & Sorkun, K. (2020). Bee bread and bee pollen of different plant sources: Determination of phenolic content, antioxidant activity, fatty acid and element profiles. *Journal of Food Measurement and Characterization,1*,1–15. https://doi.org/10.1007/s11694-020-00427-y
- Morais, M., Moreira, L., Feás, X. & Estevinho, L.M. (2011). Honeybee-collected pollen from five Portuguese Natural Parks: Palynological origin, phenolic content, antioxidant properties and antimicrobial activity.*Food and Chemical Toxicology,49,*1096–1101. https://doi.org/10.1016/j.fct.2011.01.020
- Morgan, R., Flynn, J., Sena, V. & Bull, P. (2014). Experimental forensic studies of the preservation of pollen in vehicle fires. *Science & Justice, 54,* 141–145.https://doi.org/10.1016/j.scijus.2013.04.001
- Mutsaers, M., Blitterswijk, H., Leven, L., Kerkvliet, J. & Waerdt, J.(2005). Bee products: properties, processing and marketing, Wageningen, Netherlands: Agromisa Foundation / Technical Centre for Agricultural and Rural Cooperation (CTA).
- Nagai, T., Nagashima, T., Myoda, T. & Inoue, R. (2004). Preparation and functional properties of extracts from bee bread. *Food/Nahrung, 48,* 226–229.https://doi.org/10.1002/food.200300421
- Olofsson, T.C. & Vásquez, A. (2008). Detection and identification of a novel lactic acid bacterial flora within the honey stomach of the honeybee *Apis mellifera*.*Current Microbiology, 57*,356–363.
- Özkök, A. (Ed.) (2018). Türkiye'de hızla büyüyen sektör: Arı ürünlerine genel bir bakış [Fast growing sector in Turkey: An overview of bee products].Hacettepe Üniversitesi, Ankara, Turkey: Palme Yayıncılık.
- Silici S. (2014). Bee pollen and bee bread, *Uludağ Bee Journal, 14,*99–105.https://doi.org/10.31467/uluaricilik.376901
- Southworth, D. (1990). Exine biochemistry, microspores: Evolution and ontogeny. In S. Blackmore & R.B. Knox (Eds.), *Microspores Evolution and Ontogeny*(pp.193–212). London, UK:Academic Press.
- Standifer, L. (1980). Honey bee nutrition and supplemental feeding. *Beekeeping in the United States Agriculture Handbook, 335,*39-45.
- Twiddle, C.,& Bunting, M.(2010). Experimental investigations into the preservation of pollen grains: a pilot study of four pollen types. *Review of Palaeobotany and Palynology, 162,* 621-630. https://doi.org/10.1016/j.revpalbo.2010.08.003
- Wiermann, R. & Gubatz, S. (1992). Pollen wall and sporopollenin. *International Review of Cytology, 140,*35–72.https://doi.org/10.1016/S0074-7696(08)61093-1
- Zuluaga, C.M., Serratob, J.C. & Quicazana, M.C. (2015). Chemical, nutritional and bioactive characterization of Colombian bee-bread. *Chemical Engineering Journal, 43,*175–180. https://doi.org/10.3303/CET1543030.

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Original Article

Anxiety level and COVID-19 awareness of patients in a non-COVID-19 outpatient clinic of a pandemic hospital

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ABSTRACT

Background and Aims: The aim of this study is to investigate the COVID-19 awareness and anxiety levels of patients who visited the outpatient department in a hospital.
Methods: A questionnaire for socio-demographic data and COVID-19 awareness, Beck Anxiety Scale (BAS), and Hospital Anxiety and Depression Scale (HADS) data were collected from patients who volunteered to participate in the study. Counts, percentages, independent groups t-test, and correlation analysis were used during statistical data analysis. Those patients who were over 18 years of age, literate, having no psychiatric disorder and from whom informed consent was obtained were included in the study. Patients younger than 18 years of age, patients who had psychiatric disorders, and illiterate patients were excluded.
Results: The questionnaire form and scales were distributed to 254 patients, while 210 patients were included in the study. Therefore, the response rate from the patients was 82.7%. The mean age of the patients participating in this study was 44.41 years ±16.9 SD. The BAS scores of patients over 65 years of age were significantly higher than the patients at younger ages ($p<0.001$). The percentage of female patients was 42.6% (n=89) and that of males was 57.4% (n=120). Both the BAS scores ($p<0.001$) and HADS scores ($p<0.01$) of the female patients were significantly higher than those of the male patients.
Conclusion: The BAS and HADS scores of females were found to be significantly higher than those of the males. Although the mean of the BAS scores of the patients was low, the BAS scores of those women who were at an age of 65 years or above, and those who had secondary school education or below, were significantly higher. Therefore, related research focusing on these groups should be conducted in the future.

Keywords: COVID-19, Awareness, Beck anxiety scale, Hospital anxiety and depression scale

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a rapidly spreading and deadly infectious disease affecting the respiratory tract caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Respiratory failure, acute respiratory distress syndrome, and thromboembolism are among the main complications leading to death. The spread of the infection is higher in crowded and poorly ventilated areas. These environments are the areas where the virus spreads more rapidly with respiratory droplets or aerosols (World Health Organization (WHO), 2021a).

The environment that affects the host, agent, and their interactions plays an important role in the spread of infectious diseases. Poverty, education, pollution, sanitation, crowds, availability of health services, and biological factors are among the environmental factors associated with the infection (Koley & Dhole, 2020). According to the WHO, it has been reported that, as of April 13, 2022, there were 499,119,316 confirmed cases for COVID-19 in the world and 6,185,242 people had died from this infection (WHO, 2022). The best way to prevent COVID-19 infection is to avoid exposure to the virus. Washing hands frequently, avoiding sick people and crowds, wearing masks in communal areas, and covering the mouth and nose while coughing and sneezing are among the measures that individuals can apply (Centers for Disease Control and Prevention, 2021).

Individual responsibilities are critical for the prevention of COVID-19 infection. This is related to the health literacy level of society. According to the WHO, health literacy is defined as *"the knowledge, motivation and competencies to understand, evaluate and apply health care knowledge to prevent diseases and improve health."*. Health literacy includes the ability of individuals to access accurate information and services and to use this information and service to improve both their own and public health (Kickbusch, Pelikan, Apfel, & Tsouros, 2013). Therefore, high health literacy is associated with the implementation of individual practices to prevent COVID-19 infection.

The COVID-19 pandemic is having a major impact on the capacity of health systems to deliver primary health care. While health systems around the world are struggling with increasing demand for care of COVID-19 patients, sustaining preventive and curative services is critical, especially for the most vulnerable groups, such as children, the elderly, those with chronic diseases, minorities, and the disabled. Since the emergence of the COVID-19 outbreak, people are more vulnerable to none communicable diseases and become severely ill or die from COVID-19 (WHO, 2020a).

Comprehensive measures are needed to prevent and control COVID-19 transmission in the community. Particular measures should be put into practice, especially for the elderly, healthcare professionals, and individuals who are susceptible to infection (European Centre for Disease Prevention and Control, 2020).

The current study aims to examine the anxiety level and COVID-19 awareness of patients who have visited the non-Covid-19 outpatient clinic of a pandemic hospital.

MATERIAL AND METHODS

This cross-sectional study was performed on patients who visited the orthopedics and traumatology outpatient clinic of a training and research hospital and who were examined by the same orthopedics and traumatology physician. The hospital is located in the Eastern Black Sea Region of Turkiye and the study was conducted between June 15, 2020 and October 15, 2020. During this period, the hospital served as a pandemic hospital for COVID-19.

Those patients over the age of 18, who were literate, who did not have any known psychiatric disorder, and who had voluntarily agreed to participate in this study were included in the scope of the study. Patients younger than 18 years of age, patients who had psychiatric disorders, and illiterate patients were excluded. The sample size was calculated with the sample calculation formula that is used in cases when the sample universe is uncertain (t:1.96, p:0.50, q:0.50, d:0.05). The resulting sample size was determined to be 210. This cross-sectional study complies with the principles of the Helsinki Declaration, and the study was also approved by the local ethics committee of Giresun University (Ethics Committee Number: 22.05.2020/15).

The data regarding the socio-demographic characteristics were obtained with a questionnaire form also including questions about COVID-19, which was created through a literature search (Wolf et al., 2020). The questionnaire included questions about the age, gender, and residency of the patients, in addition to questions asking the opinions of patients regarding COVID-19 infection, how it is transmitted, from which sources the patient obtains information about the infection, and protection measures for the infection.

The Beck Anxiety Scale (BAS) (Beck, Epstein, Brown, & Steer, 1988; Ulusoy, Şahin, & Erkmen, 1998) and Hospital Anxiety and Depression Scale (HADS) (Zigmond & Snaith, 1983; Aydemir, Güvenir, Küey, & Kültür, 1997) were also used for data collection. BAS was developed by Beck et al. (1988). Ulusoy et al. (1998) reported the validity and reliability of the scale for Turkish. BAS provides information about the anxiety and concerns that the individual has experienced during the previous week. The minimum and maximum scores that can be obtained from this scale (consisting of 21 questions) are 0 and 63, respectively (Beck et al., 1988; Ulusoy et al., 1998). Zigmond and Snaith (1093) developed HADS in 1983. It is a self-assessment scale regarding the anxiety and depression symptoms of patients undergoing hospital admission. The scale consists of 14 questions. Seven of the questions in the scale are about anxiety and the remaining seven are about depression. Aydemir et al. (1997) reported the validity and reliability of the scale for Turkish.

SPSS Software Package version 18 from IBM, which is based in the USA, was used for data analysis. Counts, percentages, independent groups t- test, and correlation analysis were used for statistical data analysis.

RESULTS

The data was collected from 210 patients who satisfied the requirements for the study and agreed to fill out the forms.

Patients younger than 18 years of age, patients who had psychiatric disorders, and illiterate patients were not included in the study. The questionnaire and scales were provided to 254 patients and responses from 210 of them were obtained. Therefore, the response rate was calculated as 82.7%. The socio-demographic characteristics of the patients are presented in Table 1. The mean age of the participants in this study was 44.41±16.9. 42.6% (n=89) of the participants were females and 53.6% (n=89) of them were living in the city. In this study, 41.0% (n=86) of the patients were high school graduates or had higher levels of education. 41.0% (n=86) of hospital visits took place through patients directly going to the hospital.

29.5% of the patients declared that they had researched their diseases on the internet before coming to the hospital, and the percentage of those who had visited the hospital for the same disease before was 52.4%. 32.9% of the participants stated that they were smokers. The percentage of the patients with alcohol use was 12.9%, and the percentage of those who stated that they had a nail-biting habit was 8.6%.

Table 1. Socio-demographic characteristics of the participants and their distribution according to some variables.

Characteristics		N	%
Age	18-64	179	85.3
	65↑	27	12.9
Gender	Female	89	42.6
	Male	120	57.4
Residency	Village	42	25.3
	Town	34	20.5
	City	89	53.6
Education	Secondary School and↓	101	48.1
	High School and↑	86	41.0
Method of making an appointment at the hospital	Directly and in person	86	41.0
	Central physician appointment system	42	20.0
Internet search of the current symptom	Yes	62	29.5
	No	146	69.5
Previous visits to the health institution	Yes	110	52.4
	No	93	44.3
Smoking	Yes	69	32.9
	No	135	64.3
Alcohol consumption	Yes	27	12.9
	No	167	79.5
Nail-biting	Yes	18	8.6
	No	181	86.2

A comparison of the BAS and HADS scores of the patients according to some variables is provided in Table 2. The mean BAS and HADS score of the patients was 7.92±13.3 and 13.03±6.57, respectively. An independent groups t-test was applied. Those patients aged 65 or over had significantly higher BAS scores than patients aged 64 and under ($p<0.001$). There was no significant difference in the HADS scores between these two groups ($p>0.05$).

The mean BAS and HADS scores of female patients were significantly higher than that of male patients ($p<0.001$).

The mean BAS score of the patients with an education level of secondary school or below was significantly higher than that of the patients with high school and above education ($p<0.001$). There was no significant difference between the mean HADS scores with respect to the education level ($p>0.05$).

Regarding the method of making an appointment at the hospital, there was no significant difference in the BAS scores of the patients who received an appointment remotely via the central physician appointment system and those who directly made an appointment at the hospital ($p>0.05$). In addition, HADS scores were also comparable between these two groups ($p>0.05$).

The mean BAS scores of smokers were significantly lower than that of the non-smokers ($p<0.01$). No significant difference was found between the mean HADS scores of smoking and non-smoking patients ($p>0.05$).

No significant difference was observed between the mean BAS and HADS scores among the patients who used alcohol and those who did not ($p>0.05$).

There was no significant difference between the BAS and HADS scores of the patients with and without a nail-biting habit ($p> 0.05$).

The knowledge and the attitudes of the patients regarding COVID-19 are presented in Table 3. 81.1% of the patients participating in this study considered COVID-19 infection to be a very severe public health problem. 51.9% of the patients who visited the hospital stated that they were a little worried about COVID-19 infection at the time of application, and the percentage of those who were very worried was 38.6%.

While 72.4% of the patients stated that COVID-19 was transmitted by the respiratory tract, 60.5% stated that it was transmitted by hands.

In this study, 81.4% of the patients stated that they obtained information about COVID-19 from television, 41.4% from social media, and 19.0% from announcements.

In order to protect themselves against COVID-19 infection, 78.1% of the patients stated that they used masks, 76.7% said that they stayed distant from other people, and 27.6% of them said that they prayed.

The findings showed that there was significant difference about the concern about COVID-19 infection at the hospital

Table 2. Comparison of the Anxiety (BAS) and Hospital Anxiety (HADS) levels of the patients participating in this study according to some variables.

Characteristics		BAS	p	HADS					
				total score	p	anxiety	p	depression	p
Age	64 age and↓	7.09 ±7.1	<0.001	12.97±6.5	>0.05	6.2±3.9	>0.05	6.6±3.6	>0.05
	65 age ↑	15.53 ±12.5		12.23±7.0		6.3±4.6		6.5±3.2	
Sex	Female	11.03±9.3	<0.001	14.55±6.6	<0.01	7.7±4.1	<0.001	6.7±3.4	>0.05
	Male	5.73±6.2		11.84±6.2		5.2±3.5		6.6±3.8	
Education	Secondary school and↓	10.40±9.4	<0.001	14.31±6.8	>0.05	7.5±4.3	<0.01	7.3±3.5	>0.05
	High School and ↑	6.17 ±6.7		12.04±6.4		5.8±3.7		6.2±3.7	
Method of making an appointment at the hospital	Directly and in person	7.52±8.8	>0.05	12.36±6.61	>0.05	5.9±3.7	>0.05	6.4±3.9	>0.05
	Central physician appointment system	9.48±7.3		4.41±7.7		7.0±4.9		7.1±3.6	
Internet search of the current symptom	Yes	7.97±7.5	>0.05	11.90±6.0	>0.05	5.9±3.5	>0.05	6.0±3.5	>0.05
	No	7.89±8.3		13.55±6.7		6.5±4.2		6.9±3.6	
Smoking	Yes	6.34±5.9	<0.01	12.60 ±6.6	>0.05	6.1±4.1	>0.05	6.4±3.4	>0.05
	No	8.57±8.8		13.50 ±6.4		6.3±3.5		7.2±3.9	
Alcohol consumption	Yes	12.9±6.5	>0.05	7.96±8.3	>0.05	6.0±3.6	>0.05		>0.05
	No	13.2±6.7		7.61±7.1		6.2±4.0			
Nail-biting	Yes	8.40±8.2	>0.05	12.64±5.3	>0.05	6.0±3.6	>0.05	7.0±3.7	>0.05
	No	6.33±6.8		13.15±6.7		6.3±4.0		6.7±3.6	
Place of residence	Urban	7.6±7.5	>0.05	12.9±6.3	>0.05	5.9±3.9	>0.05	6.6±3.5	>0.05
	Suburban and Rural	8.2±9.5		12.4±3.7		6.3±3.7		6.5±3.8	

between the age groups of 64 and lower, and of 65 and above (p<0.05). There was no significant difference between the education levels of the patients with respect to the concern about COVID-19 infection at the hospital (p>0.05).

While 29.5% of the patients stated that they were very worried about visiting the hospital because of COVID-19 infection, 53.8% of patients expressed that they were a little worried. With respect to age, there was no significant relationship between the levels of worry due to COVID-19 in hospital admittance (p>0.05). However, there was a significant relationship between the genders and alcohol use in the level of worry about COVID-19 (p<0.05). Additionally, the worry levels were significantly higher among patients with nail-biting habits (p<0.05).

There was a significant negative correlation between the HADS of the patients and the anxiety which they felt due to COVID-19 because of coming to the hospital (p<0.05). There was a negative correlation between the HADS score and the worry about getting COVID-19 infection. As the HADS score increases, the worry about getting COVID-19 infection decreases (p<0.05).

The percentage of those patients who thought they would definitely get COVID-19 infection was 29.5%, while the percentage of those who stated they would most likely get the infection was 53.8%. Female patients were more prone to think that they would get COVID-19 infection than the male patients (p<0.05). No significant difference was found regarding age, education level, smoking, alcohol use, and nail-biting habits between the method of making an appointment at the hospital and thinking that they might get a COVID-19 infection (p>0.05).

DISCUSSION

COVID-19 infection is known to spread faster among individuals (with respiratory droplets and aerosols) who stay in the same place with others who are infected, especially in crowded and poorly ventilated areas. Therefore, it is critical to take the necessary measures. The measures for protection from COVID-19 infection require self-responsibility, such as physi-

Table 3. Knowledge and attitudes of the patients participating in this study about the COVID-19 infection/ outbreak.

Characteristics		N	%
How severe is COVID-19 as a public health problem?	Mild	2	1.0
	Moderate	14	6.7
	Very Severe	170	81.0
How worried are you about COVID-19 infecting you?	I'm not worried	12	5.7
	I'm a little worried	109	51.9
	I am very worried	81	38.6
Were you worried about COVID-19 on your way to the hospital?	Never worried	20	9.5
	A little worried	113	53.8
	I was overly worried	62	29.5
Do you think you will get sick due to the COVID-19 outbreak?	Impossible	20	9.5
	Most probably	113	53.8
	Absolutely	62	29.5
How is COVID-19 infection transmitted?	By inhalation	152	72.4
	With body fluids	31	14.8
	With hands	127	60.5
Where did you obtain information on COVID-19 infection?	Television	171	81.4
	Social media	87	41.4
	Health personnel	81	38.6
	Announcement	40	19.0
What are the precautions you take against COVID-19 infection?	Using a mask	164	78.1
	Distance from people	161	76.7
	Washing hands	158	75.2
	Paying attention to nutrition	98	46.7
	Praying	58	27.6
	Not smoking	53	25.2

cal distance, wearing a mask, ventilating rooms, and avoiding crowds. Hence, not going to hospitals in non-emergency situations is among the individual measures.

Hospitals in Türkiye provide patients with two options to make an appointment for examination in a hospital. One of them is the central physician appointment system, which can be accessed via telephone or internet (i.e., remotely) before coming to the hospital for examination. The other choice for patients is a direct hospital appointment, where the patient has to come to the hospital in this case. Making an appointment remotely/previously via telephone or internet provides minimal time spent in the hospital, while those patients who make the appointment directly at the hospital spend more time in the hospital for examination. In this study, only 20% of the patients stated that they made their hospital appointments via the central physician appointment system. The patients who make a hospital appointment directly and in person are more likely to get a COVID-19 infection. The COVID-19 pandemic demonstrated the importance of digital health solutions during the crisis and the need to further align digital health initiatives in the future, both of which facilitates the lives of people on the front lines of the crisis (Fagherazzi, Goetzinger, Rashid, Aguayo, & Huiart, 2020). Digital technologies can facilitate the provision of healthcare services during the pandemic (WHO, 2021b; Golinelli, 2020).

The mean BAS scores of the patients were low. However, the BAS scores of those women who were at an age of 65 years or above, those who have secondary school education or below, and those who do not smoke were significantly higher. Similar to our study, in Hyland et al.'s (2020) study, the anxiety level of individuals aged 65 and over was higher than those of individuals at younger ages. This may be because COVID-19 is a severe infection and the mortality rate is higher among individuals aged 65 and over. In a related study by Özdin et al. (2020) on anxiety and depression levels during the pandemic, it was also found that women are psychologically more affected by the pandemic. The mean of the HADS scores given in Özdin et al. (2020) was lower than the corresponding mean HADS score that we observed in our study.

The HADS scores of patients were high. While there was no significant difference between the HADS scores considering other

variables, the HADS scores of the female patients were significantly higher than that of males. Similar to our results, in the study conducted by Argüder et al. (2020), the HADS scores due to COVID-19 were significantly higher among female patients.

While 81.0% of the patients participating in this study stated that they considered COVID-19 to be a very severe public health problem, 8.7% described it as a mild or moderate public health problem. In our study, no significant relationship was found between the ages of the patients and the perception of COVID-19 as a threat ($p<0.05$). Gesser-Edelsburg et al. (2020) suggested that participants aged 65 and over perceived the COVID-19 pandemic as a greater risk than those at younger ages.

In our study, the percentage of those patients who indicated that they were a little worried about the COVID-19 infection was 51.9%. More than half of the patients did not worry too much. Anxiety about getting COVID-19 differs significantly among the gender groups. A positive correlation was found between gender and worry. In Wolf et al.'s (2020) study, which is consistent with our study, it was reported that women were more concerned about the transmission of the disease than men.

COVID-19 is transmitted by close contact with the aerosols, saliva droplets, and extracts that the infected person exhales to the mouths, noses, or eyes of other people. Hands that are exposed to the droplets of infected people may cause contamination when in contact with the mouth, nose, and eyes (WHO, 2020b). In our study, the percentage of patients who stated that COVID-19 was transmitted by the respiratory tract was 72.4%. Almost one-third of the patients did not agree that it was transmitted through the respiratory tract. WHO recommends complying with physical distance, wearing masks, ventilating your environment, keeping your hands clean, and avoiding crowds in order to be protected from COVID-19 (WHO, 2021a). Although hands play an important role in transmission, 39.5% of the patients did not indicate that hands were one of the ways of COVID-19 transmission. In our study, the percentages of the participants who employed precautions such as mask-wearing, social distancing, and hand-washing against COVID-19 transmission were high. These results were in line with other related studies conducted in other countries (Alsaif et al., 2021).

The limitations of the study include the following: the research was conducted in a single clinic and the participating patients had limited time since they were waiting for examination. The study could be performed on more patients who visit different clinics.

CONCLUSION

It has been determined in this study that individuals mostly between the ages of 20 and 64 go to the hospital and half of the patients live in the city center. Both Beck Anxiety Scale and Hospital Anxiety and Depression Scale scores were significantly higher for female patients. Among the findings of the study, it is thought-provoking that one-third of the patients were not aware that COVID-19 is transmitted by the respiratory tract, and one-fifth did not include using a mask among the methods of protection. The BAS scores of those women who were at an age of 65 years or above and those who had secondary school education or below were significantly higher. Therefore, it is recommended that COVID-19-related research focusing on these groups in particular should be conducted in the future.

Ethics Committee Approval: This cross-sectional study complies with the principles of the Helsinki Declaration, and the study was also approved by the local ethics committee of Giresun University (Ethics Committee Number: 22.05.2020/15).

Peer-review: Externally peer-reviewed.

Informed Consent: Written consent was obtained from the participants.

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REFERENCES

- Alsaif, B., Elhassan, N. E. E., Itumalla, R., Ali, K. E., & Alzain, M. A. (2021). Assessing the level of awareness of covid-19 and prevalence of general anxiety disorder among the hail community, Kingdom of Saudi Arabia. *International Journal of Environmental Research and Public Health*, *18*(13), 7035.
- Argüder, E., Kılıç, H., Civak, M., Kacar, D., Kay, G., Yılmaz, A. … Karalezli, A. (2020). Anxiety and depression levels in hospitalized patients due to Covid-19 infection. *Ankara Medical Journal, 20*(4), 971–981. http://dx.doi.org/10.5505/amj.2020.80775.
- Aydemir, Ö., Güvenir, T., Küey, L., & Kültür, S. (1997). Hastane anksiyete ve depresyon ölçeği Türkçe formunun geçerlilik ve güvenilirliği [Reliability and validity of the Turkish version of Hospital Anxiety and Depression Scale]. *Türk Psikiyatri Dergisi, 8*(4), 280–287.
- Beck, A. T., Epstein, N., Brown, G., & Steer, R. A. (1988). An inventory for measuring clinical anxiety: Psychometric properties. *Journal of Consulting and Clinical Psychology, 56*(6), 893–897.
- Centers for Disease Control and Prevention. (2021, November 29). How to protect yourself & others. Retrieved from https://www.cdc.gov/coronavirus/2019-ncov/prevent-getting-sick/prevention.html
- European Centre for Disease Prevention and Control. (2020, November 6). Infection prevention and control and preparedness for COVID-19 in healthcare settings. Retrieved from https://www.ecdc.europa.eu/sites/default/files/documents/Infection-prevention-and-control-in-healthcare-settings-COVID-19_5th_update.pdf
- Fagherazzi, G., Goetzinger, C., Rashid, M. A., Aguayo, G. A., & Huiart, L. (2020). Digital health strategies to fight COVID-19 worldwide: Challenges, recommendations, and a call for papers. *Journal of Medical Internet Research, 22*(6), e19284.
- Gesser-Edelsburg, A., Cohen, R., Hijazi, R., & Abed Elhadi Shahbari, N. (2020). Analysis of public perception of the Israeli Government's early emergency instructions regarding COVID-19: Online survey study. *Journal of Medical Internet Research, 22*(5), e19370.
- Golinelli, D., Boetto, E., Carullo, G., Nuzzolese, A. G., Landini, M. P., & Fantini, M. P. (2020). Adoption of digital technologies in health care during the Covid-19 pandemic: Systematic review of early scientific literature. *Journal of Medical Internet Research, 22*(11), e22280.

- Hyland, P., Shevlin, M., McBride, O., Murphy, J., Karatzias, T., Bentall, R. P. … Vallières, F. (2020). Anxiety and depression in the Republic of Ireland during the COVID-19 pandemic. *Acta Psychiatrica Scandinavica, 142*(3), 249–256.
- Kickbusch, I., Pelikan, J. M., Apfel, F., & Tsouros, A. D. (Eds.). (2013). *Health literacy: The solid facts*. World Health Organization.
- Koley, T. K., & Dhole, M. (2020). *The COVID-19 pandemic: The deadly coronavirus outbreak*. Taylor & Francis.
- Özdin, S., & Bayrak Özdin, Ş. (2020). Levels and predictors of anxiety, depression and health anxiety during COVID-19 pandemic in Turkish society: The importance of gender. *International Journal of Social Psychiatry*, 66(5), 504-511.
- Ulusoy, M., Şahin, N., & Erkmen, H. (1998). Turkish version of beck anxiety inventory: Psychometric properties. *Journal of Cognitive Psychotherapy: An international Quaterly, 12*(2), 163–72.
- WHO. (2020a, June 1). Maintaining essential health services during the COVID-19 outbreak. Retrieved from https://www.who.int/emergencies/diseases/novel-coronavirus-2019/related-health-issues
- WHO. (2020b, June 1). Coronavirus disease (COVID-19): How is it transmitted?. Retrieved from https://www.who.int/emergencies/diseases/novel-coronavirus-2019/question-and-answers-hub/q-a-detail/coronavirus-disease-covid-19-how-is-it-transmitted
- WHO. (2021a, May 13). Coronavirus disease (COVID-19). Retrieved from https://www.who.int/emergencies/diseases/novel-coronavirus-2019/question-and-answers-hub/q-a-detail/coronavirus-disease-covid-19
- WHO. (2021b, October 1) Advice for the public: Coronavirus disease (COVID-19). Retrieved from https://www.who.int/emergencies/diseases/novel-coronavirus-2019/advice-for-public
- WHO. (2022, April 13). WHO Coronavirus (COVID-19) dashboard. Retrieved from https://covid19.who.int/
- Wolf, M. S., Serper, M., Opsasnick, L., O'Conor, R. M., Curtis, L., Benavente, J. Y. … Bailey, S. C. (2020). Awareness, attitudes, and actions related to COVID-19 among adults with chronic conditions at the onset of the U.S. outbreak: A cross-sectional survey. *Annals of Internal Medicine, 173*(2), 100–109.
- Zigmond, A. S., & Snaith, R. P. (1983). The hospital anxiety and depression scale. *Acta Psychiatrica Scandinavica, 67*(6), 361–370.

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Original Article

Are community pharmacies the best purchasing channel for cosmetic products? Prioritization of consumer preferences via analytical hierarchy process

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ABSTRACT

Background and Aims: Many different purchasing channels play an essential role in meeting cosmetic product demand worldwide. These include supermarkets, cosmetic markets, community pharmacies, beauty salons, internet retailing, and shopping complexes. This study's main objective is to prioritize consumers' cosmetic product purchasing channel preferences. The originality of this study lies in being the first study that addressed customers' choice of cosmetic product purchasing channels via the Analytical Hierarchy Process (AHP).
Methods: Firstly, a questionnaire was conducted (n=287) to determine cosmetic product purchasing channel selection criteria. Cosmetic product purchasing channel alternatives were prioritized among the first questionnaire results via the AHP with 12 consumers who reside in the city center of Van, Turkey and buy at least two cosmetic products per year.
Results: As a result of the study, community pharmacies (46.9%) were found to be the best alternative among the cosmetic product purchasing channels, followed by cosmetics stores (24.1%). The most important criterion affecting the selection of cosmetic product purchasing channels is satisfaction with the consultancy (25.2%), followed by advice from health care providers (22.1%).
Conclusion: It has been observed that consumers prefer community pharmacies more than other purchasing channels when purchasing cosmetic products. Understanding customer needs, expectations, and experiences is vital for optimizing the quality of the offered service. Thus, pharmacies with a significant market share in the cosmetics sector can further their cosmetics services by considering the consumer demands highlighted in this study.

Keywords: Analytical hierarchy process, Community pharmacy, Cosmetics, Cosmetic product purchasing channel

INTRODUCTION

Nowadays, individuals who pay attention to their physical appearance take on certain costs in order to look more beautiful, be liked, and follow innovations. Recognizing this potential, the cosmetics sector is trying to respond to consumers' demands with different product types and innovations every day. Many purchasing channels, such as beauty centers, cosmetics stores, the internet, and community pharmacies, meet the demand for cosmetic products and services. Especially in today's market conditions, where a focus on the beneficiary is at the forefront, cosmetic products and service providers must manage the purchasing process to contribute to making a profit while responding to beneficiaries' demands.

Today, the cosmetics industry shows its presence worldwide. It continues to develop day by day due to positive developments in living conditions and increased interest in and awareness about using cosmetic products and appearance. Simultaneously, individu-

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als' desire to attain these products reliably, efficiently, comfortably, and quickly has led companies to develop new marketing methods to reach consumers quickly and effectively.

The increase in demand for cosmetics and personal care products has encouraged cosmetics companies to increase their meeting points with consumers. Accordingly, the multichannel retail environment has developed over the years in the cosmetics sector, as in most sectors. Many different purchasing channels, such as supermarkets, cosmetics markets, community pharmacies, beauty salons, internet retailing, and shopping centers play an essential role in meeting cosmetics demand worldwide. It is similar in Turkey, too. Especially in recent years, an increasing number of personal care stores are becoming points where consumers can buy these products in Turkey (Agcadağ, 2017; Durmaz & Bahar, 2011). In addition, virtual (electronic) shopping, which offers consumers a different shopping environment than traditional shopping habits, is a new purchasing channel selected by many consumers (Saydan, 2008). However, it is observed that consumers are hesitant to purchase some product groups using this purchasing channel. Consumers think it would be risky to buy perfumes and personal care products on the internet that they have not used before and have not had the opportunity to experience personally. Also, virtual shopping lacks factors such as obtaining information and advice from experts on the product, testing the product, and performing skin analysis, which reduces consumers' interest in purchasing these product groups online (Cosmetics Europe, 2016).

Faced with various stimulants, the consumer is influenced by personal and environmental factors and reacts to the stimulus (or stimulants). Veuphuteh (2018) defined the variables that affect the consumer's purchasing decision process as psychological variables, socio-cultural variables, demographic variables, effects of marketing efforts, and situational effects. This situation also affects the cosmetic product purchasing behavior of individuals.

Özden, Saygılı, & Sütütemiz (2019) revealed that health awareness is effective in consumers' cosmetic product preference by evaluating many issues, such as the frequency of cosmetic product use, health awareness level, place of purchase, information sources on product groups, and packaging preference. Chen & Chen (2011) put forth factors such as brand image, sellers' experiences, customer relations, and customer satisfaction that affect consumers' intention to buy cosmetic products. Dapiapis Toros (2016) investigated the factors that impact consumers' cosmetic products purchasing decisions. The finding was that brand recognition and friends' advice are the most effective and that magazine, newspaper, and radio advertisements are the least effective.

According to Villi & Kayabaşı (2013), the store atmosphere, friends, moods, and promotions affect women's cosmetic purchase behavior. Yalçın & Gülsün (2020) evaluated five main criteria and 15 sub-criteria related to cosmetic product purchasing behavior via multi-criteria decision-making methods. They put forth that price, promotion, and quality are critical factors for Turkish women purchasing a cosmetic product. Lu & Liu (2018) examined women's preferences for information channels about cosmetic products. It was found that young women prefer online purchasing channels, and connected with this, they provide information about cosmetic products via social platforms and the internet. In contrast, older women choose physical stores more, and they prefer to get information face to face.

Kirby (2014) reported that the consumers make their choices considering reasons such as the store having too many brands together, relaxing atmosphere of the store, the store offering products with samples and gifts, and the store offering price discounts and installment opportunities. In the study conducted by Desai (2014), product quality was found to be more important than the product price on consumers' choice of cosmetic products. Accordingly, it was seen that the majority of the participants preferred to purchase products from stores that offer quality products at affordable prices. Also, medical and cosmetics stores were the purchasing channels that are mainly selected.

The fact that, pharmacists' consultancy services are not only pre-sales but also continue after-sales puts pharmacies ahead of other cosmetic product purchasing channels. Considering today's economic conditions, it should be noted that pharmacists' interest in the cosmetics sector is increasing. For pharmacists to protect their assets and improve their activities, issues such as selecting the products and services to be offered in the pharmacy, choosing the pharmacy location, and who the stakeholders are should be planned correctly. In this context, it is of great importance that pharmacists who decide to offer cosmetic product consultancy include more than one criteria in the decision process, taking into account the expectations of the beneficiaries as well as time, money, and similar criteria in light of reliable, scientific estimates. In summary, decision-making processes play a vital role in the pharmacy profession, as in every business field.

As can be seen from the studies mentioned above, factors affecting consumer preferences in cosmetic products and cosmetic product purchasing channels (CPPCs) have been evaluated. Still, to the best of the authors' knowledge, no study has addressed customers' choice of CPPC via the Analytical Hierarchy Process (AHP) approach. AHP is a well-known multi-criteria decision-making technique developed by Thomas L. Saaty in the 1970s to determine the relative weights of enabling factors via pair-wise comparisons.

In this context, the study's motivation comes from prioritizing customers' CPPC selection according to determined selection criteria. In addition, the study aims to guide pharmacists who provide or plan to provide cosmetic services by revealing what criteria the consumers of cosmetic products consider when choosing purchasing channels.

The remaining part of this paper discusses CPPC selection parameters based on questionnaire results and applies the AHP to the problem at hand, followed by the conclusion.

MATERIAL AND METHODS

This study was carried out in two phases: (i) determination of CPPC selection criteria through a questionnaire, and (ii) prioritizing CPCC alternatives in line with the results of the first questionnaire via the AHP. According to Saaty (1980), the flow diagram followed in the application of this study is given in Figure 1.

Data collection and sample size

The study was conducted in accordance with the Turkish Republic Ministry of Health, Van Provincial Health Directorate's permission number 73040253-044-E.439, the Van-Bitlis-Hakkari Chamber of Pharmacists' decision number 2019/1626, and the World Medical Association (WMA) Declaration of Helsinki Ethical Principles in Medical Research on Human Volunteers.

This study's population consists of male and female individuals over the age of 18 and under the age of 65 who reside in the city center of Van, in the eastern part of Turkey, and buy at least two cosmetic products per year. The sample size was calculated for the criteria determination questionnaire, taking the 95% confidence level and the sampling error as 0.1. The minimum sample size to be reached was calculated as 96. To increase the reliability of the results, the researchers tried to get the maximum number of individuals that could be achieved, and 287 individuals participated in the criteria determination questionnaire between 04.03.2020-15.06.2020. The questionnaires were administered to randomly selected volunteers who wanted to participate in the study in the city center using the face-to-face questionnaire technique, after getting written consent. In addition, to increase the number of participants, the questionnaires were also administered via the internet using the snowball technique.

According to Schmidt, Aumann, Hollander, Damm & von der Schulenburg (2015) and Baby (2013), there is no precise method for determining sample size in AHP studies; large sample sizes are generally not needed. Therefore, 12 consumers shopping via determined alternative CPPC were selected as decision-makers to make pair-wise comparisons in the second phase. These comparisons were conducted face-to-face from20.06.2020-30.06.2020.

Determining the CPPC selection criteria

Many factors are involved in the selection of a CPPC. Based on the researchers' experience and literature review (Villi & Kayabaşı, 2013; Özden, Saygılı, & Sütütemiz, 2019), 22 criteria were determined. The criteria were divided into three groups: (i) 8 criteria related to the purchasing channel, (ii) 3 criteria related to consulting, and (iii) 11 criteria related to the promotion. Participants were asked to score these criteria from 1 (less important) to 5 (very important). The averages of the points given for each criterion were calculated, and criteria with an average score below 3.5 points were eliminated or combined.

Determining the CPPC alternatives

In light of the structure of the cosmetics market in Turkey and the relevant literature, CPPC alternatives were identified as (i) pharmacies, (ii) cosmetics stores, (iii) supermarkets, (iv) internet stores, and (v) others.

Pair-wise comparisons

To calculate relative importance values of the criteria and alternatives, participants were asked to evaluate criteria for the second phase of the study, i.e., how much a criterion is preferable to another criterion. Saaty's priority (importance) scale was used (Table 1) to conduct pair-wise comparisons.

Table 1. Saaty's importance scale.

Importance level	Definition
1	Equal importance
3	Moderately more important than one another
5	Strong importance
7	Very strong importance
9	Extreme importance
2,4,6,8	Intermediate / average values

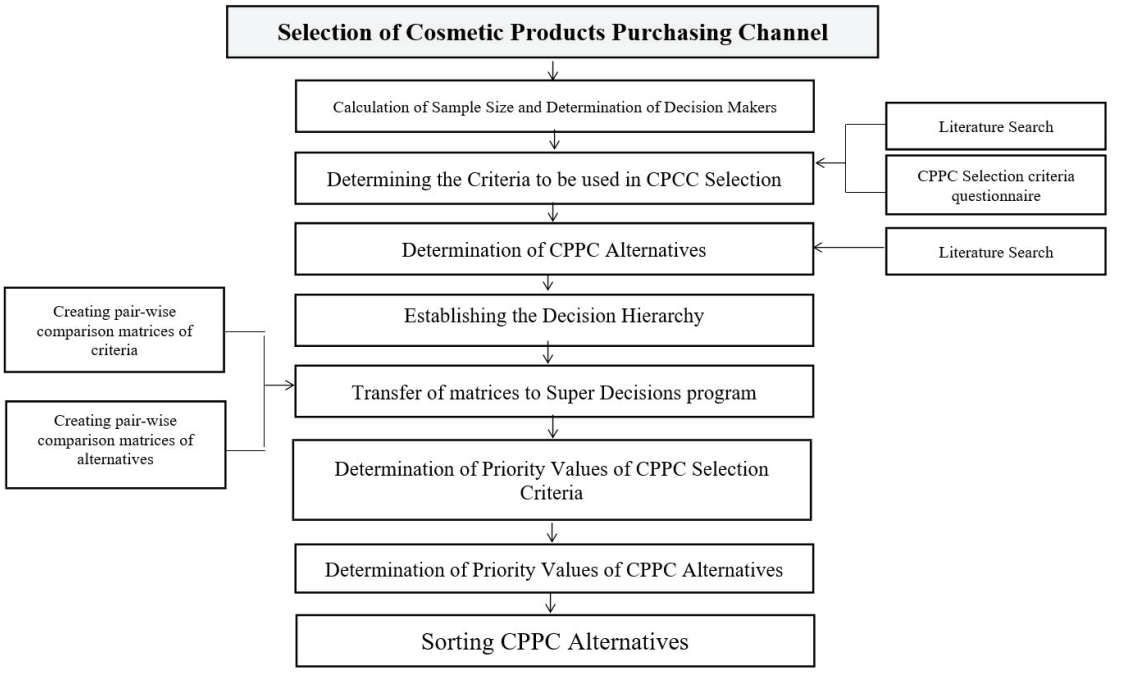

Figure 1. The flow diagram of the application.

Data analysis

The data obtained from the criteria determination questionnaire were subjected to descriptive statistical analysis via the SPSS 22.0 (IBM Electronics, USA) package program. The Super Decisions 3.2.0 package program was used to analyze paired comparison matrices obtained in the second phase of the study to determine the relative weight of criteria, alternatives, and final weights. Finally, sensitivity analysis was performed to analyze the flexibility of the final decision.

RESULTS

A total of 287 people participated in the criteria determination questionnaire applied within the scope of this study. 188 of them were women, and 99 of them were men. The average age of the participants was 25.44, and 82% of them were university graduates. Average points of the criteria are given in Table 2.

From Table 2, it is seen that the average of 13 criteria is above 3.5. In the AHP, the number of pair-wise comparisons increases with the number of criteria/alternatives (Ishizaka, Pearman & Nemery, 2012), and a higher number of them can cause complexity. Saaty (1980) recommends choosing a maximum of 7 ± 2 criteria/alternatives to avoid this situation. These 13 criteria were considered for pair-wise comparisons, and some of them were combined with decreasing the number.

Table 2. Average points of the criteria.

	Average points
Criteria related to the purchasing channel	
The reliability of the products sold	4.293
The quality of the products sold	4.328
Brand diversity	3.565
Wide product range	3.575
Selling organic/natural products	3.930
Selling magistral products	2.794
The design of the store	2.923
Accessibility of the store	3.631
Criteria related to consulting	
Providing additional services	3.450
Satisfaction from consulting	3.721
Providing free skin/hair care	3.370
Criteria related to promotion	
TV commercials	2.686
Advice from others	3.317
Advice from health care providers	3.830
Social media promotions	2.826
Price	3.770
Promotions provided	3.875
Presentation of giveaways	3.540
Possibility ofshopping over the Internet	3.289
Conditioned promotions	2.878
Possibility ofexchanging the product	3.819
Opportunity totry the product	3.794

It is seen that the criterion with the highest average is "The quality of the products sold," followed by "The reliability of the products sold." These two criteria are combined into "The quality and reliability of the products sold," because the mean of these two criteria are very close to each other and the criteria are related. The average value for the criteria for brand diversity, wide product range, and presentation of natural/organic products is higher than 3.5, and the values are close to each other, too. For the second phase of the study, these three criteria were combined and named "Product diversity (brand diversity, organic/natural product presentation, etc.)." Lastly, among the criteria related to the purchasing channel, the average of the "The accessibility of the store" criterion was above 3.5, and this criterion took place in the second phase.

According to Table 5, as the average of the "Satisfaction from consulting" criterion is above 3.5, this criterion was considered in the second phase.

The averages of price, discounts, and giveaways criteria, under the criteria for promotion, are above 3.5. Hence, for the second phase of the study, these criteria were combined into "price/promotions" to decrease the number of the criteria. The product trial and product exchange criteria averages were also high, and were incorporated into "Opportunity to try and exchange products." Additionally, the "Advice from health care providers" criterion was included in the second phase due to its average. As a result, seven criteria were determined for the paired comparison questionnaire.

The paired comparison questionnaire will be evaluated in the study's second phase. Firstly, the hierarchical structure with seven criteria and five alternatives was created (Figure 2).

After the hierarchical structure was created, the geometric averages of the responses of the 12 participants for the criteria comparison matrix were taken and rounded to the nearest integer before being transferred to the Super Decisions 3.2.0 Program (Table 3).

To make the matrix given in Table 3 more understandable, "The quality and reliability of the products sold" is six times more preferable than "Price/promotions." "Advice from health care providers" is two times more preferable than "The quality and reliability of the products sold." In addition, "The quality and reliability of the products sold" and "Satisfaction from consulting" have the same preference level.

The result of the criteria comparison is that the most crucial criterion affecting the selection of CPPC of the participants is "satisfaction with the consultancy" (25.2%); this criterion is followed by "advice from health care providers" (22.1%), "quality and reliability of the products sold" (18.3%), and "opportunity to try and exchange the products" (15.4%). The minor effective criteria are found to be price/promotions (8.1%), product diversity (6.4%), and accessibility of the store (4.5%). The inconsistency ratio of the analysis was calculated as 0.084. This value

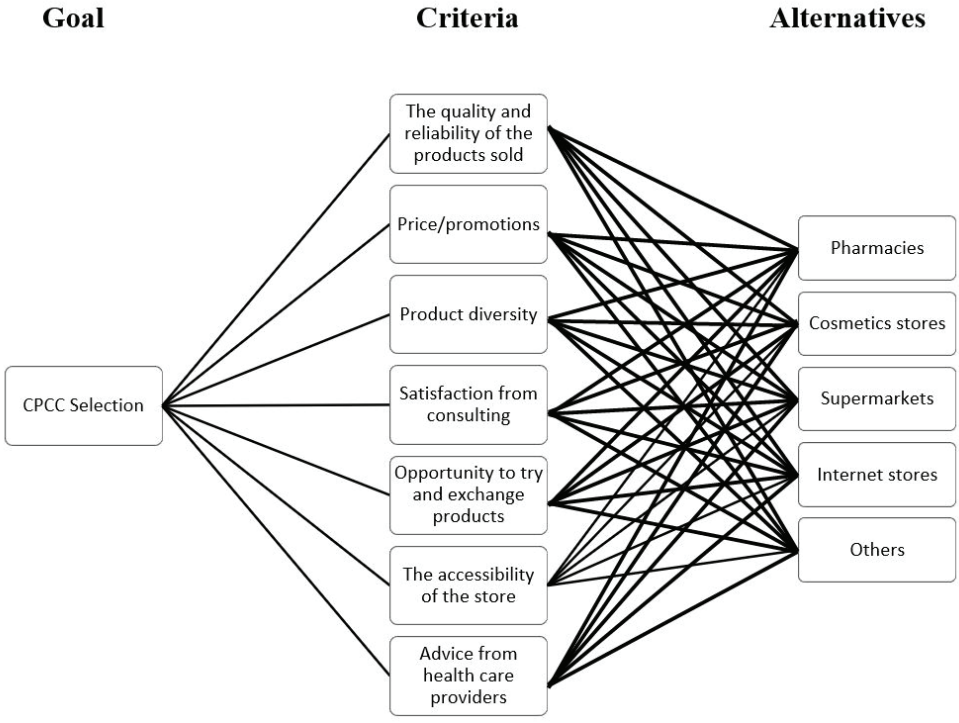

Figure 2. AHP hierarchy framework.

Table 3. Pair-wise comparison matrix for criteria.

Criteria	The quality and reliability of the products sold	Price/promotions	Product diversity	Satisfaction from consulting	Opportunity to try and exchange products	The accessibility of the store	Advice from health care providers
The quality and reliability of the products sold	1	6	2	1	1	2	½
Price/promotions	1/6	1	2	1/5	½	4	1/3
Product diversity	½	½	1	1/5	1/3	3	¼
Satisfaction from consulting	1	1/5	5	1	1	4	2
Opportunity to try and exchange products	1	2	3	1	1	3	½
The accessibility of the store	½	¼	1/3	¼	1/3	1	¼
Advice from health care providers	2	3	4	½	2	4	1

being below 0.1 shows that the comparisons made by the participants are consistent (Saaty, 1980).

In the last analysis stage, the alternatives were evaluated concerning each criterion, similar to the paired comparison of criteria. Firstly, prioritization between alternatives concerning the "The quality and reliability of the products sold" criterion was done (Table 4).

The inconsistency ratio of the analysis was calculated as 0.09265. According to calculated relative weights (0.618, 0.223, 0.037, 0.073, 0.049) based on Table 4, pharmacies were deter-

mined to be the best alternative for this criterion. A pair-wise comparison of alternatives based on the "Price/promotions" criterion is given in Table 5.

The inconsistency ratio of the analysis was calculated as 0.01656. According to calculated relative weights (0.056, 0.340, 0.110, 0.319, 0.176) based on Table 5, cosmetics stores were determined to be the best alternative for this criterion. A result of prioritization between alternatives concerning the "Product diversity" criterion is presented in Table 6.

The inconsistency ratio of the analysis was calculated as 0.09275. Due to the calculated relative weights (0.463, 0.128, 0.032, 0.244, 0.176) based on Table 6, cosmetics stores were determined to be the best alternative for this criterion. A pair-wise comparison of alternatives based on the "Satisfaction from consulting" criterion is presented in Table 7.

The inconsistency ratio of the analysis was calculated as 0.08414. According to calculated relative weights (0.617, 0.221, 0.062, 0.039, 0.062) based on Table 7, pharmacies were determined to be the best alternative for this criterion. The pair-wise comparison of alternatives based on the "Opportunity to try and exchange products" criterion is shown in Table 8.

The inconsistency ratio of the analysis was calculated as 0.0492. Relative weights of criteria are 0.122, 0.416, 0.171, 0.169, and 0.122. Accordingly, cosmetics stores were found to be the best. A pair-wise comparison of alternatives based on the "Advice from health care providers" criterion is presented in Table 9.

Table 4. Pair-wise comparison matrix for the "The quality and reliability of the products sold" criterion.

The quality and reliability of the products sold	**Pharmacies**	**Cosmetics stores**	**Supermarkets**	**Internet stores**	**Others**
Pharmacies	1	6	8	9	9
Cosmetics stores	1/6	1	6	5	6
Supermarkets	1/8	1/6	1	1/3	½
Internet stores	1/9	1/5	3	1	2

Table 5. Pair-wise comparison matrix for the "Price/promotions" criterion.

Price/promotions	**Pharmacies**	**Cosmetics stores**	**Supermarkets**	**Internet stores**	**Others**
Pharmacies	1	1/5	1/3	1/5	1/3
Cosmetics stores	5	1	4	1	2
Supermarkets	3	¼	1	1/3	½
Internet stores	5	1	3	1	2
Others	3	½	2	½	1

Table 6. Pair-wise comparison matrix for the "Product diversity" criterion.

Product diversity	**Pharmacies**	**Cosmetics stores**	**Supermarkets**	**Internet stores**	**Others**
Pharmacies	1	5	8	2	5
Cosmetics stores	1/5	1	9	½	½
Supermarkets	1/8	1/9	1	1/6	1/5
Internet stores	½	2	6	1	3
Others	1/5	2	5	1/3	1

Table 7. Pair-wise comparison matrix for the "Satisfaction from consulting" criterion.

Satisfaction from consulting	**Pharmacies**	**Cosmetics stores**	**Supermarkets**	**Internet stores**	**Others**
Pharmacies	1	7	7	8	7
Cosmetics stores	1/7	1	5	7	5
Supermarkets	1/7	1/5	1	2	1
Internet stores	1/8	1/7	½	1	½
Others	1/7	1/5	1	2	1

The inconsistency ratio of the analysis was calculated as 0.07267. According to calculated relative weights (0.638, 0.184 0.092, 0.044, 0.042) based on Table 9, pharmacies were determined to be the best alternative.

Lastly, the final weight was obtained by combining relative weights via Super Decisions 3.2.0. The program output, including priority values obtained from the analysis, is given in Figure 3.

In light of the data presented in Figure 3, it was determined that pharmacies (46.9%) were the best alternative among the cosmetic product purchasing channels, in line with the criteria discussed in the study, followed by cosmetics stores (24.1%). It was determined that the importance of supermarkets, internet stores, and other alternatives (beauty centers, herbalists, etc.) is around 10%.

In AHP, a sensitivity analysis should be done to see how the changes in the criteria weights will affect the results. Therefore, sensitivity analyses were performed for each criterion by changing criteria weights via Super Decisions 3.2.0. It was determined that prioritization of purchasing channel alternatives does not affect the change in criterion weights in general.

DISCUSSION

Within the scope of this study, the cosmetic product purchasing channel preferences of cosmetic consumers in the province of Van were discussed. In this context, the AHP method, which is frequently used in such real-life problems where more than one decision variable is involved, was used. Although there are many studies in the literature on consumers' choice of cosmetic products, the number of studies dealing with the choice of cosmetic product purchasing channel is quite limited.

As a result of the criterion determination survey, it was determined that the factors that most affect the choice of cosmetic product purchasing channel are the reliability and quality of

Table 8. Pair-wise comparison matrix for the "Opportunity to try and exchange products" criterion.

Opportunity to try and exchange products	Pharmacies	Cosmetics stores	Supermarkets	Internet stores	Others
Pharmacies	1	1/3	1	½	1
Cosmetics stores	3	1	3	3	3
Supermarkets	1	1/3	1	2	1
Internet stores	2	1/3	½	1	2
Others	1	1/3	1	½	1

Table 9. Pair-wise comparison matrix for the "Advice from health care providers" criterion.

Advice from health care providers	Pharmacies	Cosmetics stores	Supermarkets	Internet stores	Others
Pharmacies	1	7	8	8	9
Cosmetics stores	1/7	1	3	5	5
Supermarkets	1/8	1/3	1	3	3
Internet stores	1/8	1/5	1/3	1	1
Others	1/9	1/5	1/3	1	1

Name	Normalized by cluster	Limiting
The quality and reliability of the products sold	0.18257	0.091284
Price/promotions	0.08084	0.040418
Product diversity	0.06357	0.031786
Satisfaction from consulting	0.25186	0.125931
Opportunity to try and exchange products	0.15464	0.077322
The accessibility of the store	0.04527	0.022637
Advice from health care providers	0.22124	0.110621
Pharmacies	0.46982	0.234908
Cosmetics stores	0.24121	0.120607
Supermarkets	0.09131	0.045653
Internet stores	0.11862	0.059310
Others	0.07905	0.039523

Figure 3. Priorities.

the products sold. As with many products, the product's quality and reliability are fundamental reasons for the preference for cosmetic products (Huang & Foosiri, 2017, Desai, 2014). Because cosmetic products are widely used worldwide and are generally applied directly to human skin, it is essential to evaluate their safety (Loretz et al., 2005). In this context, it is expected that the quality and reliability of the products sold will affect the choice of the cosmetic product purchasing channel of the individuals. Furthermore, parallel to Chan & Tran (2016) and Desai (2014), pharmacies have been identified as the most preferred CPPC by participants in terms of product quality and reliability.

It has been determined that the criteria of brand diversity, wide product range, and presentation of natural/organic products are quite effective in choosing a cosmetic product sales channel, and these three factors were combined into "Product diversity (brand diversity, organic/natural product presentation, etc.)." Meydan (2017) revealed that although the price of an organic cosmetic product known worldwide is high compared to other cosmetic products, it is preferred over others. The study also draws attention to the fact that the products chosen in the top ranks are herbal ingredients. Zengin (2019) found that female consumers generally pay attention to the content of cosmetic products, and this affects their cosmetic product purchasing decisions. Accordingly, the findings obtained within the scope of the current study are in line with the literature. It is thought that product ingredients and diversity are important for consumers in cosmetic products because users want to protect their existing skin health while using cosmetic products. However, considering this criterion in terms of factors affecting the choice of cosmetic product purchasing channel, it is seen that the effect of product diversity is not very high.

It is known that modern-day customers are more demanding and information-seeking, especially when selecting healthcare products and healthcare product purchasing channels. According to Chan & Tran (2016), consumers accepted pharmacies as trustworthy purchasing channels for healthcare products regarding information and product. Yıldırım (2016) revealed that the sources from whom consumers get the most information and most trust are doctors, pharmacists, and their relatives, while advertising is in the last place. Görkemli, Matır, Seki, & Çelik (2016) emphasized the importance of the store's advertising, promotion, and accessibility in purchasing cosmetic products. Özden et al. (2019) stated that individuals consider friends' advice about cosmetic products, in particular; in contrast, beauticians and sales consultants are the least preferred source of information. As a result of this study, advice from health care providers and others and satisfaction in consultancy were influential factors in selecting cosmetic product purchasing channels, and price/promotions were relatively less important. Farrag, El Sayed, & Belk (2010) revealed that the shopping center's features, the discounts/promotions offered in the store, accessibility, and security factors affect consumers' decisions to go to shopping malls. In the study conducted by Kabadayı & Paksoy (2016), it was observed that, among the individual's various purposes in going to the shopping mall, finding the cheapest product attracted attention. Görkemli et al. (2016) emphasized the importance of accessibility of the store in purchasing cosmetic products. Also, Kawa, Rahmadiani & Kumar (2013) found the store's location to be a significant factor affecting consumers' imported cosmetics purchasing behavior. In contrast, the price/promotions and accessibility of the purchasing channel discussed in this study are the minor effective criteria in choosing a cosmetic product purchasing channel.

Balkan & Nardalı (2019) stated that consumers are more inclined to buy cosmetic products by trying them. Individuals prefer not to buy make-up products advertised by various famous people or internet phenomena on online platforms without trying them. Accordingly, within the scope of the current research, it is seen that the probability of participants buying a make-up product that is sold in advertisements, internet sales, supermarkets, and other purchasing channels that they have not tried is very low. In this context, pharmacies that offer the opportunity to try products are at the forefront.

Wu & Chan (2011) revealed that physical stores are more preferred than internet stores when purchasing cosmetic products. The satisfaction with the service offered is particularly effective in this preference. Özden et al. (2019) determined that women prefer cosmetics chain markets and men prefer markets, and found that men prefer pharmacies more than women. Similarly, this study has shown that pharmacies are the best alternative among the cosmetic product purchasing channels, followed by cosmetic markets. Furthermore the importance levels of other CPPS (supermarkets, the internet, and others) are very close to each other.

CONCLUSION

In this study, through the AHP, the relationship between the criteria for the evaluation of the consumer was found, their weights were determined, and the results were evaluated by ranking the criteria according to their importance. It has been observed that consumers prefer pharmacies more than other purchasing channels when purchasing cosmetic products in Van. The quality and reliability of the products offered in pharmacies, the ability to obtain information about the product via experts, the opportunity to compare different brands, and the possibility of trying and exchanging the products are the reasons for the preference for pharmacies. It should be noted that the dynamics of the province of Van were also influential in determining that pharmacies are the most preferred CPPC in this study. The number of shopping centers and cosmetic markets located in Van's city center is relatively low compared to other metropolises in Turkey. This may also have caused the study results to favor the pharmacy.

Although the number and efficiency of information sources has increased in all fields, consumers take into account health care providers' recommendations in particular when shopping for cosmetic products. This result can be seen as a sign that consumers do not sufficiently trust communication and information tools in an environment where they encounter hundreds of advertisements every day. Therefore, it would be more appropriate for cosmetic companies to set up their marketing

efforts to support pharmacies' services, which society accepts as high quality and reliable. Understanding customer needs, expectations, and experiences is vital for optimizing the quality of services offered. Thus, pharmacies with a significant market share in the cosmetics sector can further their cosmetics services by considering the consumer demands highlighted in this study.

The fact that Van is a province with high tourism potential, especially from neighboring countries, is also thought to impact the study results. Therefore, in future studies, the study criteria can be increased and the study can be expanded with participation from consumers from different cultures. In addition, future studies can be designed for other OTC product groups, such as immune enhancers, nutritional supplements, baby products, etc., via a similar approach.

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REFERENCES

- Agcadağ, İ. (2017). Popüler Kültür Bağlamında Kozmetik Ürünleri Tüketimi ve Yeni Bir Satış Kanalı Olarak Ağ Pazarlama (Network Marketing) Sistemi [The Consumption of Cosmetic Products in the Context of Popular Culture and Network Marketing as a New Way of Sales Channel]. *Sosyoloji Dergisi*, 36, 157-169.
- Arslan, M. (2020). Application of AHP method for the selection of pharmaceutical warehouse location. *Journal of Ankara University Faculty of Pharmacy*, 44, 253-264. https://doi.org/10.33483/jfpau.709528
- Baby, S. (2013). AHP modeling for multicriteria decision-making and to optimise strategies for protecting coastal landscape resources. *International Journal of Innovation, Management and Technology, 4,* 218-227.
- Chan, V. & Tran, H. (2016). Purchasing Over-the-counter medicines from Australian pharmacy: What do the pharmacy customers value and expect? *Pharmacy Practice*, *14,* No. 782 (2016 Jul-Sep). https://dx.doi.org/10.18549/PharmPract.2016.03.782
- Chen, C. W. & Chen, Y. F. (2011). Statistical analysis for consumers intentions of purchasing cosmetics. *African Journal of Business Management*, *5,* 11630-11635. https://doi.org/10.5897/AJBM11.1278
- Cosmetics Europe, Socio-Economic Contribution of the European Cosmetics Industry Report 2016. (2020, November 20). Retrieved from: https://www.cosmeticseurope.eu/files/2214/6582/1941/Cosmetics_Europe_Socio-economic_Contribution_of_the_European_Cosmetics_Industry_2016.pdf
- Desai, K. (2014). A study on consumer buying behaviour of cosmetic products in Kolhapur. *Reviews of literature, 1,* 1-11.
- Durmaz, Y. & Bahar, R. (2016). Tüketicilerin satin alma davranışları üzerinde sosyolojik faktörlerin etkisinin incelenmesine yönelik ampirik bir çalışma [A study on the impact of sociological factors on consumer spending behavior to be examined]. *Elektronik Sosyal Bilimler Dergisi*, *10*(37), 60-77.
- Farrag, D. A., El Sayed, I. M. & Belk, R. W. (2010). Mall shopping motives and activities: Multimethod approach. *Journal of International Consumer Marketing, 22,* 95-115. https://doi.org/10.1080/08961530903476113
- Görkemli, N., Matır, L., Seki, Ö. & Çelik, C. (2016). Monobrand mağazacılık ve tüketici ilişkisi: Flormar örneği [Monobrand stores and consumer relationship: Case of flormar]. *Gümüşhane Üniversitesi İletişim Fakültesi Elektronik Dergisi*, *4*(1), 104-123. https://doi.org/10.19145/guifd.93757
- Huang, Z. & Foosiri, P. (2017). Factors affecting chinese consumers' purchase intention on facial make-up cosmetics. *University of Thai Chamber of Commerce, Bangkok*, 173-189.
- Ishizaka, A., Pearman, C. & Nemery, P. (2012). AHPSort: an AHP-based method for sorting problems. *International Journal of Production Research, 50,* 4767-4784. https://doi.org/10.1080/00207543.2012.657966
- Kabadayi , S. & Paksoy, B. (2016). A segmentation of Turkish consumers based on their motives to visit shopping centers. *The International Review of Retail, Distribution and Consumer Research. 26,* 456-476. https://doi.org/10.1080/09593969.2016.1157513
- Kawa, L. W., Rahmadiani, S. F. & Kumar, S. (2013). Factors affecting consumer decision-making: a survey of young-adults on imported cosmetics in Jabodetabek, Indonesia. *SIJ Transactions on Industrial, Financial & Business Management*, *1,* 175-180. https://doi.org/10.9756/SIJASREE/V1I3/0105420101
- Kirby, J. (2004). Online Viral Marketing: The strategic synthesis in peer-to-peer brand marketing. *Brand Channel White Paper, 5,* 1-5.
- Loretz, L. J., Api, A. M., Babcock, L., Barraj, L. M., Burdick, J., Cater, K. C., ... Scrafford, C. G. (2008). Exposure data for cosmetic products: facial cleanser, hair conditioner, and eye shadow. *Food and chemical Toxicology, 46,* 1516-1524. https://doi.org/10.1016/j.fct.2007.12.011
- Lu, Y. & Liu, X. (2018). Chinese female preference of cosmetic products information channels. Global Knowledge. *Memory and Communication*, *67,* 166-179. https://doi.org/10.1108/GKMC-04-2017-0042
- Meydan, B. (2017). Etik tüketicinin kozmetik ürünü satın alma kararı: Promethee tekniği ile bir uygulama [Buying decision of ethical consumer's: Application with promethee method]. *Uluslararası Akademik Yönetim Bilimleri Dergisi, 3*(4), 233-259.
- Nardalı, S. & Balkan, E. (2019). Sosyal medya fenomenleri: Instagram ve kozmetik sektöründe bir uygulama [Marketing through social media influencers: Instagram & Cosmetic sector]. *İzmir Katip Çelebi Üniversitesi İktisadi ve İdari Bilimler Fakültesi Dergisi*, *2*(2), 176-187.
- Özden, S., Saygılı, M. & Sütütemiz, N. (2019). Kozmetik ürünlerin tüketiminde sağlık bilincinin rolü. *XI. Kongre Özel Dergisi, IBANESS Kongreler Serisi; 9-10 Mart 2019,* 791-802.
- , T. L. (1980), *The Analytic Hierarchy Process*, New York, NY: McGraw-Hill Education. Schmidt, K., Aumann, I., Hollander, I., Damm, K. & von der Schulenburg, J. M. G. (2015). Applying the Analytic Hierarchy Process in healthcare research: A systematic literature review and evaluation of reporting. *BMC medical informatics and decision making*, *15,* 1-27. https://doi.org/10.1186/s12911-015-0234-7
- Saydan, R. (2008). Tüketicilerin online alışverişe yönelik risk ve fayda algılamaları: geleneksel ve online tüketicilerin karşılaştırılması [Perceived Risks and Benefits by Consumers in Online Purchasing: A Comparative Study Between Online and Non-Online Consumers]. *Elektronik Sosyal Bilimler Dergisi,* 7(23), 386-402.
- Dapiapis Toros, N. (2016). Pazarlama iletişimi açısından internet ortamında kullanılan ağızdan ağıza pazarlama tekniklerinin marka tercihine etkisi [The effect of e-womm methods on brand preferences through marketing communıcations frame-

work]. *Üsküdar Üniversitesi Sosyal Bilimler Dergisi*, *1*, 57-182. https://doi.org/10.32739/uskudarsbd.1.1.14

- Veuphuteh, F. M. (2018). *Gıda Kalitesi, Sağlık Bilinci ve Fiyat Duyarlılığının, Fast-food Satın Alma Niyetine Etkisi: Türkiye ve Kanada'daki Tüketicilerin Karşılaştırması*. (Doctoral dissertation, Hacettepe University, Turkey). Retrieved from http://www.openaccess.hacettepe.edu.tr:8080/xmlui/handle/11655/5173
- Villi, B. & Kayabaşı, A. (2013). Kozmetik ürünlerde kadınların dürtüsel satın alma davranışlarını etkileyen faktörlerin analizi [Analysis of the factors affecting impulsive buying behaviours of women in cosmetics]. *Ekonomik ve Sosyal Araştırmalar Dergisi, 9*(9), 143-165.
- Wu, S. I. & Chan, H. J. (2011). Perceived service quality and self-concept influences on consumer attitude and purchase process: A comparison between physical and internet channels. *Total Quality Management, 22,* 43-62. https://doi.org/10.1080/14783363.2010.529645
- Yalçın, S. & Gülsün, B. (2020). Kozmetik sektöründe tüketicilerin ürün satin alma davranışlarının bulanık dematel, bulanık ANP ve vikor yöntemleri ile değerlendirilmesi [Evaluation of consumer purchases in the cosmetics sector by fuzzy Dematel, fuzzy ANP and Vikor methods]. *Niğde Ömer Halisdemir Üniversitesi Mühendislik Bilimleri Dergisi*, *9*(1), 254-271. https://doi.org/10.28948/ngumuh.681203
- Yıldırım, Y. (2016). Tüketicinin satın alma karar sürecinde bilgi kaynakları ve güvenirlikleri: Referans grubu olarak yakın çevrenin etkisinin İncelenmesi [Information resouces and their reliabilities on the process of consumer purchase decisions: investigation of the effect of near surroundings as a reference group]. *Akademik Yaklaşımlar Dergisi, 7*(1), 214-231.
- Zengin, H. (2019). Kadınların satın alma davranışları kozmetik ürünlerin pazarlanması üzerine bir araştırma / Purchasing behavior of women a research on the marketing of cosmetic products (Doctoral dissertation thesis, Maltepe University, Turkey). Retrieved from http://openaccess.maltepe.edu.tr/xmlui/handle/20.500.12415/2818.

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Original Article

A new hybrid of the genus *Origanum* L. (Lamiaceae): *Origanum × symes* Carlström

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ABSTRACT

Background and Aims: *Origanum* × *symes* was described as a new species by Carlström in 1984 on the Aegean Island of Symi (Dodecanese, Greece). In this work, a taxonomic re-evaluation of *O. symes* is proposed in light of new findings.
Methods: New specimens belonging to *Origanum symes* from the island of Symi were collected by the first author in 2017. These specimens were carefully examined and compared with the type specimen of *O. symes*. Also, the new *Origanum symes* specimen was compared morphologically with *O. calcaratum* and *O. onites*, which are possible parents, and their calyces were drawn.
Results: The new hybrid was morphologically compared with its putative parents *Origanum calcaratum* and *O. onites*. Its diagnostic features, description, figures, and distribution map were provided.
Conclusion: It has been determined that *O. symes* has intermediate characteristics between its two parents in terms of features such as leaves, calyx, and corolla. These determinations led us to hypothesize that *O. symes* is a hybrid. As a result, it was concluded that *O. symes* was a hybrid between *O. calcaratum* and *O. onites*, and was re-arranged as *Origanum* × symes Carlström.

Keywords: *Origanum symes,* hybrid, Dodecanese, Symi

INTRODUCTION

Origanum L. (family: Lamiaceae, subfamily: Nepetoideae, tribe: Mentheae, subtribe Menthinae) consists of about 42 species (49 taxa) and 22 hybrids worldwide, including the new hybrid described here (Dirmenci et al., 2021). Turkiye is a hotspot of *Origanum* diversity, with 21 species (24 taxa, 13 endemic) and 13 hybrids (12 endemics) (Ietswaart, 1980, 1982; Harley, 2004; Dirmenci, Yazıcı, Özcan, Çelenk, & Martin, 2018; Dirmenci, Özcan, Yazıcı, Arabacı, & Martin, 2018; Dirmenci et al., 2019; Arabaci, Dirmenci, & Yildiz, 2020; Dirmenci, Özcan, Arabacı, Çelenk, & Martin, 2020; Arabaci et al., 2021; Dirmenci et al., 2021). Greece counts 9 species of *Origanum* (6 endemics) and 3 hybrids: *O.* × *intercedens* Rech.f. (1961: 395), *O.* × *minoanum* P.H. Davis (1953: 137) (endemic), and the new endemic *O.* × *symes* Carlström (1984: 19) (Rechinger, 1944, 1961; Davis, 1953; Ietswaart, 1980; Carlström, 1984; Kokkini & Vokou, 1993; Dimopoulos, Raus, & Strid, 2018). Hybridization in natural habitats, as well as in cultivation, is common in Lamiaceae, especially in the genera *Origanum*, *Phlomis* L., *Thymus* L., *Salvia* L., and *Sideritis* L. (Celep, Rader & Drew, 2020; Dirmenci et al., 2021; Dadandi & Duman, 2003). Hybridization in *Origanum* is possible even between species belonging to different sections (Ietswaart, 1980; Dirmenci et al., 2019, 2020, 2021).

During a revisional study of the genus *Origanum* in Greece, it was noticed that *O. symes* is morphologically intermediate between *O. calcaratum* Juss. (1789: 115) and *O. onites* L. (1753: 590). Both taxa are characterized by the same chromosome number, 2n=30,

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and this type of diploidy is reported for most of the species belonging to the genus *Origanum*, though in a few cases, the chromosome number is 2n = 28 and/or 32 (Arabaci et al., 2021; Martin, Dirmenci, Arabaci, Yazıcı & Özcan, 2020). Ample botanical research has been carried out on Symi since the work of Orphanides in 1856, namely by Desio (1924), Rechinger (1944), Davis (1965-1985), Carlström (1987), Keitel & Remm (1991), Jahn (Strid 2016), Chilton (2010), Galanos (2016), Galanos & Tzanoudakis (2017, 2019), Burton & Tan (2017), Cattaneo & Grano (2017, 2018, 2019), and Cattaneo (2020), and only *O. onites* and *O. symes* have been detected on the island. *O. calcaratum* has not been found on Symi, but it is the only species that overlaps with *O. onites* in this area. *O. calcaratum* occurs on several islands and islets of the Cyclades and the Dodecanese, such as Keros, Anidros, Amorgos, Astypalea, Ofidoussa, Kounoupi, Safora, Sirina, and Chalki, and also in eastern Crete (Sitia). The nearest island to Symi where *O. calcaratum* occurs is Chalki (43 km), while Crete is the most distant island from Symi with a distance of 222 km. It grows on the crevices of vertical limestone cliffs at 50-500 m (Strid, 2016). *O. onites* has a wider distributional range and is mostly found in southern Greece, and western and southern Turkiye. It is a phryganic element, and grows on dry, rocky slopes at 0-800 m. A taxonomic re-evaluation of *O.* × *symes* is proposed.

MATERIAL AND METHODS

The specimens of *Origanum* × *symes* were collected from Aghios Dhysalonas Bay on Symi Island between two, shaded, vertical limestone cliffs close to the sea by the first author in 2017 (Figure 1). Carlström previously found this taxon at the same site (Carlström, 1984). It has not been found anywhere else, but considering the difficulty of conducting research at this site due to its tall, vertical limestone cliffs, the presence of a wider population of *O.* × *symes* is not excluded. The collected specimens were identified by comparing them with Carlström's samples, preserved at LD Herbarium (Figure 2). Voucher specimens were deposited in Cattaneo's personal herbarium (Figure 3). Calyx drawings are given in Figure 4. In addition, the new hybrid is morphologically compared with its putative parents *O. calcaratum* and *O. onites* in Table 1.

Figure 1. Distribution map of *Origanum* × *symes* on Symi Island.

RESULTS

Morphological observations

Description of the hybrid

Origanum × *symes* Carlström (Willdenowia 14: 19-21 (1984) (Figures 2-4)

(*Origanum calcaratum* Jussieu (Sect. *Amaracus* (Gleditsch) Bentham) × *Origanum onites* L. (Sect. *Majorana* (Miller) Bentham)

Type: [Greece] Symi, Georgiou Disalona Bay, near s.l., Carlström 8363 (holo. LD-photo!).

Description: Subshrub. Stems ascending, up to 35 cm tall, light yellow or yellow-brown, pubescent at base, hairs c. 1 mm, hairy above. Branches of first order usually present, mostly in upper part of the stems, up to 6 pairs per stem, 3–5 cm long, not rami-

Figure 2. *Origanum* × *symes* holotype (reproduced with permission of the Director of the Biological Museum of Lund).

Figure 3. Specimen of Cattaneo's personal herbarium.

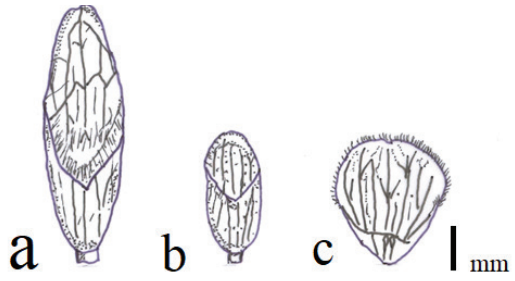

Figure 4. Calyx of *Origanum calcaratum* (a), *O.* × *symes* (b), and *O. onites* (c).

fied and with stalked glands. Leaves up to 15 pairs per stem, 13–28 mm long, 9–17 mm wide, cordate to ovate, obtuse to acute at apex, with marked veins raised at the undersides and with numerous sessile glands (up to 1000 per cm^2), lower leaves lanate with a distinct petiole, c. 1.5 cm long, upper leaves ciliate, subsessile. Spikes usually erect, oblong-ovoid, 20–30 mm long, 10–20 mm wide. Bracts 8–12 pairs per spike, imbricate, ovate to suborbicular, rounded at apex, 7–10 mm long, 7–9 mm wide, glabrous, sessile glandular, green. Flowers 2 per verticillaster, subsessile. Calyx 3.3–3.8 mm long, c. 1.3 mm wide, with colored sessile and colorless stalked glands; upper lip entire; lower lip absent; throat glabrous. Corolla 2-lipped, c. 13 mm long, pink, clearly saccate, with scattered glands; upper lip subentire or divided into 2 lobes, 0.3–0.5 mm long; lower lip divided into 3 subequal lobes, 1.5–2 mm long. Stamens 4, all stamens protruding from upper lip of corolla; filaments c.1.5–11 mm long; anthers c. 0.4 mm long. Style protruding between the filaments, up to 18 mm long. Nutlets c. 1 × 0.5 mm, light-brown, slightly tuberculate, with two depressions exclusively on one side.

Table 1. Comparison of diagnostic characters in *O. calcaratum*, *O.* × *symes*, and *O. onites*.

	O. calcaratum	*O.* × *symes*	*O. onites*
Stems	erect, up to 35 cm, hirsute or slightly lanate, branches up to 6 pairs per stem and up to 2.5 cm	ascending, up to 35 cm, pubescent at base, branches 6 pairs per stem and up to 5 cm	erect or ascending, up to 100 cm, hirsute and glandular, branches up to 8 pairs per stem and up to 7.5 cm
Leaves	(sub)sessile, roundish to ovate, 6-28 × 5-25 mm, hirsute or slightly lanate, with sessile glands up to 600 per cm^2	subsessile to petiolate, cordate-ovate, 13-28 × 9-17 mm, ciliate, lanate with numerous sessile glands up to 1.000 per cm^2	shortly petiolate, cordate, ovate or oval with an acute apex, 3-22 × 2-19 mm, hirsute and glandular-pilose with sessile glands up to 1700 cm^2
Inflorescence	spikes cylindrical or pyramidal, 10-40 × 9-17 mm	spikes oblong-ovoid, 20-30 × 10-20 mm	spikes subglobose, ovoid, or quadrigonus-cylindrical, 3-17 × 3- 5 mm
Bracts	roundish to oval, acute at apex, 5-13 × 4-10 mm, glabrous, partly slightly purple	ovate-suborbicular, rounded at apex, 7-10 × 7-9 mm, glabrous, glandular, green	oval or obovate, acute at apex, 2-5 × 1.5-4 mm, hairy, light green
Calyx	5-8 mm, 1-lipped, upper lip (sub)entire, with a pilose throat	3.3-3.8 mm, 1-lipped, upper lip entire, with a glabrous throat	2-3 mm 1-lipped, upper lip entire or denticulate
Corolla	10-17 mm, pink, saccate, more or less glabrous	13 mm long, pink, saccate	3-7 mm, white, pilosellous
Stamens	4, exserted from corolla	4, exserted from corolla	4, or absent, if present, slightly exserted from corolla

Habitat: Shaded limestone sea cliffs.

Flowering time: June

Comparison of the hybrid with the parental species

Carlström described *Origanum* × *symes* as a new species (Carlström, 1984), including it in the section *Amaracus* (Gled.) Benth.

She underlined the closeness of *O.* × *symes* with *O. calcaratum*, but also pointed out some differences, namely: its less compact inflorescences with always green, ovate to suborbicular bracts, compared to *O. calcaratum*'s usually elliptical bracts with acute apex; the calyx in *O.* × *symes* is smaller, 3.3–3.8 mm long, with a glabrous throat, whereas in *O. calcaratum* it is 5–8 mm long and the throat is usually hairy (Figure 4); and lastly, the differently shaped spikes which in *O.* × *symes* are not pyramidal as they are in *O. calcaratum*.

Moreover, *Origanum* × *symes* differs from *O. calcaratum* in several other features (Table. 1); its stems are glabrous to puberulent (not hirsute or lanate); leaves rounded to ovate, ciliate (not hirsute), up to 15 pairs per stem (not 35 pairs per stem); lower leaves with a distinct petiole ca. 1.5 cm (not (sub)sessile); sessile glands up to 1000 per cm^2 (not 600 per cm^2); bracts with rounded, very glandular apex, 7–10 × 7–9 mm (not acute apex, slightly glandular, 5–13 × 4–10 mm); calyx with glabrous throat, 3.3–3.8 mm long (not pilose throat, 5–8 mm long); and corolla 13 mm long (not 10–17 mm long). *O.* × *symes* is more similar to *O. calcaratum,* but it also has intermediate characters with *O. onites*, such as the appearance of the leaves with marked veins on the undersides, and the shape and size of the calyx. *O. symes* differs from *O. onites* in puberulent-ciliate leaves, up to 15 pairs per stem, 13–28 × 9–17 mm with sessile glands up to 1000 per cm^2 (not hirsute leaves up to 28 pairs per stem, 3–22 × 2–19 mm with sessile gland up to 1700 cm^2), bracts up to 12 pairs per spike 7–10 × 7–9 mm (not up to 34 pairs per spike 2–5 × 1.5–4 mm).

DISCUSSION

Hybridization is a common phenomenon in the genus *Origanum*. Hybrids can form between species belonging to different sections and exhibit features similar to those of one parent or that are intermediate between both parents. In particular, leaf, bract, calyx, and corolla size and shape are usually intermediate between the parents (Rechinger, 1961; Duman, Başer, & Aytaç, 1996; Bakha et al., 2017; Dirmenci et al., 2018a, 2018b, 2020). Indeed, this is clearly recognizable in examples such as *O.* × *aytacii* Dirmenci, T. Yazıcı & Arabacı (2020: 4) (Arabaci et al., 2021) and *O.* × *intermedium* P.H. Davis (1949: 410), which is more similar to *O. sipyleum* L. (1753: 589), *O.* × *intercedens*, which is closer to *O. vulgare* subsp. *hirtum* (Link) Ietsw. (1980: 112), and *O. haradjanii* Rech.f. (1952: 64), which is closer to *O. syriacum* subsp. *bevanii* (Holmes) Greuter & Burdet (1985: 301) (Dirmenci et al., 2020; Arabaci et al., 2021).

In the genus *Origanum*, most hybrids co-exist with their parents in the same area, but some of them may not share the same geographical proximity. This is the case with *O.* × *munzurense* Kit Tan & Sorger (1984: 534), *O.* × *malatyanum* Yıldız, Arabaci & Dirmenci (2020: 10), and *O.* × *intercedens* (Tan & Sorger, 1984; Kokkini & Vokou, 1993; Dirmenci et al., 2019; Arabaci et al., 2020). However, the alleged absence of one or both parents could also be related to faults in the research. In the case of *O.* × *symes,* only one putative parent (*O. onites*) has been observed in its proximity, but the complexity of investigating in the area could have been what led to the other putative parent (*O. calcaratum*) not being found. This led Carlström to identify *O.* × *symes* as a new species.

Although *O.* × *symes* shows intermediate characteristics between both parents, it is much more similar to *O. calcaratum* in terms of general appearance and from an ecological point of view. Indeed, *O. calcaratum* and *O.* × *symes* are both chasmophilous species that grow on the crevices of vertical limestone cliffs (Cattaneo & Grano, 2019). *O.* × *symes* seems to avoid direct irradiation, as it was found in a shaded, deep cleft of a cliff. *O. calcaratum* belongs to the section *Amaracus*. This section is characterized by the usual presence of branches of the first order, and seldom those of the second order; leaves are usually leathery; spikes large, usually nodding; bracts imbricate, membranous, partly purple, more or less glabrous; flowers usually 2 per verticillaster, hermaphrodite and subsessile; calyx 1- or 2-lipped, corolla saccate and all stamens long exserted from corolla (Ietswaart, 1982). *O. onites* belongs to the sect. *Majorana*, which is characterized by the consistent presence of branches of the first order, and sometimes those of the second or third order; leaves herbaceous; spikes (sub)globose, often quadrigonus-cylindrical, small, erect; bracts different from leaves, densely imbricate, ± as long as calyces, enclosing marginally, herbaceous, whitish, greyish, green, hairy; flowers hermaphrodite or female, small; calyces 1-lipped corolla usually 2-lipped flattened. Stamens unequal, shortly protruding from corolla (Ietswaart, 1982).

CONCLUSION

In this study, *Origanum* × *symes* has been taxonomically re-evaluated. Following a careful study of its morphological features, it was possible to identify the hybrid nature of the species, highlighting the similarities with and the differences from its parents *O. calcaratum* and *O. onites*.

In Symi, the presence of *Origanum onites* has been ascertained but not *O. calcaratum*, however the morphological closeness and the geographical proximity of *O. calcaratum*, which occurs on the nearby island of Chalki (southwest of Rhodes), have led the authors to believe that this is one of the putative parents. Floral characters are very important in the identification of putative parents in hybrids. In *O.* × *symes,* the corolla is very close in shape (clearly saccate), size, and color (pink) to that of *O. calcaratum*, whereas the calyx is closer in shape and size to that of *O. onites*. In *O.* × *symes,* all stamens are long-exserted from the corolla, as in *O. calcaratum*. Furthermore, while the stem size and number of branches bring *O.* × *symes* closer to *O. calcaratum*, the cordate-ovate leaves, rich in sessile glands, are reminiscent of those of *O. onites*.

Within *Amaracus* sect., in addition to *Origanum calcaratum*, *O. dictamnus* L. (1753: 589) could also be considered another putative parent of *O.* × *symes* because of its general appearance. The calyx is similar, but in *O.* × *symes* it is smaller and the corolla is more or less saccate, while in *O.* × *symes,* it is clearly saccate, as with *O. calcaratum*. Moreover, stem and leaves in *O. dictamnus* are lanate with branched hairs (exclusive feature of *O. dictamnus*), while in *O. symes*, the stem is pubescent only at the base, the upper leaves are ciliate, and the lower ones are lanate. Also, from a geographical point of view, *O. dictamnus* occurs only on Crete, which is quite distant from Symi compared to Chalki, where *O. calcaratum* occurs. In conclusion, both for morphological and geographical reasons it is assumed that *O. calcaratum* and *O. onites* are the putative parents of *O.* × *symes*.

Species belonging to the genus *Origanum* are mainly found in the Mediterranean region (Zohary, 1973) and most species occur exclusively in the Eastern Mediterranean region (Ietswaart, 1980). Hybridization is a common phenomenon in the genus *Origanum* and should be considered the most important speciation mechanism in the genus. An example is provided by *O.* × *lirium* Heldr. ex Halácsy (1899: 192), which originated from hybridization between *O. scabrum* Boiss. & Heldr. (1846: 48) and *O. vulgare* subsp. *hirtum* (Link.) Ietsw. (1980: 112). Hybrids and hybrid zones are widespread in some plant genera. Hybrids are expected to form only between closely related, genetically similar taxa. However, many such taxa are reproductively isolated from one another by very effective prezygotic isolating mechanisms such as geographic barriers, divergent phenology, divergent pollinators, and mating system differences, as well as postzygotic mechanisms involving immigrant and hybrid inviability. These barriers are likely to prevent such species from hybridizing and forming hybrid zones in sympatric and parapatric situations unless they are disrupted by natural or anthropogenic disturbance. Furthermore, hybrid zones could be a result of climate or geological change, or secondary contact after allopatric divergence (Abbott, 2017). In Greece only three hybrids: *O.* × *intercedens*, *O.* × *minoanum* P.H. Davis, and *O.* × *symes,* have been detected so far, but there may be many more in the overlapping areas of different species.

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REFERENCES

- Abbott, R., Albach, D., Ansell, S., Arntzen, J. W., Baird, S. J. E., Bierne, N. … Boughman, J. (2013). Hybridization and Speciation. *Journal of Evolutionary Biology*, *26*, 229-246. https://doi.org/10.1111/j.1420-9101.2012.02599.
- Arabaci, T., Dirmenci, T., & Yildiz, B. (2020). A new hybrid of the Genus *Origanum* L. (*Lamiaceae*): *Origanum malatyanum*. *Bağbahçe Bilim Dergisi*, *35*, 10-15.
- Arabaci, T., Çelenk, S., Özcan, T., Martin, E., Yazici, T., Açar, M. … Dirmenci, T. (2021). Homoploid hybrids of *Origanum* (*Lamiaceae*) in Turkey: morphological and molecular evidence for a new hybrid. *Plant Biosystems*, *155*(3), 1-13. https://doi.org/10.1080/11263504.2020.1762777
- Bakha, M., Al Faiz, C., Daoud, M., El Mtili, N., Aboukhalid, K., Khiraoui, A. … Silijak-Yakovlev, S. (2017). Genome size and chromosome number for six taxa of *Origanum* genus from Morocco. *Botany Letters, 164*, 361-370. https://doi.org/10.1080/23818107.2017.1395766
- Boissier, E. (1842-1859). *Diagnoses plantarum orientalium Novarum*, vol. 1, fasc. 1-7; v. 2, fasc. 8-13; ser. 2, v. 3, fasc. 1-6. Lipsiae: Hermann.
- Burton, R. M. & Tan, K. (2017). Reports 12-67. In Vladimirov, V., Aybeke, M., Matevski, V. & Tan, K. (Eds.), New floristic records in the Balkans: 34. *Phytologia Balcanica*, *23*, 413-444.
- Carlström, A. (1984). New species of *Alyssum*, *Consolida*, *Origanum* and *Umbilicus* from the SE Aegean Sea. *Willdenowia, 14*, 15-26.
- Carlström, A. (1987). A survey of the flora and phytogeography of Rodhos, Simi, Tilos and the Marmaris Peninsula (SE Greece, SW Turkey). Lund: Ph.D. thesis, University of Lund.
- Cattaneo, C. & Grano, M. (2017). Reports 68-71. In Vladimirov, V., Aybeke, M. & Tan, K. (Eds.), New floristic records in the Balkans, 34. *Phytologia Balcanica*, *23*, 413-444.
- Cattaneo, C. & Grano, M. (2018). Reports 23-27. In Vladimirov, V., Aybeke, M., Matevski, V. & Tan, K. (Eds.), New floristic records in the Balkans, 36. *Phytologia Balcanica*, *24*, 263-292.
- Cattaneo, C. & Grano, M. (2019). Checklist updating and analysis of the flora of Symi island and of the nearby island of Seskli (Dodecanese, Greece). *Bocconea, 28*, 425-463. https://doi.org/10.7320/Bocc28.001
- Cattaneo, C. (2020). Description of a new *Allium* species from Symi Island, (SE Aegean, Greece): *Allium carlstroemi* sp. nov. (*Amaryllidaceae*). *Parnassiana Archives*, *8*, 103-111.
- Celep, F., Rader, E. & Drew, B. (2020). Two new hybrid species of *Salvia* (*S.* × *karamanensis* and *S.* × *doganii*) from Turkey: evidence from molecular and morphological studies. *Turkish Journal of Botany*, *44*, 647-660.
- Chilton, L. (1999). Plant list for Symi (Greece: East Aegean Islands). Ed. 1 (slightly revised 2000-2010). Retford: Marengo.
- Dadandı, M. Y. & Duman, H. (2003). A new hybrid of *Phlomis* (*Lamiaceae*) from Turkey. *Annales Botanici Fennici*, *40,* 287-290.
- Davis, P. H. (1949). Additamenta ad floram anatoliae. I. *Kew Bulletin*, *4*, 393-426.
- Davis, P. H. (1953). Notes on the summer flora of the Aegean. *Notes from the Royal Botanic Garden*, *21*, 101-142.
- Davis, P. H. (1965-1985). Flora of Turkey and the East Aegean Islands, 1-9: Edinburgh, UK: Edinburgh University Press.
- Desio, A. (1924). Sulla costituzione geologica delle isole di Piscopi, Simi, Calimno, Lero, Lipso e Patmo (Mar Egeo). *Rend. R. Accad. Lincei, ser. 5a*, *33*, 358-361.
- Dimopoulos, P., Raus, Th. & Strid, A. (2018). Flora of Greece web. Vascular Plants of Greece. An Annotated Checklist Version III (April 2020). Retrieved from http://portal.cybertaxonomy.org/flora-greece/
- Dirmenci, T., Yazıcı, T., Özcan, T., Çelenk, Ç. & Martin, E. (2018a). A new species and a new natural hybrid of *Origanum* L. (*Lamiaceae*) from the west of Turkey. *Turkish Journal of Botany*, *42*, 73-90. https://doi.org/10.3906/bot-1704-35.
- Dirmenci, T., Özcan, T., Yazıcı, T., Arabacı, T. & Martin, E. (2018b). Morphological, cytological, palynological and molecular evidence on two new hybrids: an example of homoploid hybridization in *Origanum* (*Lamiaceae*). *Phytotaxa*, *371*, 145-167. https://doi.org/10.11646/phytotaxa.371.3.1
- Dirmenci, T., Özcan, T., Açar, M., Arabacı, T., Yazıcı, T. & Martin, E. (2019). A rearranged homoploid hybrid species of *Origanum* (*Lamiaceae*): *O.* × *munzurense* Kit Tan & Sorger. *Botany Letters*, *166*, 153-162. https://doi.org/10.1080/23818107.2019.1585283
- Dirmenci, T., Özcan, T., Arabacı, T., Çelenk, S., & Martin, E. (2020). An important hybrid zone: evidence for two natural homoploid hybrids among three *Origanum* species. *Annales Botanici Fennici*, *57*, 143-157. https://doi.org/10.5735/085.057.0120
- Dirmenci, T., Arabacı, T., Özcan, T., Drew, B., Yazıcı, T., Celep, F. & Martin, E. (2021). Taxonomy of *Origanum* L. (Lamiaceae). In Dirmenci, T. (ed.), *"Oregano" The genus Origanum (Lamiaceae): Taxonomy, Cultivation, Chemistry and Uses* (pp. 1-48). New York: Nova Science Publishers.

- Duman, H., Başer, K. H. C. & Aytaç, Z. (1996). Two new species and a new hybrid from Anatolia. *Turkish Journal of Botany*, *22*, 51-55.
- Galanos, C. J. (2016). Reports 110-118. In Vladimirov, V. & Tan, K. (Eds.), New floristic records in the Balkans: 31. *Phytologia Balcanica*, *22*, 429-467.
- Galanos, C. J. & Tzanoudakis, D. (2017). *Allium symiacum* (*Amaryllidaceae*), a new species from Symi Island (SE Aegean, Greece). *Willdenowia*, *47*, 107-113. https://doi.org/10.3372/wi.47.47202
- Galanos, C. J. & Tzanoudakis, D. (2019). *Allium panormitisi* (*Amaryllidaceae*), a new autumn-flowering species from Symi Island, SE Aegean, Greece. *Botanica Serbica*, *43*, 197-203. https://doi.org/10.2298/BOTSERB1902197G
- Greuter, W. R. & Raus, T. (1985). Med-Checklist Notulae, 11: *Origanum syriacum* subsp. *bevanii* Greuter & Burdet. *Willdenowia*, *14*, 301.
- Halácsy, E.v. (1899). Beiträge zur Flora von Griechenland. Zweiter Theil. *Verh. K.K. Zool.-Bot. Ges. Wien*, *49*, 185-195.
- Harley, R. M., Atkins, S., Budantsev, A., Cantino, P. H., Conn, B., Grayer, R., Harley, M. M., de Kok, R., Krestovskaja, T., Morales, R., Paton, A. J., Ryding, O. & Upson, T. (2004). *Labiatae*. In Kadereit J. W. (Ed.), *The families and genera of vascular plants*. Springer, 7: 167-275.
- Ietswaart, J. H. (1980). A taxonomic revision of the genus *Origanum* (*Labiatae*). Leiden Botanical Series. No. 4. Netherlands: Leiden University Press.
- Ietswaart, J. H. (1982). *Origanum* L. In Davis P. H. (Ed.), *Flora of Turkey and the East Aegean Islands*. Edinburgh, UK: Edinburgh University Press, 7: 297-313.
- Jussieu, A. L. de (1789). *Genera plantarum*: se*cundum ordines naturales disposita, juxta methodum in Horto regio parisiensi exaratam*, anno M.DCC. LXXIV.
- Keitel, Ch. & Remm, W. (1991). Die Orchideenflora der Inseln Simi, Tilos, Nisyros, *Mitt. Bl. Arbeitskr. Heim. Orch. Baden-Württ*., *23*, 81-106.
- Kokkini, S. & Vokou, D. (1993). The Hybrid *Origanum* × *intercedens* from the Island of Nisyros (SE Greece) and its Parental Taxa; Comparative Study of Essential Oils and Distribution. *Biochemical/Systematics and Ecology*, *21*, 397-403. https://doi.org/10.1016/0305-1978(93)90031-L
- Linnaeus, C. (1753). *Species Plantarum*. 2 vols. Stockholm, Sweden: Holmiae.
- Martin, E., Dirmenci, T., Arabaci, T., Yazıcı, T, Özcan, T. (2020). Karyotype studies on the genus *Origanum* L. (*Lamiaceae*) species and some hybrids defining homoploidy. *Caryologia*, *73*, 127-143. https://doi.org/0.13128/caryologia-579
- Rechinger, K. H. (1944). Flora Aegaea. Flora der Inseln und Halbinseln des ägäischen Meeres. *Akad. Wiss. Wien, Math. -Naturwiss. Kl., Denkschr*. *105*(1).
- Rechinger, K. H. (1952). Labiatae novae orientales. *Oesterreichische Botanische Zeitschrift. Gemeinnütziges Organ für Botanik*, *99*, 63-64.
- Rechinger, K. H. (1961). Die Flora von Euboea. *Bot. Jahrb*., *80*, 395-396.
- Strid, A. (2016). *Atlas of the Aegean Flora*. Part 1: Text & plates. Part 2: Maps. Berlin: Botanic Garden and Botanical Museum Berlin.
- Tan, K., & Sorger, F. (1984). New Taxa from East Anatolia: *Origanum munzurense*. *Notes from Royal Botanic Gardens*, *41*, 534.
- Zohary, M. (1973). *Geobotanical Foundation of the Middle East* 1 & 2. Stuttgard: Gustav Fischer Verlag and Amsterdam, Swets and Zeitlinger.

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Review

Obesogens: Definition, mechanisms of action, potential industrial chemicals and pharmaceuticals

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ABSTRACT

The incidence of obesity and related diseases has increased dramatically in the last decade. Some endocrine disruptors have been shown to interact with metabolic processes via various cellular mechanisms, and disrupt homeostasis in adipose tissue, resulting in weight gain and obesity. These chemicals are called "obesogens". Today, with the increased use of industrial chemicals, individuals are exposed to a mixture of chemicals at very low doses. Most of these industrial chemicals have been investigated for their possible endocrine-disrupting and obesogenic effects. Besides these chemicals, pharmaceuticals can also have similar adverse effects; however, limited studies have been performed to investigate such effects of drugs. Furthermore, there are few studies investigating the relationship between prenatal exposure to pharmaceuticals and childhood obesity. Therefore, to clarify the endocrine-disrupting and obesogenic effects of the pharmaceuticals, which are prescribed during pregnancy, mechanistic studies should be performed, and necessary precautions should be taken. In this paper, we reviewed the mechanisms of obesogens, briefly overview several well-known obesogenic industrial chemicals, and focused more on potential obesogenic pharmaceuticals.

Keywords: Endocrine disruptors, Obesogens, Adipogenesis, Lipogenesis, Pharmaceuticals

Endocrine Disruptors

The endocrine system consists of various glands that are found in many parts of the body, hormones which are secreted from these glands to the bloodstream, and receptors that respond by binding to these hormones (USEPA, 2021). Some natural (such as phytoestrogens and hormones) and/or synthetic chemicals (such as pharmaceuticals, pesticides, plasticizers) can cause adverse health effects by interacting with and disrupting normal functions of this system. These chemicals are called "endocrine disruptors" or "endocrine-disrupting chemicals (EDCs)" and defined by the World Health Organization (WHO) as "an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations" (Bashshur, Mandil, & Shannon, 2002). Recently Autrup et al. noted that "endocrine disruptors" is not a scientific term and suggested using the phrase "chemicals interfering with the endocrine system," which they believe better defines their specific effects (Autrup et al., 2020). They can be transferred to humans through the food chain and stored in fatty tissues. They can also be transferred through the placenta to the developing fetus and disrupt the developmental programming of the offspring even at very low levels (Newbold, Padilla-Banks, Snyder, Phillips, & Jefferson, 2007b). EDCs act through different mechanisms: 1) interacting / activating hormone receptors, 2) antagonizing hormone receptors, 3) modifying receptor expression, 4) altering signal transduction pathways in hormone-responsive cells, 5) leading to epigenetic modifications in hormone-producing / responsive cells, and 6) altering hormone synthesis / transport / distribution / metabolism (La Merrill et al., 2020). In the early 2000s, it was hypothesized that some EDCs may lead to the development of obesity by interacting with adipose tissue

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function. Studies have supported the hypothesis that exposure to these chemicals in the early and late stages of life can lead to the development of obesity (Casals-Casas & Desvergne, 2011; De Cock & Van de Bor, 2014; Newbold, Padilla-banks, Snyder, & Jefferson, 2005).

Obesity and Obesogens

The obesity rates in children, adolescents, and adults have been increasing drastically all around the world (Ritchie & Max, 2017). Obesity has been reported as a pandemic in the last decade (Hill, Wyatt, & Peters, 2012). Besides being an important health problem itself, obesity also is known as a contributory factor in other pathologies, such as insulin intolerance, hyperlipidaemia, depression and cardiovascular diseases (Newbold, Padilla-Banks, Snyder, & Jefferson, 2007a. Obesity is defined as "abnormal or excessive fat accumulation that may impair health" by the WHO and is assessed by Body Mass Index (BMI) (WHO, 2021). Although overeating, lack of physical activity and genetic factors are the main reasons for the development of obesity, these factors alone cannot be responsible for the dramatic increase in obesity and related diseases. Over the last decades the increase in both the use of industrial chemicals and obesity incidence has led to the hypothesis that these chemicals, especially those with endocrine disrupting effects, may be responsible for this dramatic increase by disrupting the processes involved in adipo- and lipo-genesis (Baillie-Hamilton, 2002). The chemicals that disrupt lipid metabolism and alter adipo- and lipo-genesis processes are called "obesogens" (Figure 1) (Grün & Blumberg, 2006a). Since the early stages of life, especially organogenesis, are highly sensitive to low doses of EDCs and obesogens compared to later periods, it has been suggested that exposure to these chemicals during early periods may change metabolic homeostasis and cause increased obesity in children and adolescents (La Merrill & Birnbaum, 2011). For better understanding the role of endocrine-disrupting obesogens especially in early stages of life, the possible mechanisms involved in obesity should be considered.

Mechanisms of Obesogens

Obesogens can alter adipose tissue function and increase adiposity by disrupting various cellular processes, thus they can act through distinct mechanisms. In this review, we will focus on five main mechanisms that can lead to obesity.

Adipogenesis

Adipose tissue is comprised of various cell types, such as endothelial and blood cells, fibroblasts, preadipocytes, adipocytes, and macrophages. Among these, the main adipose tissue-forming cells are mature adipocytes. The process of differentiation of preadipocytes and precursory stem cells to mature adipocytes through a transcriptional cascade is called "adipogenesis" (Sarjeant & Stephens, 2012). Various transcriptional factors are involved in this process (Figure 2). The main and crucial transcriptional factors regulating the adipogenic gene expressions are peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT enhancer-binding protein alpha (C/EBPα) (Miettinen, Sarkanen, & Ashammakhi, 2008). PPARγ is a member of the nuclear receptor superfamily of ligand-activated transcription factors and is mainly expressed in adipose tissue. After forming a heterodimer with retinoid X receptor (RXR), PPARγ binds to its response element on promoter regions of genes and regulates the transcription of target genes involved in adipogenesis by increasing their mRNA expression (Lefterova & Lazar, 2009). C/EBPβ and C/EBPδ are also the regulators of adipogenesis and all of them are expressed in adipocytes. During adipogenesis, C/EBPβ and C/EBPδ together induce the expression of the main regulators PPARγ and C/EBPα. These regulators, mainly PPARγ, bind to the promoter regions of genes and promotes adipogenesis (Moseti, Regassa, & Kim, 2016; Richard, White, Elks, & Stephens, 2000). In addition to these adipogenic regulators, there are pro-adipogenic factors that induce PPARγ expression levels and stimulate adipogenesis. There are also anti-adipogenic factors that suppress the adipogenesis processes in adipocytes (Rosen & MacDougald, 2006; Sarjeant & Stephens, 2012). In brief, obesogens can disrupt this transcriptional cascade in adipocytes and thus lead to fat accumulation, resulting in obesity.

Lipogenesis

Lipogenesis is the formation of fatty acids and triglycerides, which is regulated by hormonal, nutritional and transcriptional factors (Kersten, 2001). Triglycerides are produced mainly in the liver, but they can also be generated minorly in adipocytes. Lipogenesis takes place in adipocytes in two different pathways:

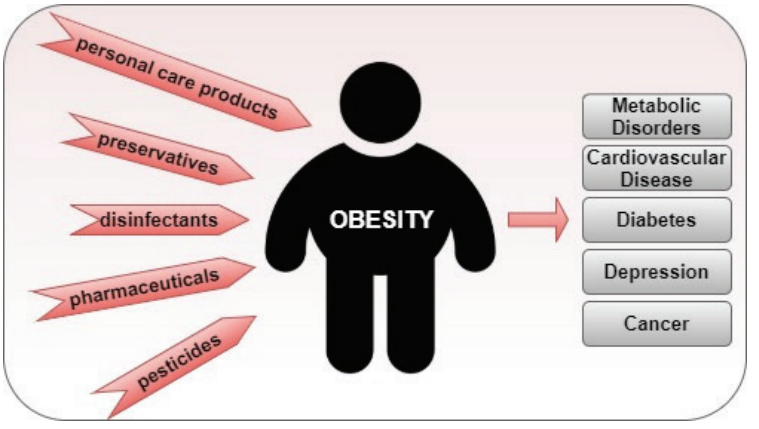

Figure 1. Obesogens and their negative impacts on human health.

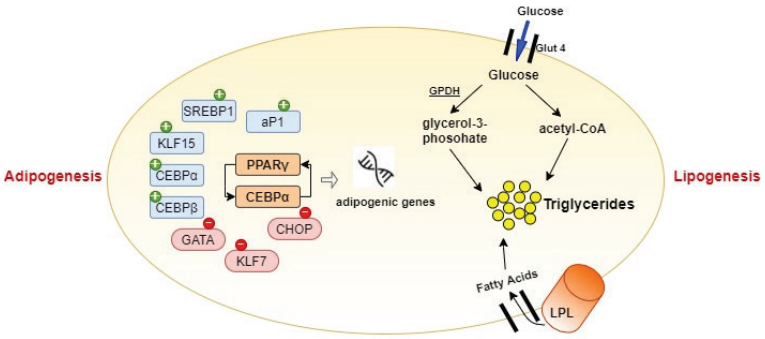

Figure 2. Adipo- and lipo-genesis pathways in adipocytes. PPARγ; peroxisome proliferator-activated receptor gamma, C/EBP; CCAAT/enhancer-binding protein, KLF; kruppel-like factor, aP1; activator protein 1, SREBP1; sterol regulatory element-binding protein 1, CHOP; transcription factor homologous to CCAAT/enhancer-binding protein, GPDH; glycerol-3-phosphate dehydrogenase, LPL; lipoprotein lipase.

1) catabolism of glucose to triglycerides, and 2) conversion of lipoproteins to fatty acids and triglycerides (Figure 2). In the first pathway, circulating glucose enters adipocytes by insulin stimulation and undergoes the glycolysis pathway. Pyruvate formed through glycolysis turns to acetyl-CoA, then enters the Krebs cycle in mitochondria and turns into malonyl CoA via various enzymatic reactions. Malonyl-CoA turns into acyl-CoA, fatty acids, and triglyceride molecules mediated by fatty acid synthase (FAS) and other multiple enzymatic reactions. In addition, glucose is converted into glycerol 3-phosphate by glycerol-3-phosphate dehydrogenase (GPDH) enzyme in the glycolysis pathway and finally turns into triglyceride molecules with further reactions (Romao & Guan, 2014; Vernon & David J. Flint, 2011). Lipoprotein lipase (LPL) is an important enzyme found in adipose tissue, secreted by adipocyte cells, and bound to capillary endothelium. In the second pathway of lipogenesis, circulating lipoproteins formed in the liver are hydrolysed by LPL and turned into fatty acids and monoacylglycerol. These fatty acids are taken into adipocyte cells and accumulate in these cells in the form of triglycerides (Richard et al., 2000). Obesogens can disrupt glucose metabolism in adipocytes and change gene transcription of key enzymes involved in lipogenesis, causing excessive lipid accumulation and increasing the size and number of adipocytes (Chamorro-García et al., 2013; Yang et al., 2018).

Estrogen and estrogen receptor mediated mechanisms

Estrogen is the key hormone involved in the development of female reproductive organs. In postmenopausal women, while estrogen synthesis in ovaries is diminished, its local synthesis in adipose tissue is increased (Mauvais-Jarvis, 2011). Physiologically, estrogen binds to the nuclear estrogen receptor (ER) in cells and the ER signaling pathway is activated by the binding of this homodimer to the estrogen response element in promoter regions of related genes. Estrogen can also regulate gene expression indirectly via binding to the G-protein coupled ER (GPER) located on the cell membrane (Sharma & Prossnitz, 2021). GPER is expressed in various tissues, such as the liver, reproductive organs and adipose tissue (Sharma & Prossnitz, 2017). While reproductive functions are regulated by nuclear ERs, energy homeostasis and metabolic processes are mainly modulated by GPER (Mauvais-Jarvis, 2011). Studies have shown increased fat accumulation, adiposity and body weight in GPER knockout mice (Davis et al., 2014; Sharma et al., 2013). The possible reason for this is reported as a decrease in thermogenic gene expression and leptin hormone sensitivity (Davis et al., 2014). Pre-adipocyte 3T3-L1 cell line also expresses GPER. One study reported that lipogenesis is inhibited and accordingly fat accumulation is reduced by incubating the cells with estradiol (Zhu, Yuen, Sham, & Cheng, 2013).

It has been shown that estrogen also regulates lipid metabolism in adipose tissue and inhibition of estrogen synthesis in ovariectomized mice, resulting in obesity (Pedersen, Børglum, Mø11er-Pedersen, & Richelsen, 1992). Estradiol treatment in 3T3-L1 cells *in vitro* and ovariectomized animals *in vivo* was found to reduce fat accumulation by inhibiting LPL expression and lipogenesis (Homma et al., 2000). Estradiol is reported to exert biphasic effects, decreasing LPL expression at high concentrations while increasing it at low concentrations (Palin et al., 2003). Obesogens can cause adiposity and lipid accumulation by changing the expression of ERs, altering estrogen hormone levels, mimicking these hormones, and thus leading to weight gain through these mechanisms.

Glucocorticoid mediated mechanisms

Glucocorticoids (GCs) are hormones that are secreted from the adrenal gland and regulated by the adrenocorticotropic hormone (ACTH) and corticotropin-releasing hormone (CRH). GCs play roles in various physiological processes, such as regulation of blood pressure, modulation of key enzymes involved in lipid and glucose metabolism, and anti-inflammatory reactions (Moraitis, Block, Nguyen, & Belanoff, 2017). GCs produce biological responses by binding to cytosolic glucocorticoid receptors (GRs). Thereafter, ligand-bound complex is transferred to the nucleus and regulates the expression of genes by binding to the specific glucocorticoid response elements (Heitzer, Wolf, Sanchez, Witchel, & DeFranco, 2007). GCs have been shown to stimulate adipogenesis locally in adipocytes and promote their proliferation (Wu, Bucher, & Farmer, 1996). Therefore, in adipogenesis assays GCs are often used as adipogenic agents. Cortisol secretion elevates as a result of the increase in positive feedback of hypothalamic pituitary adrenal (HPA) axis with obesity (Peeke & Chrousos, 1995). The underlying mechanism of hypercortisolism in obese patients is described as increased levels of 11β-hydroxysteroid dehydrogenase type-1 (11βHSD1) enzyme, which is responsible for converting the inactive form, cortisone, to the active form , cortisol (Rask et al., 2001; Wake et

al., 2003). Another possible mechanism for the involvement of the glucocorticoid pathway in obesity is corticosteroid binding globulin (CBG) deficiency in obese individuals. With the decrease in CBGs, excess cortisol levels cannot be cleared, causing increased proliferation of mature adipocytes (Janesick & Blumberg, 2012). In brief, obesogens may lead to obesity by interacting with the HPA axis, competing with ligands binding to GR, or by disrupting the GC pathway by altering expression of key enzymes and modulating lipid metabolism as described.

Epigenetics

Genetic factors are also known to play an important role in the development of obesity. However, genetics alone are unlikely to be responsible for the dramatic increase in worldwide obesity cases in such a short time. Therefore, the risk of obesity seems to be increasing as a result of the interaction between environmental and genetic factors (Obri, Serra, Herrero, & Mera, 2020). Environmental factors can induce epigenetic modifications through changing gene activities by causing structural changes in genes associated with weight gain, without any changes in DNA sequences (Stger, 2008). The main epigenetic modifications are: 1) DNA methylation (addition of a methyl group to the 5th carbon of a cytosine), 2) histone modifications (changes in chromatin structures by post-translational modifications of histone proteins), 3) interference of non-coding RNAs (regulation of post-transcriptional gene expression through different mechanisms such as negative regulation of mRNA degradation, chromatin remodeling) (Obri et al., 2020). Some obesogens can cause changes in the expression of key genes which play roles in adipo- and lipo-genesis pathways via these modifications (Janesick & Blumberg, 2012). As EDCs bind to nuclear receptors, they can alter the epigenetic programming of obesity via changes in chromatin structure. As described above, PPARγ is a key factor for adipogenesis and regulates the expression of genes involved in this pathway. Some EDCs which are PPARγ ligands can cause obesogenic effects via one of the described epigenetic modifications in PPARγ or target genes (Stel & Legler, 2015). For example, tributyltin, a well-known obesogen, has been demonstrated to cause a decrease in methylation of PPARγ or target genes and consequently results in abnormal genomic responses and obesogenic effects (Bastos Sales et al., 2013). Furthermore, exposure to obesogens in the early stages of life may cause epigenetic changes that lead to transgenerational effects (Rissman & Adli, 2014).

Detection of Obesogens

To investigate the mode of action of possible obesogens, various *in vitro* and *in vivo* models are used. In this review we summarized the frequently used models. *In vitro* models are commonly preferred because of their simplicity, cost effectiveness and advantage of high-throughput screening. 3T3-L1 mouse embryo fibroblast cells are pre-adipocytes and commonly used in *in vitro* obesogen screening studies. In a proper growth media, these cells have the ability to differentiate into mature adipocytes which accumulate triglycerides (OECD, 2012). Mouse bone marrow-derived mesenchymal stem cells are also used widely in adipogenesis assays (Janesick et al., 2016). The difference of species can cause difficulties in extrapolation of the results to humans and might be a disadvantage for the use of 3T3-L1 and mouse-derived stem cells (Griffin, Pereira, DeBari, & Abbott, 2020). Human adipose-derived mesenchymal stem cells are also preferred in the *in vitro* model (Cohen, Cohenour, Harnett, & Schuh, 2021; Foley, Clewell, & Deisenroth, 2015) which does not need an extrapolation step. Besides *in vitro* models, animal models are important to study the kinetics and systemic effects of the obesogenic compounds. Rodents are widely used to detect the obesogenic effects of chemicals. Frequently C57BL/6J (Chu, Malinowska, Jura, & Kozak, 2017; Koya & Kanasaki, 2011) and CD1 mice (Moazzam, Jarmasz, Jin, Siddiqui, & Cattini, 2021; Newbold, Padilla-Banks, Jefferson, & Heindel, 2008) are used as *in vivo* models for obesity studies. For better understanding the mechanisms of the action of the obesogenic compounds, all information from *in vitro* and *in vivo* models should be combined.

Industrial Chemicals as Possible Obesogens

The number and use of industrial chemicals are increasing day by day. Thus, the chances of individuals being exposed to these chemicals at very low doses in their daily lives are getting higher (Figure 1). Some chemicals such as pesticides, food preservatives and plasticizers are investigated largely in terms of their endocrine-disrupting and obesogenic effects. Comprehensive reviews have been published summarizing all studies (Darbre, 2017; Gupta et al., 2020). Therefore, only the well-known obesogens bisphenol A, phthalates, and tributyltin are reviewed in this section (Table 1).

Tributyltin

Tributyltin (TBT) is used in various fields of industry and is the most studied organotin compound with obesogenic effects. Due to its lipophilic properties, TBT bioaccumulates in animal and human tissues and can be transferred to the developing fetus through the placenta. TBT has one of the best-understood modes of action among obesogens, as a nanomolar affinity ligand of the PPARγ and RXR (Darbre, 2017). *In vitro* studies have shown that TBT increases preadipocyte differentiation and this effect is mediated by the activation of PPARγ (Kanayama, Kobayashi, Mamiya, Nakanishi, & Nishikawa, 2005; Kirchner, Kieu, Chow, Casey, & Blumberg, 2010; Li, Ycaza, & Blumberg, 2011). *In vivo* studies with TBT have shown that prenatal exposure in mice induces obesity in male and female offspring by increasing adipogenesis through PPARγ activation (Grün et al., 2006b. In addition, transgenerational obesogenic effects were observed in a multigenerational study *in vivo*. After female mice were exposed to TBT during pregnancy, an increase in adipose tissue weight, adipocyte hypertrophy and hyperplasia, and hepatic lipid accumulation were observed in the exposed F1 and F2 generations as well as the unexposed F3 generation (Chamorro-Garcia et al., 2017; Chamorro-García et al., 2013). To summarize, as an industrial product, TBT is a well-known obesogen that has been shown to exert its obesogenic effects through PPARγ and RXR activation.

Bisphenol A

Bisphenols are synthetic lipophilic chemicals widely used in the plastic industry. The most studied member, bisphenol A (BPA), is found in water bottles and food containers (Darbre, 2017). BPA was shown to induce the differentiation of preadi-

pocyte 3T3-L1 cells into mature adipocytes *in vitro* (Ahmed & Atlas, 2016; Masuno et al., 2002). Prenatal BPA exposure in mice showed adipogenic effects in the offspring (Manikkam, Tracey, Guerrero-Bosagna, & Skinner, 2013; Rubin et al., 2017; Somm et al., 2009). Somm et al. showed that increased adipose tissue weight of the female pups is associated with the overexpression of proadipogenic transcription factors. The majority of these proadipogenic genes upregulated by exposure to BPA are transcriptional targets of PPARγ itself, thus this connection between BPA and PPARγ needs to be better explored (MacKay & Abizaid, 2018). Like tributyltin, BPA induced transgenerational adipogenic effects *in vivo* (Manikkam et al., 2013; Rubin et al., 2017). A prospective epidemiological study has found no association between BPA levels and adipose tissue mass or lipid distribution, but a positive correlation between BPA levels and circulating levels of leptin and adiponectin. Changes in blood levels of these hormones suggest that BPA might exert its obesogenic effects by interfering with the hormonal control of satiety and hunger (Rönn et al., 2014). In summary, the obesogenic effect of BPA is demonstrated in experimental studies; however, the mechanism(s) underlying the obesogenic effect of BPA needs to be better elucidated.

Phthalates

Phthalates are used as industrial plasticizers. Important routes of human exposure include oral, inhalation and dermal routes, and placental transfer from the mother to the developing fetus. Experimental studies have focused mainly on diethylhexyl phthalate (DEHP) and its metabolite monoethylhexyl phthalate (MEHP). DEHP is short-lived and rapidly converted to MEHP by hydrolysis of one of the ester groups in the body (Casals-Casas & Desvergne, 2011). MEHP is a potent agonist of PPARγ (Maloney & Waxman, 1999) and was shown to increase preadipocyte differentiation *in vitro* (Feige et al., 2007). Prenatal exposure to DEHP caused increased body weight, visceral fat mass, circulating leptin, insulin, and glucose levels in mice (Hao, Cheng, Guo, Xia, & Ma, 2013). In a cross-sectional study, blood levels of several phthalate metabolites showed a positive correlation with abdominal obesity and insulin resistance in males (Stahlhut, Wijngaarden, Dye, Stephen, & Swan, 2007). In conclusion, phthalates are found in many products and are shown to induce adipogenesis mainly through PPARγ activation.

Table 1. Summary of the known obesogenic chemicals / pharmaceuticals and their mode of actions.

Obesogens	Mode of action	References
Tributyltin	-PPARγ and RXR activator adipogenesis ↑ -transgenerational effects	(Kanayama et al., 2005; Li et al., 2011) (Chamorro-Garcia et al., 2017; Chamorro-García et al., 2013)
Bisphenol A	-adipogenesis ↑ -increased expression of proadipogenic factors -transgenerational effects -elevated serum leptin and adiponectin levels	(Ahmed & Atlas, 2016; Masuno et al., 2002) (Somm et al., 2009) (Manikkam et al., 2013; Rubin et al., 2017) (Rönn et al., 2014)
Phthalates	-PPARγ activator -adipogenesis↑ -weight gain -visceral fat mass, serum leptin, insulin, glucose levels ↑ -abdominal obesity, insulin resistance	(Maloney & Waxman, 1999) (Feige et al., 2007) (C. Hao et al., 2013) (Stahlhut et al., 2007)
Diethylstilbestrol	-PPARγ activator -adipogenesis↑ -lipogenesis↑ -weight gain -serum TG, leptin, glucose ↑	(Newbold, Padilla-Banks, Snyder, Phillips, et al., 2007b) (Hao, Cheng, Xia, & Ma, 2012b)
Thiazolidinediones	-PPARγ activator -adipogenesis ↑ -LPL ↑ -weight gain -BMI ↑	(Lehmann et al., 1995) (McTernan et al., 2002) (Mori et al., 1999)
Selective Serotonin Reuptake Inhibitors	-weight gain	(Arterburn et al., 2016; Blumenthal et al., 2014; Gafoor et al., 2018)
Atypical Antipsychotics	-lipogenesis↑ -impaired glucose, insulin tolerance -weight gain	(Raeder, Fernø, Vik-Mo, & Steen, 2006; Yang, Chen, Yu, & Chen, 2007) (Lord et al., 2017; Albaugh et al., 2011) (Verhaegen & Van Gaal, 2017)

PPARγ; peroxisome proliferator-activated receptor gamma, RXR; retinoid X receptor, TG; triglyceride, LPL; lipoprotein lipase, BMI; body mass index.

Pharmaceuticals as Possible Obesogens

Besides industrial chemicals, pharmaceuticals, which are generally used intentionally, might be potential obesogens and cause weight gain and obesity. Although several pharmaceuticals are reported to have either endocrine mediated adverse effects (such as gynecomastia, cryptorchidism, and sexual dysfunction) or effects on body weight, limited studies are performed to investigate their possible endocrine-related obesogenic potential. In this section, the potential pharmaceutical obesogens diethylstilbestrol, thiazolidinediones, selective serotonin reuptake inhibitors and atypical antipsychotics have been reviewed (Table 1).

Diethylstilbestrol (DES)

Diethylstilbestrol, a synthetic estrogen, is a well-known endocrine disruptor that was used against miscarriages during pregnancy between the 1940s – 1970s. Although it was reported to be ineffective for miscarriages in 1953, it continued to be prescribed until the 1970s (Schrager & Potter, 2004). Mothers who used DES during pregnancy were found to have an increased risk of breast cancer (Greenberg et al., 1984). Daughters exposed to DES *in utero* were demonstrated to develop vaginal and cervical cancers (Hoover et al., 2011). Moreover, urogenital abnormalities such as cryptorchidism and inflammation of the testes were associated with prenatal DES exposure in sons of the exposed mothers (Palmer et al., 2009). Recently, DES is also suggested to be a potent obesogen by disrupting lipid and glucose metabolism (Alonso-Magdalena et al., 2005; Newbold, Padilla-Banks, Snyder, & Jefferson, 2007a). Hao et. al. found that DES significantly induces adipogenesis and preadipocyte differentiation in 3T3-L1 cell lines and induces lipogenesis and triglyceride accumulation via increasing GPDH activity (Hao, Cheng, Xia, & Ma, 2012a). They also treated C57BL/6J mice prenatally with DES and observed increases in body weight, serum triglyceride and glucose levels, and induced expression of PPARγ and adipogenic genes. Newbold et. al. indicated that exposure to DES in early stages of development results in obesity later in life (Newbold, Padilla-Banks, Snyder, Phillips, et al., 2007b). They found enhanced body weight, serum insulin, triglyceride, leptin and adiponectin levels, and impaired glucose tolerance in prenatally treated CD1 mice. In an epidemiological cohort study, an association between prenatal DES exposure and obesity development in adult women was reported (Hatch et al., 2014). In another prospective pregnancy cohort study, use of DES was found to be associated with childhood obesity (Jensen & Longnecker, 2014). Consequently, DES is a model pharmaceutical product for obesity-related studies, and it has been suggested that exposure to some pharmaceuticals prenatally may cause obesogenic effects and related metabolic disorders later in life.

Thiazolidinediones (TZDs)

Thiazolidinediones (TZDs) are insulin-sensitizing agents that were developed against type 2 diabetes mellitus. Rosiglitazone, troglitazone and pioglitazone are members of TZDs that decrease insulin resistance. Troglitazone has been withdrawn due to its hepatotoxicity and rosiglitazone use has been restricted because of its potential to increase cardiovascular risks (Lebovitz, 2019). *In vitro* studies have shown that TZDs are potent PPARγ agonists that promote adipogenesis in preadipocytes and mesenchymal stem cells (Fürnsinn & Waldhäusl, 2002; Hausman, Poulos, Pringle, & Azain, 2008; Lehmann et al., 1995). Troglitazone was shown to induce the differentiation and proliferation of 3T3-L1 cell lines in the presence of a suitable hormone cocktail and increase the expression of the adipogenic key regulator C/EBPα (Tafuri, 1996). *In vitro* studies with rosiglitazone demonstrated that this anti-diabetic agent promotes differentiation of the preadipocytes via increasing the expression levels of LPL and this can be the possible reason behind weight gain with rosiglitazone treatment (McTernan et al., 2002). Krichner et.al. showed an increase in lipid accumulation and differentiation capacity of adipose-derived stromal stem cells in 8-week old C57BL/6j mice, which were exposed to rosiglitazone *in utero* (Kirchner et al., 2010). Haliakon et. al. demonstrated increased body weight in rats treated with pioglitazone and they commented that pioglitazone stimulates the adipocyte differentiation with the induction of adipogenic factors, especially C/EBPα (Haliakon et al., 1997). In a study with type 2 diabetes mellitus patients, troglitazone treatment caused a significant increase in BMI and subcutaneous fat rather than visceral fat (Mori et al., 1999). Moreover, epidemiological studies have shown that treatment with pioglitazone also causes significant increases in the body weight of the patients (Aghamohammadzadeh et al., 2015; Chawla, Kaushik, Singh, Ghosh, & Saxena, 2013). Thus, this group of antidiabetics is known to increase body weight due to their agonistic effects, especially on PPARγ in adipose tissue.

Selective serotonin reuptake inhibitors (SSRIs)

Selective serotonin reuptake inhibitors (SSRIs) are among the most prescribed antidepressants worldwide, and members of this pharmaceutical group are sertraline, citalopram (and its S-stereoisomer escitalopram), fluoxetine, fluvoxamine, and paroxetine (Hansen, Larsen, Sørensen, Halling-Sørensen, & Styrishave, 2017). SSRIs exert their pharmacological effect by inhibiting the reuptake of serotonin in the presynaptic neuron, thus elongating the effect of serotonin in the synaptic cleft. Recent *in vitro* and *in vivo* studies have demonstrated that SSRIs might have ED effects. In two different microsome-based *in vitro* activity assays, five SSRIs were shown to reduce the estrogen level by inhibiting the aromatase enzyme (Jacobsen, Hansen, Nellemann, Styrishave, & Halling-Sørensen, 2015). All five SSRIs were found to disrupt steroid hormone synthesis in *in vitro* steroidogenesis assay performed with the H295R cell line (Hansen et al., 2017; Jacobsen et al., 2015). In male rats, sertraline was found to inhibit the production of steroid hormones in the testis and adrenal glands (Munkboel, Larsen, Weisser, Kristensen, & Styrishave, 2018), and cause a decrease in sperm count and motility *in vivo* (Atli et al., 2017). Müller et al. reported *in vivo* and *in vitro* estrogenic activity of fluoxetine (Müller et al., 2012). Following the results by Müller et al., Montagnini et al. conducted a similar experiment and showed that sertraline and escitalopram had no effect on the uterus weight, thus they suggested that sertraline and escitalopram did not show *in vivo* estrogenic activity described for fluoxetine (Montagnini et al., 2013). In epidemiological studies, sexual dysfunction was reported in 30-60% of the patients using SSRIs (Gre-

gorian Jr, Golden, Bahce, Goodman, & Kwong, 2002). Another epidemiological study reported alteration in hormone levels such as a decrease in testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH) levels and an increase in prolactin hormone levels in adult men receiving SSRI treatment (Safarinejad, 2008). Epidemiological studies also showed long-term treatment with SSRIs causes weight gain in adults (Arterburn et al., 2016; Blumenthal et al., 2014; Gafoor, Booth, & Gulliford, 2018). However, there are no *in vitro* nor *in vivo* studies in the literature investigating the mechanism of these effects or showing a relationship between prenatal exposure to SSRIs and their possible effects on obesity later in life.

Atypical antipsychotics (AAs)

Atypical antipsychotics (AAs) are relatively newer antipsychotics, preferred in the treatment of various psychiatric disorders due to their limited neurological side effects. AAs exert their pharmacological effects by antagonizing 5-HT2 receptors as well as dopamine D2 receptors, and thus, have a better side-effect profile than typical antipsychotics. However, *in vitro* studies have demonstrated that clozapine and olanzapine induce SREBP mediated lipogenesis in cultured human liver cells (Raeder, Fernø, Vik-Mo, & Steen, 2006) and 3T3-L1cells (Yang, Chen, Yu, & Chen, 2007). Olanzapine administration resulted in impaired glucose tolerance, increased food intake, altered physical activity and energy expenditure in female mice (Lord et al., 2017). Another *in vivo* study with male rats showed that olanzapine administration impaired glucose and insulin tolerance, and increased adipose tissue mass, however, did not increase body weight or food intake (Albaugh et al., 2011). In epidemiological studies, AAs are shown to cause weight gain in 80% of the patients. The mechanisms behind these effects are unknown, although a recent study suggested that AAs might affect the hormonal control of satiety and hunger through the hypothalamus and alter food intake, leading to excessive calorie consumption and obesity, as well as exacerbation of insulin resistance (Verhaegen & Van Gaal, 2017).

Future Directions

All around the world, people are exposed to chemicals and pharmaceuticals both unintentionally and intentionally. These exposures are not only limited to adults, but children and even developing fetuses can also be affected. Many pharmaceuticals, such as analgesics, antibiotics and antidepressants, can be used during pregnancy, and many of these drugs are known to pass through the placenta to the developing fetus. Possible teratogenic effects of the pharmaceuticals are tested preclinically during the drug development process. However, prenatal exposure may lead to adverse effects like endocrine modulation and/or obesogenic effects, that may emerge later in life. In the early 2000s, it was hypothesized that prenatal exposure to chemicals and/or pharmaceuticals may play an important role in childhood obesity and related metabolic syndrome (Casals-Casas & Desvergne, 2011; De Cock & Van de Bor, 2014; Janesick & Blumberg, 2011).

Paracetamol is known as a "safe" drug and is commonly prescribed during pregnancy for pain relief. However, recent *in vitro* and epidemiological studies have shown it that may have endocrine-disrupting effects, such as decreased testosterone secretion, impaired semen quality in men, and reduced anogenital distance in sons exposed prenatally (Albert et al., 2013; Buck Louis, Chen, Kim, Smarr, & Kannan, 2017; Lind et al., 2017). In addition, an epidemiological study has demonstrated a positive relationship between prenatal exposure to paracetamol and childhood obesity (Murphy et al., 2015). However, contradictory results are also reported; Liew et. al. have found no significant association between the use of paracetamol and childhood obesity (Liew et al., 2019). Therefore, there are conflicts about the relationship between prenatal paracetamol exposure and obesity, and no *in vitro* and *in vivo* mechanistic studies have been reported so far. Similarly, as detailed above, various epidemiological studies have reported that some SSRIs might cause weight gain in adults but no reports showing the relationship between prenatal exposure and childhood obesity have been reported (Arterburn et al., 2016; Blumenthal et al., 2014; Gafoor et al., 2018). Another drug, used commonly during pregnancy against preeclampsia, is alpha-methyldopa and is shown to have endocrine-related adverse effects such as gynecomastia in epidemiological studies (Piersma et al., 2009). However, its potential for endocrine-disrupting and obesogenic effects in children who were exposed prenatally have not been investigated. As such, to prevent childhood obesity and related diseases, the endocrine-disrupting and obesogenic effects of other pharmaceuticals, especially the ones widely used during pregnancy, should be investigated.

In conclusion, many studies have mostly focused on the potential endocrine-disrupting and obesogenic effects of industrial/environmental chemicals and their adverse effects on human health but there are few studies investigating such effects of commonly prescribed drugs during pregnancy and their role in childhood obesity. With further studies, effects of these drugs should be investigated, and mechanistic studies should be performed to enlighten their mode of action.

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REFERENCES

- Aghamohammadzadeh, N., Niafar, M., Dalir Abdolahinia, E., Najafipour, F., Mohamadzadeh Gharebaghi, S., Adabi, K., … Ahadi, H. (2015). The effect of pioglitazone on weight, lipid profile and liver enzymes in type 2 diabetic patients. *Therapeutic Advances in Endocrinology and Metabolism*, *6*(2), 56–60. https://doi.org/10.1177/2042018815574229

- Ahmed, S., & Atlas, E. (2016). Bisphenol S- and bisphenol A-induced adipogenesis of murine preadipocytes occurs through direct peroxisome proliferator-activated receptor gamma activation. *International Journal of Obesity (2005)*, *40*(10), 1566–1573. https://doi.org/10.1038/IJO.2016.95
- Albaugh, V. L., Judson, J. G., She, P., Lang, C. H., Maresca, K. P., Joyal, J. L., & Lynch, C. J. (2011). Olanzapine promotes fat accumulation in male rats by decreasing physical activity, repartitioning energy and increasing adipose tissue lipogenesis while impairing lipolysis. *Molecular Psychiatry*, *16*(5), 569–581. https://doi.org/10.1038/MP.2010.33
- Albert, O., Desdoits-Lethimonier, C., Lesné, L., Legrand, A., Guillé, F., Bensalah, K., … Jégou, B. (2013). Paracetamol, aspirin and indomethacin display endocrine disrupting properties in the adult human testis in vitro. *Human Reproduction (Oxford, England)*, *28*(7), 1890–1898. https://doi.org/10.1093/humrep/det112
- Alonso-Magdalena, P., Laribi, O., Ropero, A. B., Fuentes, E., Ripoll, C., Soria, B., & Nadal, A. (2005). Low doses of bisphenol A and diethylstilbestrol impair Ca2+ signals in pancreatic α-cells through a nonclassical membrane estrogen receptor within intact islets of Langerhans. *Environmental Health Perspectives*, *113*(8), 969–977. https://doi.org/10.1289/ehp.8002
- Arterburn, D., Sofer, T., Boudreau, D., Bogart, A., Westbrook, E., Theis, M., … Haneuse, S. (2016). Long-Term Weight Change after Initiating Second-Generation Antidepressants. *Journal of Clinical Medicine*, *5*(4), 48-60. https://doi.org/10.3390/jcm5040048
- Atli, O., Baysal, M., Aydogan-Kilic, G., Kilic, V., Ucarcan, S., Karaduman, B., & Ilgin, S. (2017). Sertraline-induced reproductive toxicity in male rats: Evaluation of possible underlying mechanisms. *Asian Journal of Andrology*, *19*(6), 672–679. https://doi.org/10.4103/1008-682X.192637
- Autrup, H., Barile, F. A., Berry, S. C., Blaauboer, B. J., Boobis, A., Bolt, H., … Vermeulen, N. P. (2020). Human exposure to synthetic endocrine disrupting chemicals (S-EDCs) is generally negligible as compared to natural compounds with higher or comparable endocrine activity. How to evaluate the risk of the S-EDCs? *Toxicology in Vitro*, *67*, 104861. https://doi.org/10.1016/J.TIV.2020.104861
- Baillie-Hamilton, P. F. (2002). Chemical toxins: A hypothesis to explain the global obesity epidemic. *Journal of Alternative and Complementary Medicine*, *8*(2), 185–192. https://doi.org/10.1089/107555302317371479
- Bashshur, R. L., Mandil, S. H., & Shannon, G. W. (2002). Global assessment of the state-of-the-science of endocrine disruptors - Chapter 1: Executive summary. *Telemedicine Journal and E-Health*, *8*(1), 95–107. https://doi.org/10.1089/15305620252933437
- Bastos Sales, L., Kamstra, J. H., Cenijn, P. H., van Rijt, L. S., Hamers, T., & Legler, J. (2013). Effects of endocrine disrupting chemicals on in vitro global DNA methylation and adipocyte differentiation. *Toxicology in Vitro*, *27*(6), 1634–1643. https://doi.org/10.1016/J.TIV.2013.04.005
- Blumenthal, S. R., Castro, V. M., Clements, C. C., Rosenfield, H. R., Murphy, S. N., Fava, M., … Perlis, R. H. (2014). An electronic health records study of long-term weight gain following antidepressant use. *JAMA Psychiatry*, *71*(8), 889–896. https://doi.org/10.1001/jamapsychiatry.2014.414
- Buck Louis, G. M., Chen, Z., Kim, S., Smarr, M. M., & Kannan, K. (2017). Male urinary paracetamol and semen quality. *Andrology*, *5*(6), 1082–1088. https://doi.org/10.1111/andr.12413
- Casals-Casas, C., & Desvergne, B. (2011). Endocrine Disruptors: From Endocrine to Metabolic Disruption. *Annual Review of Physiology*, *73*(1), 135–162. https://doi.org/10.1146/annurev-physiol-012110-142200
- Chamorro-Garcia, R., Diaz-Castillo, C., Shoucri, B. M., Käch, H., Leavitt, R., Shioda, T., & Blumberg, B. (2017). Ancestral perinatal obesogen exposure results in a transgenerational thrifty phenotype in mice. *Nature Communications*, *8*(1). https://doi.org/10.1038/s41467-017-01944-z
- Chamorro-García, R., Sahu, M., Abbey, R. J., Laude, J., Pham, N., & Blumberg, B. (2013). Transgenerational inheritance of increased fat depot size, stem cell reprogramming, and hepatic steatosis elicited by prenatal exposure to the obesogen tributyltin in mice. *Environmental Health Perspectives*, *121*(3), 359–366. https://doi.org/10.1289/ehp.1205701
- Chawla, S., Kaushik, N., Singh, N. P., Ghosh, R. K., & Saxena, A. (2013). Effect of addition of either sitagliptin or pioglitazone in patients with uncontrolled type 2 diabetes mellitus on metformin: A randomized controlled trial. *Journal of Pharmacology and Pharmacotherapeutics*, *4*(1), 27–32. https://doi.org/10.4103/0976-500X.107656
- Chu, D. T., Malinowska, E., Jura, M., & Kozak, L. P. (2017). C57BL/6J mice as a polygenic developmental model of diet-induced obesity. *Physiological Reports*, *5*(7), e13093. https://doi.org/10.14814/phy2.13093
- Cohen, I. C., Cohenour, E. R., Harnett, K. G., & Schuh, S. M. (2021). BPA, BPAF and TMBPF Alter Adipogenesis and Fat Accumulation in Human Mesenchymal Stem Cells, with Implications for Obesity. *International Journal of Molecular Sciences*, *22*(10), 5363. https://doi.org/10.3390/ijms22105363
- Darbre, P. D. (2017). Endocrine Disruptors and Obesity. *Current Obesity Reports*, *6*(1), 18–27. https://doi.org/10.1007/s13679-017-0240-4
- Davis, K. E., Carstens, E. J., Irani, B. G., Gent, L. M., Hahner, L. M., & Clegg, D. J. (2014). Sexually dimorphic role of G protein-coupled estrogen receptor (GPER) in modulating energy homeostasis. *Hormones and Behavior*, Vol. 66, pp. 196–207. Academic Press Inc. https://doi.org/10.1016/j.yhbeh.2014.02.004
- De Cock, M., & Van de Bor, M. (2014). Obesogenic effects of endocrine disruptors, what do we know from animal and human studies? *Environment International*, *70*, 15–24. https://doi.org/10.1016/j.envint.2014.04.022
- Feige, J. N., Gelman, L., Rossi, D., Zoete, V., Métivier, R., Tudor, C., … Desvergne, B. (2007). The endocrine disruptor monoethylhexyl-phthalate is a selective peroxisome proliferator-activated receptor gamma modulator that promotes adipogenesis. *The Journal of Biological Chemistry*, *282*(26), 19152–19166. https://doi.org/10.1074/JBC.M702724200
- Foley, B., Clewell, R., & Deisenroth, C. (2015). Development of a Human Adipose-Derived Stem Cell Model for Characterization of Chemical Modulation of Adipogenesis. *Applied In Vitro Toxicology*, *1*(1), 66–78. https://doi.org/10.1089/aivt.2014.0007
- Fürnsinn, C., & Waldhäusl, W. (2002). Thiazolidinediones: Metabolic actions in vitro. *Diabetologia*, Vol. 45, pp. 1211–1223. https://doi.org/10.1007/s00125-002-0899-1
- Gafoor, R., Booth, H. P., & Gulliford, M. C. (2018). Antidepressant utilisation and incidence of weight gain during 10 years' follow-up: Population based cohort study. *BMJ (Online)*, *361*. https://doi.org/10.1136/bmj.k1951
- Greenberg, E. R., Barnes, A. B., Resseguie, L., Barrett, J. A., Burnside, S., Lanza, L. L., … Colton, T. (1984). Breast Cancer in Mothers Given Diethylstilbestrol in Pregnancy. *New England Journal of Medicine*, *311*(22), 1393–1398. https://doi.org/10.1056/nejm198411293112201
- Gregorian Jr, R. S., Golden, K. A., Bahce, A., Goodman, C., & Kwong, W. J. (2002). Antidepressant-Induced Sexual Dysfunction. The Annals of pharmacotherapy, 36(10), 1577–1589. https://doi.org/10.1345/aph.1A195.
- Griffin, M. D., Pereira, S. R., DeBari, M. K., & Abbott, R. D. (2020). Mechanisms of action, chemical characteristics, and model systems of obesogens. *BMC Biomedical Engineering*, *2*(1), 1–13. https://doi.org/10.1186/s42490-020-00040-6

- Grün, F., & Blumberg, B. (2006a). Environmental obesogens: Organotins and endocrine disruption via nuclear receptor signaling. *Endocrinology*, Vol. 147. https://doi.org/10.1210/en.2005-1129
- Grün, F., Watanabe, H., Zamanian, Z., Maeda, L., Arima, K., Cubacha, R., … Blumberg, B. (2006b). Endocrine-disrupting organotin compounds are potent inducers of adipogenesis in vertebrates. *Molecular Endocrinology*, *20*(9), 2141–2155. https://doi.org/10.1210/me.2005-0367
- Gupta, R., Kumar, P., Fahmi, N., Garg, B., Dutta, S., Sachar, S., … Vimaleswaran, K. S. (2020). Endocrine disruption and obesity: A current review on environmental obesogens. *Current Research in Green and Sustainable Chemistry*, *3*. https://doi.org/10.1016/J.CRGSC.2020.06.002
- Haliakon, S., Doare, L., Foufelle, F., Kergoat, M., Guerre-Millo, M., Berthault, M.-F., … Ferre, P. (1997). Pioglitazone Induces In Vivo Adipocyte Differentiation in the Obese Zucker fa/fa Rat. *Diabetes*, *46*(9), 1393–1399. https://doi.org/10.2337/diab.46.9.1393
- Hansen, C. H., Larsen, L. W., Sørensen, A. M., Halling-Sørensen, B., & Styrishave, B. (2017). The six most widely used selective serotonin reuptake inhibitors decrease androgens and increase estrogens in the H295R cell line. *Toxicology in Vitro*, *41*, 1–11. https://doi.org/10.1016/j.tiv.2017.02.001
- Hao, C., Cheng, X., Guo, J., Xia, H., & Ma, X. (2013). Perinatal exposure to diethyl-hexyl-phthalate induces obesity in mice. *Frontiers in Bioscience (Elite Edition)*, *5*(2), 725–733. https://doi.org/10.2741/E653
- Hao, C. J., Cheng, X. J., Xia, H. F., & Ma, X. (2012a). The endocrine disruptor diethylstilbestrol induces adipocyte differentiation and promotes obesity in mice. *Toxicology and Applied Pharmacology*, *263*(1), 102–110. https://doi.org/10.1016/j.taap.2012.06.003
- Hao, C. J., Cheng, X. J., Xia, H. F., & Ma, X. (2012b). The endocrine disruptor diethylstilbestrol induces adipocyte differentiation and promotes obesity in mice. *Toxicology and Applied Pharmacology*, *263*(1), 102–110. https://doi.org/10.1016/j.taap.2012.06.003
- Hatch, E. E., Troisi, R., Palmer, J. R., Wise, L. A., Titus, L., Strohsnitter, W. C., … Hoover, R. N. (2014). Prenatal diethylstilbestrol exposure and risk of obesity in adult women. *Journal of Developmental Origins of Health and Disease*, *6*(3), 201–207. https://doi.org/10.1017/S2040174415000033
- Hausman, G. J., Poulos, S. P., Pringle, T. D., & Azain, M. J. (2008). The influence of thiazolidinediones on adipogenesis in vitro and in vivo: potential modifiers of intramuscular adipose tissue deposition in meat animals. *Journal of Animal Science*, Vol. 86. https://doi.org/10.2527/jas.2007-0219
- Heitzer, M. D., Wolf, I. M., Sanchez, E. R., Witchel, S. F., & DeFranco, D. B. (2007). Glucocorticoid receptor physiology. *Reviews in Endocrine and Metabolic Disorders*, Vol. 8, pp. 321–330. Springer. https://doi.org/10.1007/s11154-007-9059-8
- Hill, J. O., Wyatt, H. R., & Peters, J. C. (2012). Energy balance and obesity. *Circulation*, *126*(1), 126–132. https://doi.org/10.1161/CIRCULATIONAHA.111.087213
- Homma, H., Kurachi, H., Nishio, Y., Takeda, T., Yamamoto, T., Adachi, K., … Murata, Y. (2000). Estrogen suppresses transcription of lipoprotein lipase gene. Existence of a unique estrogen response element on the lipoprotein lipase promoter. *Journal of Biological Chemistry*, *275*(15), 11404–11411. https://doi.org/10.1074/jbc.275.15.11404
- Hoover, R. N., Hyer, M., Pfeiffer, R. M., Adam, E., Bond, B., Cheville, A. L., … Troisi, R. (2011). Adverse Health Outcomes in Women Exposed In Utero to Diethylstilbestrol. *New England Journal of Medicine*, *365*(14), 1304–1314. https://doi.org/10.1056/nejmoa1013961
- Jacobsen, N. W., Hansen, C. H., Nellemann, C., Styrishave, B., & Halling-Sørensen, B. (2015). Effects of selective serotonin reuptake inhibitors on three sex steroids in two versions of the aromatase enzyme inhibition assay and in the H295R cell assay. *Toxicology in Vitro*, *29*(7), 1729–1735. https://doi.org/10.1016/j.tiv.2015.07.005
- Janesick, A., & Blumberg, B. (2012). Obesogens, stem cells and the developmental programming of obesity. *International Journal of Andrology*, Vol. 35, pp. 437–448. NIH Public Access. https://doi.org/10.1111/j.1365-2605.2012.01247.x
- Janesick, A. S., Dimastrogiovanni, G., Vanek, L., Boulos, C., Chamorro-García, R., Tang, W., & Blumberg, B. (2016). On the utility of ToxCast™ and ToxPi as methods for identifying new obesogens. *Environmental Health Perspectives*, *124*(8), 1214–1226. https://doi.org/10.1289/ehp.1510352
- Janesick, Amanda, & Blumberg, B. (2011). Endocrine disrupting chemicals and the developmental programming of adipogenesis and obesity. *Birth Defects Research Part C: Embryo Today: Reviews*, *93*(1), 34–50. https://doi.org/10.1002/BDRC.20197
- Jensen, E. T., & Longnecker, M. P. (2014). Pharmacologic sex hormones in pregnancy in relation to offspring obesity. *Obesity*, *22*(11), 2406–2412. https://doi.org/10.1002/oby.20778
- Kanayama, T., Kobayashi, N., Mamiya, S., Nakanishi, T., & Nishikawa, J. I. (2005). Organotin compounds promote adipocyte differentiation as agonists of the peroxisome proliferator-activated receptor γ/retinoid X receptor pathway. *Molecular Pharmacology*, *67*(3), 766–774. https://doi.org/10.1124/mol.104.008409
- Kersten, S. (2001). Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO Reports*, *2*(4), 282–286. https://doi.org/10.1093/embo-reports/kve071
- Kirchner, S., Kieu, T., Chow, C., Casey, S., & Blumberg, B. (2010). Prenatal exposure to the environmental obesogen tributyltin predisposes multipotent stem cells to become adipocytes. *Molecular Endocrinology*, *24*(3), 526–539. https://doi.org/10.1210/me.2009-0261
- Koya, D., & Kanasaki, K. (2011). Biology of obesity: Lessons from animal models of obesity. *Journal of Biomedicine and Biotechnology*, Vol. 2011. https://doi.org/10.1155/2011/197636
- La Merrill, M. A., Vandenberg, L. N., Smith, M. T., Goodson, W., Browne, P., Patisaul, H. B., … Zoeller, R. T. (2020). Consensus on the key characteristics of endocrine-disrupting chemicals as a basis for hazard identification. *Nature Reviews Endocrinology*, *16*(1), 45–57. https://doi.org/10.1038/s41574-019-0273-8
- La Merrill, M., & Birnbaum, L. S. (2011). Childhood obesity and environmental chemicals. *Mount Sinai Journal of Medicine*, *78*(1), 22–48. https://doi.org/10.1002/msj.20229
- Lebovitz, H. E. (2019). Thiazolidinediones: the Forgotten Diabetes Medications. *Current Diabetes Reports*, Vol. 19, pp. 1–13. Springer. https://doi.org/10.1007/s11892-019-1270-y
- Lefterova, M. I., & Lazar, M. A. (2009). New developments in adipogenesis. *Trends in Endocrinology and Metabolism*, *20*(3), 107–114. https://doi.org/10.1016/j.tem.2008.11.005
- Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M., & Kliewer, S. A. (1995). An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). *Journal of Biological Chemistry*, *270*(22), 12953–12956. https://doi.org/10.1074/jbc.270.22.12953
- Li, X., Ycaza, J., & Blumberg, B. (2011). The environmental obesogen tributyltin chloride acts via peroxisome proliferator activated receptor gamma to induce adipogenesis in murine 3T3-L1 preadipocytes. *Journal of Steroid Biochemistry and Molecular Biology*, *127*(1–2), 9–15. https://doi.org/10.1016/j.jsbmb.2011.03.012
- Liew, Z., Nohr, E. A., Morgen, C. S., Ernst, A., Li, J., Sørensen, T. I. A., & Olsen, J. (2019). Prenatal Exposure to Acetaminophen and Overweight in Childhood. *Obesity*, *27*(8), 1314–1322. https://doi.org/10.1002/oby.22526
- Lind, D. V., Main, K. M., Kyhl, H. B., Kristensen, D. M., Toppari, J., Andersen, H. R., … Jensen, T. K. (2017). Maternal use of mild analgesics during pregnancy associated with reduced anogenital distance in sons: A cohort study of 1027 mother-child pairs. *Human Reproduction*, *32*(1), 223–231. https://doi.org/10.1093/humrep/dew285

- Lord, C. C., Wyler, S. C., Wan, R., Castorena, C. M., Ahmed, N., Mathew, D., … Elmquist, J. K. (2017). Atypical antipsychotic causes weight gain by targeting serotonin receptor 2C. *Clinical Pharmacist*, *9*(10), 3402–3406. https://doi.org/10.1211/CP.2017.20203665
- MacKay, H., & Abizaid, A. (2018). A plurality of molecular targets: The receptor ecosystem for bisphenol-A (BPA). *Hormones and Behavior*, *101*(November), 59–67. https://doi.org/10.1016/j.yhbeh.2017.11.001
- Maloney, E. K., & Waxman, D. J. (1999). trans-Activation of PPARalpha and PPARgamma by structurally diverse environmental chemicals. *Toxicology and Applied Pharmacology*, *161*(2), 209–218. https://doi.org/10.1006/TAAP.1999.8809
- Manikkam, M., Tracey, R., Guerrero-Bosagna, C., & Skinner, M. K. (2013). Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. *PloS One*, *8*(1). https://doi.org/10.1371/JOURNAL.PONE.0055387
- Masuno, H., Kidani, T., Sekiya, K., Sakayama, K., Shiosaka, T., Yamamoto, H., & Honda, K. (2002). Bisphenol A in combination with insulin can accelerate the conversion of 3T3-L1 fibroblasts to adipocytes. *Journal of Lipid Research*, *43*(5), 676–684. https://doi.org/10.1016/S0022-2275(20)30108-5
- Mauvais-Jarvis, F. (2011). Estrogen and androgen receptors: Regulators of fuel homeostasis and emerging targets for diabetes and obesity. *Trends Endocrinol Metab*, *22*(1), 24–33. https://doi.org/10.1016/j.tem.2010.10.002
- McTernan, P. G., Harte, A. L., Anderson, L. A., Green, A., Smith, S. A., Holder, J. C., … Kumar, S. (2002). Insulin and rosiglitazone regulation of lipolysis and lipogenesis in human adipose tissue in vitro. *Diabetes*, *51*(5), 1493–1498. https://doi.org/10.2337/diabetes.51.5.1493
- Miettinen, S., Sarkanen, J. R., & Ashammakhi, N. (2008). Adipose Tissue and Adipocyte Differentiation: Molecular and Cellular Aspects and Tissue Engineering Applications. *Topics in Tissue Engineering*, *4*, 1–26.
- Moazzam, S., Jarmasz, J. S., Jin, Y., Siddiqui, T. J., & Cattini, P. A. (2021). Effects of high fat diet-induced obesity and pregnancy on prepartum and postpartum maternal mouse behavior. *Psychoneuroendocrinology*, *126*, 105147. https://doi.org/10.1016/j.psyneuen.2021.105147
- Montagnini, B. G., Bortolan, S., Santos, B. D. Dos, Moreno, A. P., De Azevedo Camin, N., Gerardin, D. C. C., & Moreira, E. G. (2013). Evaluation of escitalopram, sertraline, and methylphenidate in the immature rat uterotrophic assay. *International Journal of Toxicology*, *32*(6), 426–430. https://doi.org/10.1177/1091581813509674
- Moraitis, A. G., Block, T., Nguyen, D., & Belanoff, J. K. (2017, January 1). The role of glucocorticoid receptors in metabolic syndrome and psychiatric illness. *Journal of Steroid Biochemistry and Molecular Biology*, Vol. 165, pp. 114–120. Elsevier Ltd. https://doi.org/10.1016/j.jsbmb.2016.03.023
- Mori, Y., Murakawa, Y., Okada, K., Horikoshi, H., Yokoyama, J., Tajima, N., & Ikeda, Y. (1999). Effect of troglitazone on body fat distribution in type 2 diabetic patients. *Diabetes Care*, *22*(6), 908–912. https://doi.org/10.2337/diacare.22.6.908
- Moseti, D., Regassa, A., & Kim, W. K. (2016). Molecular regulation of adipogenesis and potential anti-adipogenic bioactive molecules. *International Journal of Molecular Sciences*, *17*(1), 1–24. https://doi.org/10.3390/ijms17010124
- Müller, J. C., Imazaki, P. H., Boareto, A. C., Lourenço, E. L. B., Golin, M., Vechi, M. F., … Dalsenter, P. R. (2012). In vivo and in vitro estrogenic activity of the antidepressant fluoxetine. *Reproductive Toxicology*, *34*(1), 80–85. https://doi.org/10.1016/j.reprotox.2012.04.001
- Munkboel, C. H., Larsen, L. W., Weisser, J. J., Kristensen, D. M., & Styrishave, B. (2018). Sertraline suppresses testis and adrenal steroid production and steroidogenic gene expression while increasing LH in Plasma of male rats resulting in compensatory hypogonadism. *Toxicological Sciences*, *163*(2), 609–619. https://doi.org/10.1093/toxsci/kfy059
- Murphy, R., Stewart, A. W., Braithwaite, I., Beasley, R., Hancox, R. J., & Mitchell, E. A. (2015). Association between paracetamol use in infancy or childhood with body mass index. *Obesity*, *23*(5), 1030–1038. https://doi.org/10.1002/oby.21045
- Newbold, R. R., Padilla-Banks, E., Jefferson, W. N., & Heindel, J. J. (2008). Effects of endocrine disruptors on obesity. *International Journal of Andrology*, *31*(2), 201–207. https://doi.org/10.1111/j.1365-2605.2007.00858.x
- Newbold, R. R., Padilla-banks, E., Snyder, R. J., & Jefferson, W. N. (2005). *Developmental Exposure to Estrogenic Compounds and Obesity*. *480*, 478–480. https://doi.org/10.1002/bdra.20147
- Newbold, R. R., Padilla-Banks, E., Snyder, R. J., & Jefferson, W. N. (2007a). Perinatal exposure to environmental estrogens and the development of obesity. *Molecular Nutrition and Food Research*, Vol. 51, pp. 912–917. https://doi.org/10.1002/mnfr.200600259
- Newbold, R. R., Padilla-Banks, E., Snyder, R. J., Phillips, T. M., & Jefferson, W. N. (2007b). Developmental exposure to endocrine disruptors and the obesity epidemic. *Reproductive Toxicology*, Vol. 23, pp. 290–296. https://doi.org/10.1016/j.reprotox.2006.12.010
- Obri, A., Serra, D., Herrero, L., & Mera, P. (2020). The role of epigenetics in the development of obesity. *Biochemical Pharmacology*, Vol. 177, p. 113973. Elsevier Inc. https://doi.org/10.1016/j.bcp.2020.113973
- OECD. (2012). OECD Detailed review paper on the state of the science on novel in vitro and in vivo screening and testing methods and endpoints for evaluating endocrine disruptors OECD Environment, Health and Safety Publications Series on Testing and Assessment 2012 Par. Retrieved November 6, 2021, from https://www.oecd-ilibrary.org/docserver/9789264221352-en.pdf?expires=1636196089&id=id&accname=guest&checksum=960E616120F388C1D44E902319C840AC
- Palin, S. L., McTernan, P. G., Anderson, L. A., Sturdee, D. W., Barnett, A. H., & Kumar, S. (2003). 17β-Estradiol and anti-estrogen ICI:compound 182,780 regulate expression of lipoprotein lipase and hormone-sensitive lipase in isolated subcutaneous abdominal adipocytes. *Metabolism: Clinical and Experimental*, *52*(4), 383–388. https://doi.org/10.1053/meta.2003.50088
- Palmer, J. R., Herbst, A. L., Noller, K. L., Boggs, D. A., Troisi, R., Titus-Ernstoff, L., … Hoover, R. N. (2009). Urogenital abnormalities in men exposed to diethylstilbestrol in utero: A cohort study. *Environmental Health: A Global Access Science Source*, 8, 37. https://doi.org/10.1186/1476-069X-8-37
- Pedersen, S. B., Børglum, J. D., Mø11er-Pedersen, T., & Richelsen, B. (1992). Effects of in vivo estrogen treatment on adipose tissue metabolism and nuclear estrogen receptor binding in isolated rat adipocytes. *Molecular and Cellular Endocrinology*, *85*(1–2), 13–19. https://doi.org/10.1016/0303-7207(92)90120-U
- Peeke, P. M., & Chrousos, G. P. (1995). Hypercortisolism and Obesity. *Annals of the New York Academy of Sciences*, *771*(1), 665–676. https://doi.org/10.1111/j.1749-6632.1995.tb44719.x
- Piersma, A. H., Luijten, M., Popov, V., Tomenko, V., Altstein, M., Kagampang, F., & Schlesinger, H. (2009). Pharmaceuticals. In *Endocrine-Disrupting Chemicals in Food* (pp. 459–518). Elsevier Ltd. https://doi.org/10.1533/9781845695743.4.459
- Raeder, M. B., Fernø, J., Vik-Mo, A. O., & Steen, V. M. (2006). SREBP activation by antipsychotic- and antidepressant-drugs in cultured human liver cells: Relevance for metabolic side-effects? *Molecular and Cellular Biochemistry*, *289*(1–2), 167–173. https://doi.org/10.1007/s11010-006-9160-4

- Rask, E., Olsson, T., Söderberg, S., Andrew, R., Livingstone, D. E. W., Johnson, O., & Walker, B. R. (2001). Tissue-specific dysregulation of cortisol metabolism in human obesity. *Journal of Clinical Endocrinology and Metabolism*, *86*(3), 1418–1421. https://doi.org/10.1210/jcem.86.3.7453
- Richard, A. J., White, U., Elks, C. M., & Stephens, J. M. (2000). Adipose Tissue: Physiology to Metabolic Dysfunction. In *Endotext*. MDText.com, Inc. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/32255578
- Rissman, E. F., & Adli, M. (2014). Minireview: Transgenerational Epigenetic Inheritance: Focus on Endocrine Disrupting Compounds. *Endocrinology*, *155*(8), 2770. https://doi.org/10.1210/EN.2014-1123
- Ritchie, H., & Max, R. (2017). Obesity - Our World in Data. Retrieved November 4, 2021, from https://ourworldindata.org/obesity
- Romao, J. M., & Guan, L. L. (2014). Adipogenesis and Obesity. In *MicroRNA in Regenerative Medicine* (pp. 539–565). Elsevier Inc. https://doi.org/10.1016/B978-0-12-405544-5.00021-6
- Rönn, M., Lind, L., Örberg, J., Kullberg, J., Söderberg, S., Larsson, A., … Lind, P. M. (2014). Bisphenol A is related to circulating levels of adiponectin, leptin and ghrelin, but not to fat mass or fat distribution in humans. *Chemosphere*, *112*, 42–48. https://doi.org/10.1016/J.CHEMOSPHERE.2014.03.042
- Rosen, E. D., & MacDougald, O. A. (2006). Adipocyte differentiation from the inside out. *Nature Reviews Molecular Cell Biology*, *7*(12), 885–896. https://doi.org/10.1038/nrm2066
- Rubin, B. S., Paranjpe, M., DaFonte, T., Schaeberle, C., Soto, A. M., Obin, M., & Greenberg, A. S. (2017). Perinatal BPA exposure alters body weight and composition in a dose specific and sex specific manner: The addition of peripubertal exposure exacerbates adverse effects in female mice. *Reproductive Toxicology (Elmsford, N.Y.)*, *68*, 130–144. https://doi.org/10.1016/J.REPROTOX.2016.07.020
- Safarinejad, M. R. (2008). Evaluation of endocrine profile and hypothalamic-pituitary-testis axis in selective serotonin reuptake inhibitor-induced male sexual dysfunction. *Journal of Clinical Psychopharmacology*, *28*(4), 418–423. https://doi.org/10.1097/JCP.0b013e31817e6f80
- Sarjeant, K., & Stephens, J. M. (2012). Adipogenesis. *Cold Spring Harbor Perspectives in Biology*, *4*(9), a008417. https://doi.org/10.1101/cshperspect.a008417
- Schrager, S., & Potter, B. E. (2004). *Diethylstilbestrol Exposure - American Family Physician*. Retrieved from www.aafp.org/afp.
- Sharma, G., Hu, C., Brigman, J. L., Zhu, G., Hathaway, H. J., & Prossnitz, E. R. (2013). GPER deficiency in male mice results in insulin resistance, dyslipidemia, and a proinflammatory state. *Endocrinology*, *154*(11), 4136–4145. https://doi.org/10.1210/en.2013-1357
- Sharma, G., & Prossnitz, E. R. (2017). G-protein-coupled estrogen receptor (GPER) and sex-specific metabolic homeostasis. In *Advances in Experimental Medicine and Biology*. 1043, 427–453. https://doi.org/10.1007/978-3-319-70178-3_20
- Sharma, G., & Prossnitz, E. R. (2021). Targeting the G protein-coupled estrogen receptor (GPER) in obesity and diabetes. *Endocrine and Metabolic Science*, *2*, 100080. https://doi.org/10.1016/j.endmts.2021.100080
- Somm, E., Schwitzgebel, V. M., Toulotte, A., Cederroth, C. R., Combescure, C., Nef, S., … Hüppi, P. S. (2009). Perinatal exposure to bisphenol A alters early adipogenesis in the rat. *Environmental Health Perspectives*, *117*(10), 1549–1555. https://doi.org/10.1289/ehp.11342
- Stahlhut, R. W., Wijngaarden, E. van, Dye, T. D., Stephen, C., & Swan, S. H. (2007). Concentrations of urinary phthalate metabolites are associated with increased waist circumference and insulin resistance in adult U.S. males. *Environmental Health Perspectives*, *115*(6), 876–882. https://doi.org/10.1289/EHP.9882
- Stel, J., & Legler, J. (2015). The role of epigenetics in the latent effects of early life exposure to obesogenic endocrine disrupting chemicals. *Endocrinology*, *156*(10), 3466–3472. https://doi.org/10.1210/EN.2015-1434
- Stger, R. (2008). Epigenetics and obesity. *Pharmacogenomics*, Vol. 9, pp. 1851–1860. https://doi.org/10.2217/14622416.9.12.1851
- Tafuri, S. R. (1996). *Troglitazone Enhances Differentiation, Basal Glucose Uptake, and Glut1 Protein Levels in 3T3-Ll Adipocytes*. Retrieved from https://academic.oup.com/endo/article/137/11/4706/2499032
- USEPA. (2021). What is the Endocrine System? | US EPA. Retrieved November 6, 2021, from https://www.epa.gov/endocrine-disruption/what-endocrine-system
- Verhaegen, A. A., & Van Gaal, L. F. (2017). Drug-induced obesity and its metabolic consequences: a review with a focus on mechanisms and possible therapeutic options. *Journal of Endocrinological Investigation*, *40*(11), 1165–1174. https://doi.org/10.1007/s40618-017-0719-6
- Vernon, R. G., & David J. Flint. (2011). Adipose tissue. *Journal Fur Asthetische Chirurgie*, *4*(1), 28–34. https://doi.org/10.1007/s12631-010-0116-x
- Wake, D. J., Rask, E., Livingstone, D. E. W., So¨derberg, S., So¨derberg, S., Olsson, T., & Walker, B. R. (2003). *COMMENT Local and Systemic Impact of Transcriptional Up-Regulation of 11-Hydroxysteroid Dehydrogenase Type 1 in Adipose Tissue in Human Obesity*. https://doi.org/10.1210/jc.2003-030286
- WHO. (2021). WHO. Retrieved May 15, 2021, from World Health Organization website: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight
- Wu, Z., Bucher, N. L., & Farmer, S. R. (1996). Induction of peroxisome proliferator-activated receptor gamma during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPbeta, C/EBPdelta, and glucocorticoids. *Molecular and Cellular Biology*, *16*(8), 4128–4136. https://doi.org/10.1128/mcb.16.8.4128
- Yang, C., Lee, H. K., Kong, A. P. S., Lim, L. L., Cai, Z., & Chung, A. C. K. (2018). Early-life exposure to endocrine disrupting chemicals associates with childhood obesity. *Annals of Pediatric Endocrinology and Metabolism*, Vol. 23, pp. 182–195. Korean society of pediatric endocrinology. https://doi.org/10.6065/apem.2018.23.4.182
- Yang, L.-H., Chen, T.-M., Yu, S.-T., & Chen, Y.-H. (2007). Olanzapine induces SREBP-1-related adipogenesis in 3T3-L1 cells. *Pharmacological Research*, *56*(3), 202–208. https://doi.org/10.1016/J.PHRS.2007.05.007
- Zhu, P., Yuen, J. M. L., Sham, K. W. Y., & Cheng, C. H. K. (2013). GPER mediates the inhibitory actions of estrogen on adipogenesis in 3T3-L1 cells through perturbation of mitotic clonal expansion. *General and Comparative Endocrinology*, *193*, 19–26. https://doi.org/10.1016/j.ygcen.2013.07.004

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Review

Check-list of additional taxa to the supplement of flora of Turkey X

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ABSTRACT

The 10th in the checklist series, this "Check-list of additional taxa to the supplement flora of Turkey", contains 225 taxa from 193 manuscripts published between January 2019 and December 2021. These taxa are not found in any of the other 11 volumes of 'Flora of Turkey' nor in the nine previously published check-lists. The following taxa are now added to the Turkish flora as a result of this paper: 180 taxa new to science and 45 taxa with new records for the Turkish flora.

Keywords: Additional taxa, Turkish flora, New species, New records

INTRODUCTION

In terms of plant diversity, Turkey is one of the most significant temperate countries on the planet. The country's vascular plant diversity has been documented in the "Flora of Turkey and the East Aegean Islands", which was published in nine volumes (Davis, 1965-1985). With the publication of this flora, Turkish and other country botanists, both scientists and amateurs, have gained a newfound interest in the rich plant diversity of Turkey. As a result of this interest, the flora of Turkey is now better known. The identification of these additional taxa necessitated the publication of supplementary volumes to the Flora of Turkey; vol. 10 (Davis, Tan, & Mill, 1988) and vol. 11 (Güner, Özhatay, Ekim, & Başer, 2000). The 11 volumes describe 8,796 species from Turkey in total (excluding an additional 192 species restricted to the east Aegean Islands). Of these, a total of 2,941 species are Turkish endemics.

Following the publication of the Flora of Turkey, subsequent research has greatly increased our understanding of the flora, resulting in the addition of numerous new taxa. These additional taxa were published as a series of papers titled "Checklist of additional taxa to the supplement flora of Turkey'. These checklists were published in a series of papers starting in the year 1994 based on all the original publications of these taxa. They were later compiled by the authors of this paper as a collection housed in the library of the Department of Pharmaceutical Botany, Istanbul University (ISTE Herbarium Library).

Following the publication of the 11th volume of Flora of Turkey, an additional 717 new taxa were added in the period up to 2011 (Özhatay & Kültür, 2006; Özhatay, Kültür, & Aslan, 2009; Özhatay, Kültür, & Gürdal, 2011), including 497 new taxa described with an additional 220 taxa new to Turkey (Table 1). In 2012, a book was published which included all the vascular plants listed by Güner, Aslan, Ekim, Vural, & Babaç (2012) in their document in Turkish, titled "Turkey Bitkileri Listesi (Damarlı Bitkiler)" and 12 new species described for plant science. After the publication of this book, checklists VI, VII, VIII and IX were printed in quick succession (Özhatay, Kültür, & Gürdal, 2013; 2015; 2017; 2019). During the period 2013 to 2019, a total of 638 new taxa were added to the Turkish flora, including 508 new taxa to plant science and an additional 130 taxa new to Turkey.

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Table 1. Statistical table of summary data of additional taxa for the checklists. (N: new taxa for science, R: new record for Turkish flora).

	Check-list III (2006)		Check-list IV (2009)		Check-list V (2011)		Check-list VI (2013)		Check-list VII (2015)		Check-list VIII (2017)		Check-list IX (2019)		Check-list X	
	N	R	N	R	N	R	N	R	N	R	N	R	N	R	N	R
Sp.	154	75	85	40	158	53	141	32	76	19	110	21	102	33	153	33
Subsp.	12	16	23	13	20	4	29	5	1	2	12	2	10	7	8	8
Var.	15	13	9	1	9	3	8	4	-	-	3	-	2	2	9	2
Hyb.	8	2	3	-	1	-	8	1	1	-	2	2	3	-	10	2
Total	189	106	120	54	188	60	186	42	78	21	127	25	117	42	180	45

Vol. 11 (2000) based on Check-list I & II

Güner et al. (2012)

This paper is the 10th in the series of publications based on 190 published papers on additional taxa to the Turkish flora from 2019 to 2021 together with any missing records. Nine publications that were overlooked in the previous checklists have been added to this checklist. Since these publications came out during this period, they have been included in this list.

In this paper, 225 taxa are listed, 180 of them as new taxa to science (comprising 153 species, 8 subspecies, 9 varieties, 10 hybrids), and a further 45 taxa as new records for the country (33 species, 8 subspecies, 2 varieties, 2 hybrids) (Figure 1). *Allium* L., *Centaurea* L., *Crocus* L., *Muscari* Mill., and *Ornithogalum* L. are the five genera which are represented by the most taxa in this study (Figure 2).

The list of the additional taxa

Information regarding the type of examined specimens is written in the same way as in the original references. e= endemic species; * = new record for Turkish flora; **= new taxon for science; ■ = new genus for Turkish flora; ■■= new genus for science.

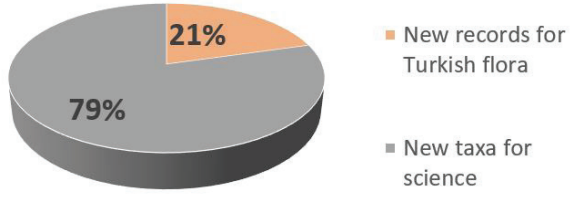

Figure 1. Additional taxa in Check-list X.

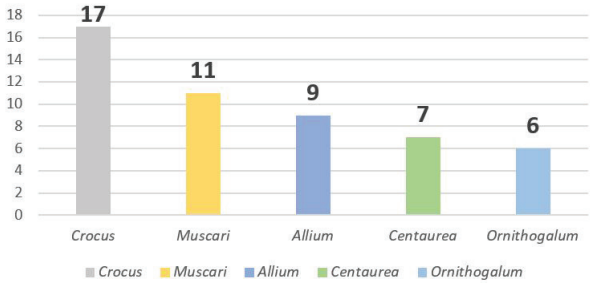

Figure 2. The most species have been added in Check-list X.

PTERIDOPHYTA

DRYOPTERIDACEAE

***Polystichum* roth**

e**P. asiae-minoris*** Tunçkol & Li Bing Zhang in Phytotaxa 447 (4): 296 (2020) [Tunçkol, Aytaç, Aksoy, & Fişne, 2020]

Type: Turkey. Kastamonu: Küre Mountains National Parks, Horma Canyon, Limestone Bedrock, 41°38'04"N, 33°08'35"E, elev. 800 m, 20 July 2018, *B. Tunçkol T4500* (holotype CDBI, isotypes ISTO-38328).

DICOTYLEDONS

AMARANTHACEAE

***Amaranthus* L.**

A. crassipes Schltdl. in Linnaea 6: 757 (1831). [Uygur, Tetik, & Doğru-Koca, 2021]

Examined specimen: Adana: Yumurtalık, turunçgil bahçesi, 25 vii 2018, S. Uygur CUBK-1AMAF-1672 (Çukurova Üniv. Bitki Koruma Bölümü, HUB).

BORAGINACEAE

***Buglossoides* Moench**

B. incrassata (Guss.) I.M.Johnst. subsp. ***splitgerberi*** (Guss.) E. Zippel & Selvi in Taxon 58(2): 624 (2009). [Raab-Straube & Raus, 2020]

Examined specimens: Burdur, macchie, 17 Apr 1985, *Nydegger 40250* (BASBG); ibid., Antalya, Campus University Akdeniz, ruderal, 7 Apr 1998, *Even 50.3* (B). Turkey-in-Europe: Umgebung von Konstantinopel, San Stefano, 6 Apr 1895, *Nemetz* (WU 2001-12328).

***Omphalodes* Mill.**

e***O. nedimeae* Aykurt & Sümbül in Phytotaxa, 498(4), 247 (2021). [Aykurt, Sümbül, Gülben, Sarı, & Konuralp, 2021]

Type: Turkey. Antalya: Kumluca, Bey Mountains in Sarıkaya Wildlife Development Area, between Kırkmuar and Kızlar Sivrisi, shady rock crevices, 2100 m elevation, 07.06.2019, *C. Aykurt 5123 & H. Sümbül* (holotype: AKDU; isotype: AKDU).

***Onosma* L.**

e***O. onur-koyuncui* Sezer in Pak. J. Bot., 53(4): 1317 (2021). [Sezer, 2021]

Type: Turkey. B3 Kütahya: Sökmen village, Calcareous rock and soils, 1050 m, 39º 35' 15" N - 30º 11'43" E, 31.05.2017, *O. Sezer*, 16481 (OUFE).

e***O. satensis* Fırat & Binzet in Adansonia 43(16): 188 (2021). [Fırat & Binzet, 2021]

Type: Turkey. C9 Hakkâri, Yüksekova Province, Sat mountains, Oremar region, Zozana Herduav plateau, rocky, stone, and calcareous areas, 1376 m, 37°22'41"N, 44°10'08"E, 7.VII.2018, *M. Fırat 34040* (holo-, VANF; iso- ANK, in the personal herbarium of the first author [Herb. M. Fırat] and the Herbarium of Mersin University).

***Rindera* Pallas**

e***R. cetineri* Yıldırım in Phytotaxa 427(4): 252 (2019). [Yıldırım, 2019]

Type: Turkey. Denizli: Çameli District, Akdağ Mountain, Karkın Platue, northern slopes, 2200 m, 05 July 2018, *H. Yıldırım 7671 & R. Çetiner* (holotype EGE 42452, isotype EGE, NGBB).

CAMPANULACEAE

***Asyneuma* Griseb. & Schenk**

e***A. hasandaghense* Hamzaoğlu in Türler ve Habitatlar 2(1): 70 (2021). [Hamzaoğlu, 2021b]

Type: Turkey. B5 Aksaray: Helvadere, Hasan Dağı, Turizm Merkezi güneyi, zirve yakını, volkanik kayalı yamaçlar, 2900 m, 29.06.2019, Hamzaoğlu 7610 (GAZI; izotip: GAZI, ANK, HUB, AYBU [Ankara Yıldırım Beyazıt Üniversitesi Herbaryumu]).

***Campanula* L.**

e***C. phitosiana* Yıldırım & Şentürk in Phytotaxa 399 (1): 30 (2019). [Yıldırım, Şentürk, Özdöl & Pirhan, 2019]

Type: Turkey. İzmir: Tire, Dallık to Dibekçi road, 5 km to Dibekçi, in gorge, schistose rock cracks, 30°01'N, 27°50'E, 1175 m, 07.06.2017, *H.Yıldırım 5866, O.Şentürk & A.F.Pirhan* (holotype: EGE 42450, isotypes: EGE 42451, ANK, HUB, NGBB).

CARYOPHYLLACEAE

***Arenaria* L.**

e***A. goekyigitii* M. Dinç & S. Doğu in Phytotaxa 459 (1): 70 (2020). [Dinç & Doğu, 2020]

Type: Turkey. C5 Karaman: Ermenek, Büyükkarapınar plateau, Dulavrat Hill, rocky area, under canopy, 1750 m, 26 june 2013, M.Dinç 3552 & S. Doğu (holotype KNYA, isotypes GAZI and personal herbarium of Şinasi Yıldırımlı, named "Yıldırımlı Herbarium").

***Bolanthus* (Ser.) Reichb.**

e***B. aziz-sancarii* Koç & Hamzaoğlu in Systematic Botany 44(1):195 (2019) [Koç, Hamzaoğlu, & Büyük, 2019]

Type: Turkey. Afyonkarahisar: between Bayat and Iscehisar, 1500 m, fissures of tuffaceous volcanic rocks, 02 Jul 2010, Koç 1209 & Hamzaoğlu (holotype: GAZI, isotypes: GAZI, ANK, HUB).

***Bufonia* Sauvages**

e***B. ali-nihatii* Özdeniz in Fresenius Environ. Bull. 28(12): 9176 (2019). [Ozdeniz, 2019]

Type: Turkey. B6 Sivas: 8 km from BEypinari to Zara, step slopes, 07.08.1982, P.H. Davis & Ekim 68799 (holotype: ANK, isotypes: Bozok Univ. Herb., GAZI).

***Cerastium* L.**

e***C. comatum* Desv. var. ***longipedicellatum*** M.Keskin in Front Life Sci RT 2(2): 36 (2021). [Keskin, 2021a]

Type: Turkey. Balıkesir, Ayvalık, Maden Adası, 29.iv.1997, 30 m, K. Alpınar ISTE 74000.

e***C. inflatum* Link ex Boiss. var. ***longum*** M.Keskin in Front Life Sci RT 2(2): 39 (2021). [Keskin, 2021a]

Type: Turkey. Bitlis, Tatvan, Zulül kaya, çayırlık ve kenarı, ca.1850 m, 20.vi.1986, Ö.Seçmen 3489 (EGE 17976).

e***C. semidecandrum* L. var. ***delicatum*** M.Keskin in Front Life Sci RT 2(2): 39 (2021). [Keskin, 2021a]

Type: Turkey. İzmir: Bergama-Kozak, çayırlık, 400 m, 27.ii.1986, G.Görk (EGE 315601).

******C. szowitsii* Boiss., Fl. Or. I: 717 (1867) [Karaer, Terzioğlu, & Kutbay, 2020]

Examined specimens: A8 Artvin - Hatila Valley National Park, rocky places, roadsides, 559 m, 19.v.2012, KATO 18913 KTUB 1097 ibid., FK 17305 (OMUB), FK 18250 ibid., 23.v.2011 (OMUB).

***Dianthus* L.**

e***D. anatolicus* Boiss. var. ***afyonensis*** Uğurlu, M. Koch & Dönmez in Ann. Bot. Fennici 58: 21 (2020). [Uğurlu Aydın, Koch, & Dönmez, 2020]

Type: Turkey. Afyonkarahisar: Çay, from Armutlu village to Kaşıkara village, crevices on limestone, 42°51´13.9´´N, 31°04´27´´E, 1306 m a.s.l., 20 June 2018 *A.A. Dönmez 20098-Z. Uğurlu Aydın* (holotype HUB, isotypes GAZI, HEID).

******D. azkurensis* Sosn. in Vestn. Tiflissk. Bot. Sada n.s., 1: 74 (1923). [Hamzaoğlu & Koç, 2019]

Examined specimen: Turkey. A9 Ardahan: Çıldır, Yıldırımtepe köyü, Şeytankalesi civarı, 1950 m, kayalık otlu yerler, 4.7.2013, Hamzaoğlu 6815 & Koç (GAZI, ANK, HUB).

e***D. demirkushii* Yıld. & Kılıç in Ot Sistematik Botanik Dergisi, 26(2): 60 (2019). [Yıldırımlı & Kılıç, 2019i].

Type: C7 Adıyaman: Merkez, Kuyulu (Turuş) köyü üstü, Atatürk barajı karşısı, çam ormanı altı ve açıklığı, 700-710 m, 28.04.2018, Ş. Yıldırımlı 44154 & Ö. Kılıç (holo. Yıldırımlı Otluk'u; iso. HUB).

e***D. dumanii* Hamzaoğlu in Türler ve Habitatlar 1(1): 22 (2020). [Hamzaoğlu, 2020b]

Type: Turkey. Eskişehir: Günyüzü, Kavuncu ve Fatih köyleri arası, yaklaşık 3. km, 750 m, 36S 405276 D - 4363002 K, jipsli bozkır, 25.06.2019, E.Hamzaoğlu 7624 (holotip: GAZI, isotip: GAZI, ANK, HUB).

e***D. hamzaoglui* Koç in Phytotaxa 439(1): 58 (2020). [Koc, 2020]

Type: Turkey. Sivas: Between Sivas and Kangal, Gürün junction, around abandoned quarry, igneous rocky steppes, 1650 m a.s.l., 14 July 2018, *Hamzaoğlu & Koç* 3431 (holotype GAZI, isotypes GAZI, ANK, HUB).

e***D. hymenolepis* Boiss. subsp. ***bingolensis*** Hamzaoğlu & Behçet in KSU J. Agric Nat 23 (6): 1530 (2020). [Hamzaoğlu, Behçet, & Yapar, 2020]

Type: Turkey. B8 Bingöl: Bingöl, S. of Alıncık village, 38°52'09"K - 40°26'02"D, *Quercus petrae* openings, rocky slopes, 1440 m a.s.l., 01.07.2018, L.Behçet and Y.Yapar 15493 (holo. GAZI, iso. ANK, Bingöl Univ. Herb.)

******D. orientalis* Adams subsp. ***aphanoneurus*** Rech.f. in Pl. Syst. Evol. 151: 290 (1986). [Hamzaoğlu & Koç, 2019]

Examined specimens: Turkey. B6 Kayseri: Sarız, Kıskaçlı köyü, Kayseri- Kahramanmaraş yolu, dinlenme tesisleri civarı, 1830 m, akışkan kalker kayalıklar, 8.8.2018, Hamzaoğlu & Koç 3491 (GAZI, ANK, HUB); Malatya: Darende, Aşağıulupınar köyü güneyi, 1300 m, kumlu taşlı yamaçlar, 12.7.2010, Koç 1245 & Hamzaoğlu (GAZI, ANK, HUB); aynı yer, taşlı kumlu yamaç, 25.7.2013, Hamzaoğlu 6947 & Koç (GAZI); C5 Mersin: Toroslar, Arslanköy-Mersin arası, c. 20 km, 980 m, orman açıklığı, 19.8.2013, Hamzaoğlu 6988 & Koç (GAZI, ANK, HUB).

e***D. yilmazii* Hamzaoğlu & Koç in Kew Bulletin 76: 524 (2021). [Hamzaoğlu, Koç, & Büyük, 2021]

Type: Turkey. Kayseri, Felahiye, Silahtar village, vineyard path, 1260 m, 11 July 2017, Hamzaoğlu & Koç 3185 (holotype GAZI; isotypes ANK, GAZI, HUB).

***Gypsophila* L.**

e***G. malyerii* Hamzaoğlu & Koç in Kew Bulletin 76(3): 533 (2021). [Hamzaoğlu, Koç, & Topal, 2021]

Type: Turkey. B4 Ankara: Beypazarı, between Harmanak-Uşakbükü, 36T0392454-4430814, 510 m, 22 Aug. 2020, marn areas, Hamzaoğlu & Koç 7825 (holotype GAZI; isotypes ANK, GAZI).

***Lepyrodiclis* Fenzl ex Endl.**

e***L. alinihatii* Menemen, Yalçınkaya & Erden in Nord J Bot 39(3)-e03037: 2 (2021). [Menemen, Yalçınkaya, & Erden, 2021]

Type: Turkey. Kleinasien [Anatolia], Bitlis to Tatvan, 1700–1900 m a.s.l., 5 Jul 1951, Renz s.n. (holotype: G00446762 [two sheets]; isotype: ADO [small parts removed from the holotype]).

***Minuartia* L.**

e***M. alpuensis* Çilden & Altınözlü in Phytotaxa 443 (1): 81 (2020). [Çilden & Gençay Çelemli, 2020]

Type: Turkey. B3 Eskişehir: Alpu, Kireçköy, rocky areas southwest of the village, 990 m elevation, 4404500N, 309547E, calcareous fields, 27 Aug 2019, E. Çilden 1947 (holotype: HUB, isotypes: ANK, GAZI).

******M. granuliflora* (Fenzl) Grossh. in Fl. Kavk. II: 381 (1842). [Koç, Hamzaoğlu, & Aksoy, 2020]

Examined specimen: Turkey. A8 Bayburt: Between Bayburt and Aşkale, Kop Mountain, Çalıdere village, 1815 m a.s.l., 12.07.2009, M.Koç 600, Hamzaoğlu & Budak (GAZI).

e***M. torosensis* Koç & Hamzaoğlu in Phytotaxa 391 (2): 124 (2019). [Koç, Hamzaoğlu, & Aksoy,2019]

Type: Turkey. C3 Antalya, Gebiz district, Bozburun Mountain, between Boğazağzı-Tozluçukur plateu, 1500 m, 18.06.2015, 37 20 N-31 02 E, M. Koç 2690, Hamzaoğlu & Budak (holotype GAZI, isotype ANK, GAZI, HUB).

***Minuartiella* Dillenberger & Kadereit**

e***M. serpentinicola* Koç & Hamzaoğlu in Phytotaxa 409(3): 164 (2019). [Koc, Hamzaoğlu, & Aksoy, 2019]

Type: Turkey. Yeşilova village, Eşeler mountain, 41°53'N–027°29'E, 2260 m, 18.08.2014, stony area with serpentines, *Koç*, *Hamzaoğlu* & *Aksoy 1847* (holotype GAZI, isotypes GAZI, ANK, HUB).

***Moenchia* Ehrh.**

e***M. akmanii* M.Keskin in Biodicon 14(1): 125 (2021). [Keskin, 2021b]

Type: Turkey, Ankara: Beypazarı, Eğriova, yavaş akan dereciklerde, 1550 m, 26 vi 1973, Y.Akman (ANK 8518).

***Sabulina* Rchb.**

e***S. nerimanae* Koç & Hamzaoğlu in Phytotaxa 498 (4): 284 (2021). [Koç, Hamzaoğlu, & Aksoy, 2021]

Type: Turkey. C2 Burdur, Altınyayla district, between Ören to Ballıköy, calcereous rocks, 1320 m, 26.06.2015, *Koç* 2054, *Hamzaoğlu & Aksoy* (holotype GAZI, isotype ANK, GAZI).

e***S. tortumensis* Koç & Hamzaoğlu in Folia Geobotanica, 54: 286 (2019). [Koç, Hamzaoğlu, & Aksoy, 2019]

Type: Turkey. A8 Erzurum, Tortum district, Meydanlar village, 2,020 m a.s.l., 40°21′N; 041°25′E, 14 July 2016, volcanic rocks, Koç et. al. 2351 (holotype: GAZI; isotypes: GAZI, ANK, HUB).

***Silene* L.**

e***S. arsuzensis* Özbek & Uzunh. in Phytotaxa 397 (1): 75 (2019). [Özbek & Uzunhisarcıklı, 2019]

Type: Turkey. C6 Hatay: İskenderun, Arsuz, around Kale Village, 0–5 m, serpentine stony sandy places, 36° 17′ 04″ N, 35° 47′ 23″ E, 09.04.2008, *E.Uzunhisarcıklı* & *U.Özbek* 2698 (holotype GAZI, isotype ANK).

e***S. bocquetiana* Yıld. & Kılıç in Ot Sistematik Botanik Dergisi, 26(2): 65 (2019). [Yıldırımlı & Kılıç, 2019i].

Type: B8 Bingöl, Bingöl-Sancak arası, çayır, bayır, yamaç, meşelik (*Quercus brandii*), 1550-1600 m, 04.05.2018, Ş. Yıldırımlı 44474 & Ö. Kılıç (holo. Yıldırımlı Otluk'u iso. EGE, GAZI, HUB).

e***S. karakotchanensis* Yıld. & Kılıç in Ot Sistematik Botanik Dergisi, 26(2): 69 (2019). [Yıldırımlı & Kılıç, 2019i].

Type: B7 Elazığ, Karakoçan, Golan kaplıcası, Peri suyu kıyısı ve yolu boyunca, orman, ağaçlık, 960-975 m, 01.05.2018, Ş. Yıldırımlı 44349 & Ö. Kılıç (holo. Yıldırımlı Otluk'u (YO); iso. GAZI, YO).

CHENOPODIACEAE

***Atriplex* L.**

e***A. turcica* Başköse & Yaprak in Phytotaxa 424(4), 233 (2019). [Başköse & Yaprak, 2019]

Type: Turkey. Sivas, Şarkışla, N of Ortatopaç village, Kızılırmak river edge, 1183 m, 30 September 2014, 039º 22.948′ n–036º15.047′ E, *Yaprak et Başköse 2781* (holotype and isotype, ANK).

***Suaeda* Forssk. ex J.F. Gmelin**

******S. aegyptiaca* (Hasselq.) Zohary in J. Linn. Soc., Bot. lv. 635 (1957). [Başköse & Yaprak, 2021]

Examined specimen: Turkey. C7 Şanlıurfa province, Akçakale district**,** Akçakale-Ceylanpınar road, ŞUSKİ wastewater treatment facility, on the road of Öncül village, approximately 1-1.5 km, irrigation channel, road and field edges**,** 344 m. a.s.l, 22.09.2018, 28.10.2018, 20.07.2019, N 36° 42′ 52.14″ - E 38° 58′ 48.37″ E, coll. İ. Başköse 4435, 4456, 4746 (ANK).

CISTACEAE

***Cistus* L.**

******C.* × *florentinus* Lam. in Encycl. 2(1): 17 (1786). [Şen Gökmen & Duman, 2021]

Cistus × *florentinus* Lam.= *Cistus monspeliensis* L. and *Cistus salviifolius* L.

Examined specimens: Turkey. Muğla: Bodrum, Kızılağaç-Bodrum yolu 1.km., yol kenarı, maki, frigana, 35 S 541848-4100769, 80-83 m, 28. İv. 2021, F. Şen 1099, H. Duman; Bodrum, Kızılağaç-Bodrum yolu 1.km., yol kenarı, maki, frigana, 35 S 541848-4100769, 80-83 m, 28. İv. 2021, F. Şen 1100, H. Duman (çiçekte erkek organlar körelmiş) ; Bodrum-Demirçiftliği, maki, v 1966, H. Peşmen (EGE 3911 b); (EGE 5120b).

CRASSULACEAE

***Crassula* L.**

******C. vaillantii* (Willd.) Roth in Enum. 1: 992 (1827). [Bozyel, Pelit, Şenol, 2021]

Examined specimen: İzmir: Çeşme Gölobası mevkii, dönemsel sulak alanlar, 38°16′10.95″K, 26°25′57.05″D; 100m., 23.03.2019, leg. Duygu Bozyel, Bahar Pelit, Orsa Yüzbaş, (EGE 43223).

***Rosularia* (DC.) Stapf**

e***R. paluensis* Yıld. & Kılıç in Ot Sistematik Botanik Dergisi, 26(2): 98 (2019). [Yıldırımlı & Kılıç, 2019d].

Type: B7 Elazığ, Palu, kale, kayalık, taşlık, çayırlık, tepe, bayır, 1150-1250 m, 10.06.2015, Ş. Yıldırımlı 41332 & Ö. Kılıç (holo. Yıldırımlı Otluk'u; iso. HUB).

COMPOSITAE

***Achillea* L.**

e***A. kirschneri* Yıld. & Kılıç in Ot Sistematik Botanik Dergisi, 26(1): 80 (2019). [Yıldırımlı & Kılıç, 2019g].

Type: B7 Elazığ, Karakoçan, Golan kaplıcası, Peri suyu kıyısı ve yolu boyunca, orman, ağaçlık, 960-975 m, 01.05.2018, Ş. Yıldırımlı 44325 & Ö. Kılıç (holo. Yıldırımlı Otluk'u (YO); iso. GAZI, YO).

***Anthemis* L.**

e***A. ekicii* Özbek, H.Duman & Aytaç in Turk J Bot 45: 60 (2021). [Özbek, Duman, Özbek, & Aytaç, 2021]

Type: Turkey. B4 Aksaray: Eskil, 3–4 km East of Eskil, semihalophytic steppe, 926 m, 06.06.2008, H.Duman 9745 et al. (Holotype: GAZI, isotypes: ANK, HUB, GAZI).

e***A. rumelica* (Velen.) Stoj. & Acht. in Notizbl. Bot. Gart. Berlin-Dahlem 13: 516 (1937). [Vladimirov, Aybeke, & Tan, 2020]

Examined specimens: In collibus arenosis circa Constantinoplem ad stationem Bijuk-Han, 14.05.1913, coll. B. Davidov (SOM 78448).

***Aytacia* Yıld.**

Type species: *Aytacia turkica* Yıld.

e*****A. turkica*** Yıld. in Ot Sistematik Botanik Dergisi 27(1-2): 20 (2020). [Yıldırımlı, 2020a]

Type: Turkey. B6 Kayseri: Sarız, Yalak, Binboğa dağı, Körkuyu-Sıçak yaylası arası, 2400-2600 m, 21.07.1992, Z. Aytaç & H. Duman (5444) at fruiting time (holo. GAZI).

***Bidens* L.**

***B. pilosa** L. in Sp. Pl. 2: 832 (1753). [Yıldırım, Özdöl, & Yaşayacak, 2019]

Examined specimens: Turkey. Osmaniye: Kırmıtlı ile Yeniköy arası, Kırmıtlı içmesuyu sondaj alanı yanı, taşkın seddesi kenarı, sulama arkı çevresi, tarlalar arası, yol kenarı, sulama arkı boyunca, 37°9′41.31″K, 36°8′42.46″ D21.08.2018, H. Yıldırım 7694 (EGE).

***Centaurea* L.**

e***C. akcadaghensis** Uysal & Şirin in Mediterranean Botany 41 (2): 174 (2020). [Şirin, Uysal, Bozkurt, & Ertuğrul, 2020]

Type: Turkey. Malatya, Akçadağ, Levent Canyon, Çayözü village, open *Quercus* forest, 1171 m, 28 April 2018, T. Uysal 3612 (holotype KNYA, isotype ANK).

e***C. ermenekensis** Uysal & Şirin in Mediterranean Botany 41 (2): 176 (2020). [Şirin et al., 2020]

Type: Turkey. Karaman, Ermenek, West of Göktepe, 1300 m, steppe, 19 June 2017, K. Ertuğrul 5377, T. Uysal & M. Bozkurt (holotype KNYA, isotype ANK).

***C. gulissashwilii** Dumbadze in Dokl. Akad. Nauk Armyanskoi S.S.R. 5(2): 49 (1946). [Hamzaoğlu & Koç, 2020]

Examined specimen: Turkey. A9 Ardahan: Posof, Sarıdarı köyü, Posof Çayı karşısı, TANAP Boru Hattı civarı, 38 T 0318759 – 4603548, 1215 m a.s.l., taşlı yamaçlar, bozkır, 26.08.2020, Koç & Hamzaoğlu 7830 (GAZI, ANK).

e***C. hekimhanensis** Şirin & Yıldırım in Botanica Serbica 45 (1): 14 (2021). [Şirin, Yildirim, Uysal, & Ertuğrul, 2021]

Type: Turkey. Malatya, Southwestern slopes of the Yamadağı, stony-gravely areas, 2545 m, 19.VI.2015, H. Yıldırım 3974 (holo. KNYA; iso. ANK).

e***C. isilae** H. Duman, Uzunh. & Y. Bahadır in Nord J Bot 39(10)-e03244: 2 (2021). [Duman, Uzunhisarcıklı, & Bahadır, 2021]

Type: Turkey. Samsun: Vezirköprü, Küçükale village, around of Kuyuma HES regulator, 1050 m a.s.l., limestone cliffs, 21 Jul 2020, H. Duman 10662 (holotype GAZI; isotypes ANK, HUB).

e***C. kirikkalensis** Özbek in Nord J Bot 39(7)-e03235: 2 (2021). [Özbek, 2021]

Type: Turkey. A4/5 Kırıkkale: Delice, above Baraklı village, *Quercus pubescens* Willd. clearings and steppe, 1100–1150 m a.s.l., 03 Jul 2016, Hamzaoğlu 7239 et al. (holotype: GAZI; isotypes: ANK HUB).

e***C. uysalii** Şirin & Çeçen in Turkish Journal of Botany 43:810 (2019). [Şirin, Çeçen, Bozkurt, & Ertuğrul, 2019]

Type: Turkey. C4 Karaman: Çakırdağı, between Kavurgalık Hill and Pelitli Stream, rocky area, 1270-1390 m, 37°23′11″ N, 33°29′ 26″ E, 01 May 2018, E. Şirin 733 and Ö. Çeçen (holotype KNYA, isotype ANK).

***Cirsium* Mill.**

e***C. ayasii** H.Duman & Dirmenci in Bağbahçe Bilim Dergisi 7(3): 36 (2020) [Dirmenci, Duman, & Arabaci, 2020]

Type: Turkey. Kütahya, Türkmenbaba Dağı, Başören köyü-İncik köyü arası, 1,5 km, karaçam ormanı açıklığı, 39.54473 N 30.29153 E, 10.08.2018, *H.Duman* 10519 (Holotip: GAZI; izotip: ANK, HUB).

e***C. × erzincanicum** Yıldız, Dirmenci & Arabacı in Turk J Bot 43: 369 (2019). [Dirmenci et al., 2019]

Type: Turkey. B7 Erzincan: between Erzincan and Kelkit, Pöske pass, steppe, 39.89030°N, 39.36216°E, 2100 m, 02.09.2016, Dirmenci 4661, Yıldız & Arabacı (Holotype: ISTE, isotype: ANK)

e***C. × kelkitensis** Yıldız, Arabacı & Dirmenci in Turk J Bot 43: 369 (2019). [Dirmenci et al., 2019]

Type: Turkey. B7 Erzincan: between Erzincan and Kelkit, Pöske pass, steppe, 39.89030°N, 39.36216°E, 2100 m, 02.09.2016, Dirmenci 4656, Yıldız & Arabacı (Holotype: ISTE, isotype: ANK).

e***C. × nezaketiae** Yıldız, Dirmenci & Arabacı in Turk J Bot 43: 368 (2019). [Dirmenci et al., 2019]

Type: Turkey B7 Erzincan: Between Erzincan and Kelkit, Pöske pass, steppe, 39.89030°N, 39.36216°E, 2100 m, 02.09.2016, Dirmenci 4659, Yıldız & Arabacı (Holotype: ISTE, isotype: ANK)

***Cousinia* Cass.**

e***C. agridagensis** Tugay, Ertuğrul & Ulukuş in Phytotaxa 427 (4): 260 (2019). [Tugay et al., 2019]

Type: Turkey. B9/10 Ağrı: Doğubeyazıt, Ortadirek village, steppe, 1800 m, 18 June 2014, O. Tugay 8055, K. Ertuğrul, D. Ulukuş (holotype and isotypes KNYA).

**C. gigantosphaera* Rech. f. in Anz. Österr. Akad. Wiss., Math.-Naturwiss. Kl. 101: 345 (1964). [Fidan, Pınar, & Eroğlu, 2019]

Examined specimens: Turkey, C9 Şırnak: Beytüşşebap-Ayvalık köyü arası, Ayvalık'tan Beytüşşebap'a doğru 6-8. km, kayalık yamaçlar, meşe açıklıkları, 37° 31› 55» K 43° 06› 42» D, 1240 m, 19.07.2019, *M.Fidan, M.Pınar, H.Eroğlu MMH1710*; Beytüşşebap, Feraşin yaylası yolu 6. km, kayalık yamaçlar, 37° 55′ 26″ K 43° 11′ 09″ D 1623 m, 12.07.2018, *M.Fidan, M.Pınar, H.Eroğlu MMH915*.

***Crepis* L.**

C. setosa** Haller f. subsp. ***topaliana Babc. Univ. Calif. Publ. Bot. 19: 403 (1941). [Inceer & Kalmuk, 2019]

Examined specimens: A1 Kırklareli: Between Kırklareli-Edirne, near Edirne, 150 m a.s.l., 12 June 2015, Inceer 1174 (KTUB); Edirne: Near Lalapasa, 100 m a.s.l., 12 June 2015, Inceer 1181 (KTUB); Tekirdağ: Çorlu, 170 m a.s.l, 13 June 2015, Inceer 1188 (KTUB); B1 Izmir: Between Bayındır-Odemis, 70 m a.s.l., 25 May 2011, Inceer 819 (KTUB); B2 Izmir: Near Bozdağ, 350 m a.s.l., 25 May 2011, Inceer 820 (KTUB).

e***C. vuralii** Yıld. in Ot Sistematik Botanik Dergisi 28(1-2): 151 (2021). [Yıldırımlı, 2021b]

Type: Turkey. A4 Ankara: Ayaş, Aysantı beli, bozkır, marnlı topraklar, dik bayır, 1150-1210 m, 29.06.2020, Ş. Yıldırımlı 46274 (holo. Yıldırımlı Otluk'u Hb. Yıldırımlı; iso. GAZI, HUB, Yıldırımlı Otluk'u).

***Gamochaeta* Wedd.**

***G. coarctata** (Willdenow) Kerguélen in Lejeunia. 120: 104 (1987). [Yılmaz, 2021]

Examined specimen: Turkey. A2 İstanbul: Eyüp-Kemerburgaz. The Kemerburgaz City Forest, walking paths near Alibeyköy Dam, 31.05.2021, 65 m, Yılmaz H., ISTO 38866, 38865.

***Helichrysum* Miller**

e***H.* × *kani-isikii*** Semiz, Şenol & Günal in Phytotaxa 507(4): 285 (2021). [Semiz, Şenol, Günal, Cicek, & Eroğlu, 2021]

Type: Turkey. C2 Denizli: Above Kızılcabölük, Babadağ, near Evran Hill, on calcareous rocks, 1968 m, 37°41´53.94´´N, 28°59´27.85´´E, 29 July 2018, *G. Semiz, S. G. Şenol & B. Günal* (holotype PAUB GSE 2055; isotype EGE).

***Hirtellina* Cass.**

***H. kurdica** (Merxm. & Rech. f.) Dittrich in Boissiera 51: 75 (1996). [Fırat, 2019a]

Examined specimens: Turkey. C9 Şırnak, Cudi Mountains, Göndek region, Rocky slopes, 969 m, 38°25′11″ N, 42°38′57″ E, 15.10.2013, *M. Fırat* 30474 VANF!; C9 Şırnak, Silopi, Cudi Mountain, Hessena region, rock crevices, 870 m, 37°20′56″ N, 42°25′38″ E, 18.10.2013, *M. Fırat* 30484, C9 Siirt; From Siirt to Eruh 10. km, Dry limestone bushy slopes, 974 m, 37°44′25″ N, 42°13′13″ E, 20.10.2013, *M. Fırat* 30490,; C9 Şırnak; Cizre, Gabar Mountain, Dry limestone bushy slopes, 1343 m, 37°33′48″ N, 42°12′21″ E, 06.10.2014, *M. Fırat* 31701

***Lactuca* L.**

e***L. anatolica*** Behçet & Yapar in Phytotaxa 455 (4): 288 (2020). [Behçet & Yapar, 2020]

Type: Turkey. B8 Bingöl: Çapakçur Valley, south of Aşağıköy village, steppe slope, 1720 m, 38°50′42.36″N, 40°21′46.90″E, 25 August 2019, *Behçet* and *Yapar* 17939 (holotype: Bingöl Univ. Biology Dept. Herbarium; isotypes: Bingöl Univ. Biology Dept. Herbarium and ANK).

***Onopordum* L.**

***O. blancheanum** (Eig) Danin in Israel J. Bot. 37: 57 (1988). [Eroğlu, 2021]

Examined specimen: Turkey. C6: Hatay: Yayladağı, Yayladağı-Antakya yolu, Ayışığı köyü yol ayrımı, yol ve tarla kenarı, 35° 59´ 15"K, 36° 07´ 08"D, 910 m, 27.05.2021, H. Eroğlu 1721.

e***O. nezaketianum*** Pınar in Turkish Journal of Botany 43:127 (2019). [Pınar & Eroğlu, 2019b]

Type: Turkey. B5 Nevşehir, Ürgüp-Göreme, Cappadocia, 7 km northeast of Göreme (zelve), 1150 m, marly steppe, 25.07.2015, 3840579 N, 3451808 E, M. Pınar 6896, H. Eroğlu (holotype: VANF, isotypes: GAZI, ANK).

***Podospermum* DC.**

e***P. aksarayense*** Yıld. in Ot Sistematik Botanik Dergisi 27(1-2): 63 (2020). [Yıldırımlı, 2020c]

Type: Turkey. B4 Aksaray: Eskil, Tuzgölü'ne doğru 3. ve 5. km, çorak ve tuzcul alanlar, y. 900 m, 27.07.2014, Ş. Yıldırımlı 40408 (holo. Yıldırımlı Otluk'u (Hb. Yıldırımlı); iso. HUB).

e***P. dincii*** Yıld. in Ot Sistematik Botanik Dergisi 27(1-2): 69 (2020). [Yıldırımlı, 2020c]

Type: Turkey. B3 Eskişehir: Sivrihisar, Aşağıkepen köyü yakını, jipizli ve kireçli yerler, tepe, bayır, y. 1000 m, 18.05.2013, Ş. Yıldırımlı 38872 & H. Işıl Yıldırımlı (holo. Yıldırımlı Otluk'u, Hb. Yıldırımlı).

e***P. omeri*** Yıld. in Ot Sistematik Botanik Dergisi 27(1-2): 73 (2020). [Yıldırımlı, 2020c]

Type: Turkey. B10 Iğdır: Aralık, Nahçıvan sınır kapısına 12 km beride, yol kıyısı, 825 m, 31.05.1998, Ş. Yıldırımlı 21973 & A. Polat (holo. Yıldırımlı Otluk'u (Hb. Yıld.); iso. HUB, Hb. Yıld.).

***Tripleurospermum* Schultz Bip.**

e***T. fissurale*** (Sosn.) E. Hossain var. ***radiata*** Özbek in Biodicon 13(2): 138 (2020). [Özbek & Onaylı, 2020]

Type: Turkey. A8 Artvin. Yusufeli, 1.5 km from Dereiçi village, rocky slopes, 40°51′43″E, 41°31′30″N, 25 May 2019, 745 m, U.Özbek 3117 & M. Ekici (holotype GAZI, isotype AEF, ANK, NGBB).

CRUCIFERAE

***Aethionema* R. BR.**

e***A. aytachii*** Ertuğrul & Hamzaoğlu in Turk J Bot 45: 564 (2021). [Ertuğrul et al., 2021]

Type: Turkey. B4 Ankara: Ayaş, around Aysantı Pass, marly hills along roadsides, 1190 m, 31.v.2019, *K.Ertuğrul* 5757 & *T.Körüklü* (Holotype KNYA; Isotypes GAZI, ANK).

e***A. sancakense*** Yıld. & Kılıç in Ot Sistematik Botanik Dergisi, 26(2): 54 (2019). [Yıldırımlı & Kılıç, 2019a].

Type: B8 Bingöl, Merkez, Aşağıköy'e doğru 7. km, meşe ormanı açıklığı, yamaç, 1450-1550 m, 19.05.2018, Ö. Kılıç 5687 & Ş. Yıldırımlı (holo. Yıldırımlı Otluk'u (YO); iso. GAZI, YO).

e***A. erzincanum*** Kandemir & Aytaç in Ann. Bot. Fenn. 54(1–3): 1 (2017). [Kandemir, Aytaç, & Fişne, 2017]

Type: Turkey. Erzincan: Kemah-íliç, Yahçiler village, 3rd km, 1226 m a.s.l., gypsum steppes, 13 June 2013 Kandemir 10376 (holotype GAZI; isotype ANK).

***Descurainia* Webb & Berth.**

e***D. tugayi*** Yıld. in Ot Sistematik Botanik Dergisi, 26(1): 94 (2019). [Yıldırımlı, 2019b].

Type: B4 Konya, Kulu, Zincirlikuyu, ahıllar arası, yol ve ekin kıyısı, 870-945 m, 04.05.2007, Ş. Yıldırımlı 33687 (holo. Yıldırımlı Otluk'u).

***Heldreichia* Boiss.**

e**H. bupleurifolia* Boiss. subsp. ***malatyana*** Özüdoğru & Yıldırım in Bağbahçe Bilim Dergisi 6 (3): 2 (2019). [Özüdoğru & Yıldırım, 2019]

Type: Malatya: Akçadağ, Levent Kanyonu, Kanyona iniş yolu, küçük kırık taşlık yamaçlar, 1275 m, 30.vi.2010, H. Yıldırım 1745 (holotip: EGE-43206, izotip: EGE-43207, HUB).

***Lepidium* L.**

e**L. tuzgoeluense* Yıld. in Ot Sistematik Botanik Dergisi, 26(1): 97 (2019). [Yıldırımlı, 2019b].

Type: B4 Konya, Kulu, Tuzyaka (Sütkanlı), Bozan köyü, Kaldırım yolu, Tuzgölü kıyısı, tuzcul yerler, 945 m, 04.05.2007, Ş. Yıldırımlı 33663 (holo. Yıldırımlı Otluk'u; iso. GAZI)

***Noccaea* Moench**

e**N. atila-ocakii* Özgişi in Phytotaxa 432 (1): 96 (2020). [Özgişi, 2020]

Type: Turkey. B3 Eskişehir: Sivrihisar, Karacaören village, Arayit (Eryiğit) Mountain, marble rocks and scree, 1670–1820 m, 39°17′47″ N, 31°45′08″ E, 24.vi. 2012, holotype: OUFE 16472, (isotype: HUB).

***Ricotia* L.**

e**R. candiriana* A.Özçandır, Aykurt & Özüdoğru in Phytotaxa 388 (4): 291 (2019). [Özçandır, Aykurt, & Özüdoğru, 2019]

Type: Turkey. Antalya: Kumluca, upper part of Alakır valley, close to the Dibek Nature Conservation Area, screes, 1160 m elevation, 15 June 2017, A. Özçandır 1885 & C. Aykurt (holotype: AKDU; isotype: HUB).

DIPSACACEAE

***Cephalaria* Schrader ex Romer & Schultes**

e**C. adiyamanensis* Yıld. in Ot Sistematik Botanik Dergisi, 26(2): 106 (2019). [Yıldırımlı & Kılıç, 2019c].

Type: C7 Adıyaman, Kahta, Ulupınar köyü, Merdi mezrası, Fırat ırmağı kıyısı, GAP Atatürk barajı altında kalacak yerler, Köyönü harlaları, Duman deresi, Ömerisa çevreleri, 500 m, 28.06.1988, Ş. Yıldırımlı 10867 (holo. Yıldırımlı Otluk'u; iso. GAZI).

e**C. kutahyaensis* Yıld. in Ot Sistematik Botanik Dergisi, 26(2): 109 (2019). [Yıldırımlı & Kılıç, 2019c].

Type: B2 Kütahya, Gümüş (Acem) dağı, radara doğru, *Juniperus excelsa* birliği, kalker kaya tabakaları, 1200-1300 m, 24.09.1999, Ş. Yıldırımlı 24712, D. Pavlova & D. Dimitrov (holo. Yıldırımlı Otluk'u (YO); iso. GAZI, YO).

***Knautia* L.**

***K. arvensis* (L.) Coult. in Mém. Dipsac. 41. 1823: 41 (1823). [Tunçkol, Aksoy, & Yaşacak, 2021]

Examined specimens: Turkey, Kastamonu, Küre Mountains National Park, Pınarbaşı district, 19 May 2019 (B. Tunçkol 4505, DUOF 1946).

***Scabiosa* L.**

e**S. ispartaca* Yıld. in Ot Sistematik Botanik Dergisi, 26(1): 65 (2019). [Yıldırımlı, 2019a].

Type: C3 Isparta, Gölcük, göl kıyısı, kumullar, 1350 m, 2107.1991, Ş. Yıldırımlı 14645 (holo. Yıldırımlı Otluk'u (YO); iso. GAZI, YO).

***S. lucida* Vill. in Prosp. Hist. Pl. Dauphiné 18 (1779). [Aksoy, Çelik, Bozkurt, & Uysal, 2020]

Examined specimens: Turkey. Bayburt: Soğanlı Geçiti'nden Çaykara'ya iniş, alpin çayır, 40°31′451″ K / 040°14′027″ D, 2318 m, 29.08.2020, *Aksoy* 3159, *Uysal* & *J. Çelik* (AKDU 5971); Kuşmer yaylasına gidiş yolu, alpin çayır, 40°30′098″ K / 040°09′883″ D, 2555 m, 29.08.2020, *Aksoy* 3163, *Uysal* & *J. Çelik* (AKDU 5970). Gümüşhane: Zigana Dağı, tesislerin sol tarafı, çayırlık yamaç, 40°38′207″ K, 039°24′567″ D, 2060-2150 m, 25.07.2018, *Aksoy* 2958 & *J. Çelik* (AKDU 5974); aynı yer,, 40°38′237″ K, 039°24′520″ D, 1900-2060 m, 19.08.2019 *Aksoy* 3109 & *J. Çelik* (AKDU 5972). Trabzon: Bayburt-Çaykara yolu, alpin çayırlar, 40°32′832″ K, 040°13′978″ D, 2055 m, 30.08.2020, *Aksoy* 3171, *Uysal* & *J. Çelik* (AKDU 5973).

e**S. sirnakia* Yıld. in Ot Sistematik Botanik Dergisi, 26(1): 70 (2019). [Yıldırımlı, 2019a].

Type: C9 Şırnak, Kumçatı, yol kıyısı, killi dere ve tepe, 600 m, 26.06.1997, Ş. Yıldırımlı 20352 & A.A. Dönmez (holo. Yıldırımlı Otluk'u).

e ****S. sivrihisarica* Yıld. in Ot Sistematik Botanik Dergisi, 26(1): 73 (2019). [Yıldırımlı, 2019a].

Type: B3 Eskişehir, Sivrihisar, Böğürtlen köyü, elma (Malus) ve asma (Vitis) harlaları, kumlu yerler, 1050 m, 13.09.1995, Ş. Yıldırımlı 19075 & Görkem Yıldırımlı (holo. Yıldırımlı Otluk'u; iso. GAZI, HUB).

e**S. tuzluca* Yıld. in Ot Sistematik Botanik Dergisi, 26(1): 75 (2019). [Yıldırımlı, 2019a].

Type: B10 Iğdır, Tuzluca çıkışı, kırmızı topraklı yerler, tepe, dere, bayır, 1000 m, 31.05.1998, Ş. Yıldırımlı 21930 & A. Polat (holo. Yıldırımlı Otluk'u; iso. GAZI, HUB).

FABACEAE

***Astragalus* L.**

e**A. askaleensis* Hamzaoğlu in Türler ve Habitatlar 1(2): 115 (2020). [Hamzaoğlu, 2020a]

Type: Turkey. Erzurum: Aşkale, Yeşilova köyü kuzeyi, 1745 m, jipsli bozkır, 14.6.2019, E.Hamzaoğlu 7594 (GAZI; izotip: GAZI, ANK).

e**A. aybarsii* H. Duman & Aytaç in Turk J Bot 44: 662 (2020). [Duman, Aytac, & Ozbek, 2020]

Type: Turkey. A5 Kastamonu: Hanönü to Taşköprü 3. km, 500 m, in open areas of *Pinus brutia* forest, 26.vi.2019, H. Duman 10558 (Holo: GAZI; iso: HUB, ANK).

e***A. bartinense* Aytaç, Tunçkol et N. Aksoy in Acta Botanica Croatica, 79(2), 132 (220). [Tunçkol, Aytaç, Aksoy, & Fişne, 2020]

Type: Turkey. A4 Bartın, Ulus, Küre Mountains National Park, above Abdurrahman village, 550 m a.s.l., limestone rocky areas, 11 June 2016, Tunçkol 2271. (Holotype: GAZI; Isotypes: ANK, DUOF).

e***A. nurhakdagensis* Uzun, Aytaç & Tülücü in Turk J Bot 45: 577 (2021). [Uzun, Aytac, & Tülücü, 2021]

Type: Turkey. B6 Kahramanmaraş: Nurhak Mountain, 21 km from the centre of Nurhak, 2000 m, steppe, calcareous rocks, 17 June 2020, *Tülücü* 46 & *Uzun* (holo. GAZI, iso. KASOF, ANK).

e***A. sertavulensis* Aytaç & Çeçen in Nordic Journal of Botany, 38(9)-e02829: 2 (2020). [Aytaç, Çeçen, & Fişne, 2020]

Type: Turkey. C4 Karaman: 22 km on the highway from Karaman to Mut, around Lale village, forest clearings and southwest of motorway limestone rocky areas, 1460 m, 20 Jun 2018, *Çeçen 4688* (holotype: GAZI, isotypes: ANK, HUB, YILDIRIMLI, KNYA).

***Dorycnium* Mill.**

******D. pentaphyllum* Scop. Fl. Carniol. ed. 2. 2: 87 (1771). subsp. ***pentaphyllum*** [Vladimirov, Aybeke, & Tan, 2021]

Examined specimens: A1(E) Edirne: Budakdoğanca, in grasslands, 98 m, 41°46'09.1"N, 26°22'10.2"E, 13.06.2017, coll. & det. M. Aybeke (EDTU 16810). A1(E) Tekirdağ: Hayrabolu, between Hayrabolu – Tekirdağ, 4th km, in cemetery, 17 m, 41°12'47"N, 27°06'25"E, 22.06.1987, coll. G. Dalgıç & A. Asan, det. M. Aybeke (EDTU 1518).

***Hedysarum* L.**

e***H. nallihanse* Aytaç, Kaptaner İğci & Körüklü in Phytotaxa 471 (3): 277 (2020). [Aytaç, Kaptaner-İğci, & Körüklü, 2020]

Type: Turkey. A3 Ankara: Beypazarı-Nallıhan road, Solta pass, gypsum steppes, Körüklü 1286 (holo. GAZI, iso. ANK, HUB).

e***H. turcicum* Hamzaoğlu & Koç in Phytotaxa 428 (1): 2 (2020). [Hamzaoğlu & Koç, 2020]

Type: Turkey. B5 Yozgat: Boğazlıyan, around Yazıkışla village, marly steppe, 1280 m, 39° 04'N, 35° 27'E, 6 June 2017, *Hamzaoğlu* and *Koç* 2942 (holotype GAZI, isotypes GAZI, ANK, HUB).

***Lathyrus* L.**

e***L. cirpicii* F. Güneş in Biodicon 12(2): 162 (2019). [Güneş, 2019]

Type: Turkey. C6 Hatay: Küçükkaraçay, road and field sides, 50–110m, 19 April 2009, *F. Güneş 2016*. (holotype NGBB, isotype MUFE, HUB, NGBB).

***Onobrychis* Adans.**

e***O. silvanensis* Aytaç, Rabaute & Coulot in Phytotaxa 477 (2): 254 (2020). [Aytac, Rabaute, & Coulot, 2020]

Type: Turkey. Diyarbakır: Diyarbakır- Silvan, 1–2 km, 683 m, steppe, *P. Coulot & Ph. Rabaute s.n*., 27 May 2009 (holotype MPU1019256; isotypes GAZI, personal herbaria of P. Coulot and Ph. Rabaute).

FUMARIACEAE

***Fumaria* L.**

******F. pugsleyana* (Maire ex Pugsley) Liden in Anal. Jard. Bot. Madrid 41: 222 (1984). [Orcan & Binzet, 2003]

Examined specimen: Turkey. C5: Mersin: Arslanköy-Mersin, 9 km, Yavca-Kavalpınar, field, roadside, 1400 m, N 37° 00´ 857", E 034° 21´ 624", 07.05.2001, N. Orcan, R. Binzet, AK-29; 17.05.2001, N. Orcan, R. Binzet, AK-26.

GLOBULARIACEAE

***Globularia* L.**

e***G. orientalis* L. var. ***nevshehirensis*** Akgül in Ot Sistematik Botanik Dergisi, 26(2): 35 (2019). [Akgül & Kılıçkaya, 2019].

Type: B5 Nevşehir, Nevşehir-Avanos arası, Nevşehir'den Avanos'a 5-7 km, 1200 m, taşlık yamaçlar, 14.04.2017, G. Akgül 2841 (holo. ANK).

HYPERICACEAE

***Hypericum* L.**

e***H. alacamdaglariense* H. Duman & E.G.Çakır in Phytotaxa 470 (2): 177 (2020). [Duman & Çakır-Dindar, 2020]

Type: Turkey. B1 Balıkesir: Bigadiç, Hacıömer Deresi village, South of Sarıkaya, 1184 m, on metamorphic cliffs, 355 635659 4357103, 28.07.2015, H. Duman 10377 (Holo: GAZI; iso: HUB, ANK, DUP).

e***H. cuisinii* Barbey subsp. ***anatolicum*** Dirmenci & N.Robson in Kew Bulletin 74: 66 (2019). [Dirmenci & Robson, 2019]

Type: Turkey. Aydın: Kuşadası, Dilek Peninsula National Park, rocky slopes, 1177 m, 22 July 2016, Dirmenci 4585 (holotype BM; isotypes ANK, BM, EGE, GAZI, HUB, ISTE).

e***H. kemaliyensis* Dirmenci & N.Robson in Kew Bulletin 74: 66 (2019). [Dirmenci & Robson, 2019]

Type: Turkey. B7 Erzincan: Between Kemaliye and Divriği road (old road), entrance of Bülent Ecevit Tunnel, 900 m, 15 June 2016, Dirmenci 4566 & Kahraman (holotype: BM; isotypes: ANK, Hb Dirmenci).

e***H. malatyanum* Peşmen subsp. ***nurhakense*** Özgişi & Ocak in Phytotaxa 479 (1): 130 (2021). [Özgişi & Ocak, 2021]

Type: Turkey. B6 Kahramanmaraş: Nurhak district, Nurhak Mountain, above Karadede Tableland, 38°2'25.04"N 37°24'46.50"E, 2010 m elevation, 19 july 2020, K. Özgişi K. O. 1400 (holotype: OUFE 16500; isotypes: HUB And EGE).

e***H. turcicum* Özbek & Hamzaoğlu in Turkish Journal of Botany, 43: 695 (2019). [Özbek, Koç, & Hamzaoğlu, 2019]

Type: Turkey. A3 Ankara: Beypazarı district, between Kırbaşı to Uşakbükü, salty floors on gypsum hills, 36 T 0395037-4429963, 810 m, 11.06.2016, Koç 2308 & Hamzaoğlu (Holotype: GAZI, isotypes: ANK, HUB, GAZI).

LABIATAE

***Lamium* L.**

e***L. ponticum* Miller subsp. ***anatolicum*** Celep in Phytotaxa 511(1): 73 (2021). [Celep, Karaer, & Duman, 2021]

Type: Turkey. Kastamonu. Küre, from Kastamonu to Küre, 23 km before Küre, 1 km before Ödemiş village, 08 May 2014, 1147m, open *Pinus nigra* subsp. *pallasiana* forest, 41° 40' 36,879" N, 33° 42' 32,308" E, *F. Celep 2332*. (holo. GAZI, iso. ANK, ADO).

***Origanum* L.**

e***O.* × ***aytacii*** Dirmenci, T. Yazıcı & Arabacı in Plant Biosystems 155 (3): 473 (2021). [Arabaci et al., 2021]

Type: Turkey. Denizli. Tas¸ Ocağı place, dry stream beds, rocky slopes, 550 m., 24.07.2016, Dirmenci 4596 (Holotype GAZI, isotypes ANK, EGE, ISTE-116810, HUB).

e***O.* × ***malatyanum*** Yıldız, Arabacı & Dirmenci in Bağbahçe Bilim Dergisi 7(1): 11 (2020). [Arabacı, Dirmenci, & Yıldız, 2020]

Type: Turkey. Malatya, Beydağı, around Eskiçamurlu village, dry/streamside, 1500-1600 m, 01 vii 1995, *B.Yıldız.* 12948 (Holotype: ANK. isotypes: GAZI, ISTE, Hb. Dirmenci).

***Salvia* L.**

e***S.* × ***doganii*** Celep & B.T.Drew in Turk J Bot 44: 652 (2020). [Celep, Raders, & Drew, 2020]

Type: Turkey. Sivas, between Altınyayla and Şarkışla, ca. 15 km before Şarkışla, greyish-brownish rock screes, steppe with some shrubs and trees, 14.7.2012, 1323 m, F.Celep 3884 (holotype ADO, isotype GAZI).

e***S.* × ***karamanensis*** Celep & B.T.Drew in Turk J Bot 44: 651 (2020). [Celep et al., 2020]

Type: Turkey. Karaman: between Bucakkışla and Ermenek, about 5–7 km from Bucakkışla, *Quercus coccifera* L. forest, growing among calcareous rocks at the edge of the road. 17 June 2017, 862 m, F.Celep 4027 & B.T. Drew (holotype ADO, isotype GAZI).

***Satureja* L.**

******S. metastasianta*** Rech.f. in Fl. Iranica 150: 497 (1982). [Dirmenci, Yıldız, & Öztekin, 2019]

Examined specimen: Turkey. Hakkâri: 10 km from Hakkâri to Çukurca, ca. 1200 m, 06.xi.2001, Dirmenci 1317, M. Koyuncu, N. Adıgüzel, M. Fırat (Hb. M. Öztekin).

***Scutellaria* L.**

e***S. topcuoglui* Yıldırım, Çiçek & Akbaş in Phytotaxa 528 (3): 181 (2021) [Yıldırım, Çiçek, Akbaş, & Şeker, 2021]

Type: Turkey. C2 Muğla: Ula district, upper of Çörüş neighbourhood, eastern part of Balan Mountain series, Mount Muslu foothills, *Pinus brutia* forest clearings, serpentine areas, 230 m, 25 May 2021, M. Çiçek 2021-400, K. Akbaş & B. Topçuoğlu (holotype EGE-43217, isotypes ANK, EGE-43218, GAZI, HUB, NGBB, M. Çiçek Herbarium).

***Stachys* L.**

e***S. ahmet-savranii*** Doğu & Bağcı in Bangladesh J. Bot. 50(2): 320 (2021). [Doğu & Bağcı, 2021]

Type: Turkey. C5 Niğde: Çamardı, Cimbar Valley, on rocky, 1900-2000 m, 25.07.2018, Y. Bağcı 4166, S. Doğu, and A. Savran (holotype: KNYA, isotype: ANK).

******S. distans* Benth. var. ***distans*** in DC., Prodr. 12: 472 (1848). [Akçiçek, 2020]

Examined specimen: Turkey. C5 Mersin: Erdemli, Limonlu, Ömerçayı district, macchie, 36o 34' 08.81"N / 34o 14' 28.03" E, 20 m, 22 June 2015, *Akçiçek 5807 & Ö. Güner* (GAZI, Hb. Akçiçek).

e***S. semsurensis* Fırat in Phytotaxa 511 (3): 276 (2021). [Fırat, 2021]

Type: Turkey. C7 Adıyaman (Semsur): Kâhta (Kolik), around Nemrut Mountain opposite Karadut (Qeredut), crevices of limestone rocks, 37o54'44" N, 38o48'55" E, 1142 m, 14 May 2015, *M.Fırat 32525* (holotype VANF, isotypes VANF, HUB and Hb. M. Fırat).

e***S. siirtensis* Ö. Güner & Akçiçek in Phytotaxa 516 (3): 253 (2021). [Güner, Akcicek, & Dirmenci, 2021]

Types: Turkey. Siirt, Eruh to Şırnak, close to the Şırnak border, Botan Valley, rocky slopes, 700–750 m, 11.06.2013, *Ö.Güner 2333, Akçiçek, Dirmenci* (holotype GAZI, isotypes ISTE, ANK, HUB).

***Teucrium* L.**

e***T. semrae*** Aksoy, Dirmenci & Özcan in Turk J Bot, 44(3), 326 (2020). [Aksoy, Özcan, Girişken, Çelik, & Dirmenci, 2020]

Type: Turkey. Muğla: Seydikemer district, Minare village, Pınara ancient city, Aşı stream, 450-650 m, 28.04.2019, Aksoy 2993 (Holotype: GAZI, isotypes ANK, EGE, ISTE, Akdeniz University Herbarium)**.**

e***T. turcicum* Çeçen & Özcan in Turk J Bot 45: 356 (2021). [Çeçen & Özcan, 2021]

Type: Turkey. Mersin: Anamur, 13th km from Abanoz village to Boğuntu village, around Çukurabanoz village, eastern slopes of calcareous rocky areas, 650–905 m, 12 May 2020, *Çeçen* 6012. (holo. GAZI, iso. ANK, HUB, KNYA).

LINACEAE

***Linum* L.**

e***L. aksehirense*** Tugay & Ulukuş in PhytoKeys 136: 25 (2019). [Tugay & Ulukuş, 2019]

Type: Turkey. B3 Konya; Akşehir, Sultan Mountains, slopes in *Pinus nigra* forest, 1150 m alt., 38°19.230'N, 31°23.181'E, 01 August 2017, *O.Tugay* 14.542 & *D.Ulukuş* (holotype KNYA, isotypes KNYA 28.229).

MALVACEAE

***Kitaibela* Willd.**

***K. vitifolia** Willd. in Neue Schriften Ges. Naturf. Freunde Berlin ii. 108 (1799). [Tugay, Ertuğrul, Aslan, & Ulukuş, 2019]

Examined specimen: C6 Osmaniye; Amanos Dağları, *Pinus* ormanı açıklıkları, 1400 m, 26.vii.2014, O. Tugay 10.138 (KNYA).

***Malvastrum* A. Gray**

***M. coromandelianum** (L.) Gracke in Bonplandia 5(18): 295 (1857). [Yıldırım, Özdöl, & Yaşayacak, 2019]

Examined specimen: Osmaniye: Alibekirli Mahallesi, Bahçe içleri ve tarım arazileri, 37° 03'29.70" K, 36°16'09.08" D; 176 m, 21.08.2018, M. Çelik, H. Yıldırım 7690 (EGE).

OROBANCHACEAE

***Phelipanche* Pomel**

***P. gussoneana** (Lojac.) Domina, Raab-Straube, Rätzel & Uhlich in Willdenowia 48(2): 209 (2018). [Raab-Straube & Raus, 2021]

Examined specimens: Turkey. 20 km W of Trabzon, mountain slope, on *Plantago* sp., 8 May 1970, *Horreüs de Haas 171* (L.2816621; det. Uhlich 12 Jul 2021).

***P. olbiensis** (Coss.) Carlón, G.Gómez, M.Laínz, Moreno Mor., Ó.Sánchez & Schneew., Doc. Jard. Bot. Atlántico 6: 79 (2008). [Raab-Straube & Raus, 2020]

Examined specimens: Turkey. Bolkar Daghlari, Karagol-Kar, Hange westlich Meydan, 37°25'N, 34°37'E, 2450 m, Zwergstrauchflur, karbonatisches Substrat, 7 Aug 1992, *Hein 89-7a* (B 10 0666639, det. Parolly 1996 as *Orobanche mutelii* F. Schultz, rev. Ratzel 11 Mar 2020).

PLUMBAGINACEAE

***Limonium* Mill.**

***L. davisii** Doğan in Plant Systematics and Evolution 306(6): 89 (2020). [Doğan, Akaydın, & Erdal, 2020]

Type: Turkey. B4 Aksaray: Aksaray-Konya road, around Sultanhanı, 915 m a. s. l., salt steppe, 3 Oct 2004. *M. Doğan* and *G. Akaydın 10120* (ANK)

L. globuliferum** Kuntze var. ***subglobosum Akaydın & Doğan in Plant Systematics and Evolution 306(6): 89 (2020). [Doğan et al., 2020]

Type: Turkey. B4 Aksaray: Aksaray-Adana road, around Yukarı Göndelen, 1040 m a. s.l., 18 Jun 2003, Doğan and Akaydın 7884 (ANK).

L. lilacinum** (Boiss.) Wagenitz var. ***laxiflorum Doğan & Akaydın in Plant Systematics and Evolution 306(6): 89 (2020). [Doğan et al., 2020]

Type: Turkey. B4 Aksaray: Aksaray, around Aksaray University Campus, 930 m a. s. l., 17 Aug 2003, Doğan and Akaydın 8182 (ANK).

POLYGONACEAE

***Persicaria* (L.) Miller**

***P. hydropiperoides** (Michaux) Small in Fl. S.E. U.S. 378, 1330 (1903). [Keskin & Severoğlu, 2021a]

Examined specimen: Istanbul, Sancaktepe, Paşaköy, center, in valley, fountain area, wet place, N 41° 03' 49.7" and E 28° 44' 57.4", 23.ix.2019, M. Keskin 7899.

P. lapathifolia** (L.) Delarbre subsp. ***brittingeri (Opiz) Soják in Preslia 46: 153 (1974). [Keskin & Severoğlu, 2020]

Examined specimen: Istanbul: Tuzla, Akfırat, Against the Formula-1 race ground, meadows and old humid areas, 25.vi.2020, M.Keskin 8019.

P. lapathifolia** (L.) Delarbre subsp. ***nodosa (Pers.) Á.Löve, Rit Landbúnaoard. Atvinnud. Háskólans, B 3: 109 (1948). [Keskin & Severoğlu, 2021b]

Examined specimens: Istanbul, Çekmeköy: Hüseyinli köyü girişi, 7 m, N 41° 07' 07,5" ve 29° 17' 58,8", 11.vii.2020, M. Keskin 8032; Sarıyer: Bahçeköy, Belgrad ormanı, Karanlık bendi, su kenarı, 6.ix.2020, M. Keskin 8071; Tuzla: Akfırat beldesi, Formula 1 alanı karşısı, çayırlık, 7.viii.2019, M.Keskin 7861; Tuzla: Akfırat beldesi, Formula 1 Yarış alanı karşısı, kurumuş dere yatağı, kumlu toprak, 11.viii.2020, M.Keskin 8062.

***Reynoutria* Houtt.**

***R. japonica** Houtt., Nat. Hist. 2(8): 639 (1777). [Karaer, Terzioğlu, & Kutbay, 2020]

Examined specimen: A6 Samsun - Terme, Bazlamaç district, field margins, roadsides, 210-315 m, 16.10.2014, F. Karaer 30135 (OMUB), Ibid. 250-425 m, 15. xi. 2014, F. Karaer 30375 (OMUB), (KATO 19251)

***Rheum* L.**

***R. telianum** İlçim in Phytotaxa, 477(1): 82 (2020). [İlçim & Karahan, 2020]

Type: Turkey. Adıyaman (C7): Kayatepe (Rezip) village, rocky serpentine soils, 37°87' N, 38°27' E, 1350 m, 18 April 2018, *İlçim1945* (holotype ANK, isotype Herbarium Hatay Mustafa Kemal Univ.).

PRIMULACEAE

***Androsace* L.**

***A. azizsancarii** Sefalı in Nord J Bot 39(7)-e03208: 4 (221). [Sefali, 2021]

Type: Turkey. Bayburt: Soganlı Mountains, south of Anzer Mountain, on moraines, 2831 m a.s.l., 40°30′N, 40°30′E, 1 July 2020, A.Sefalı 507 (holotype: ISTE 117267; isotypes: ANK 60610, BIN 9405, EGE 43195).

***Dionysia* Fenzl**

e***D. zeynepiae* Güzel in Phytotaxa 525(4): 282 (2021). [Güzel, 2021]

Type: Turkey. C6 Hatay: Antakya, Habib-i Neccar Mountain, Demirkapı vicinity, rock crevices of the cliffs, 36°12′26″N 36°10′53″E, 195 m elevation, 2 February 2021, *Y. Güzel 3450* (holotype: HUB; isotype: ANK).

RANUNCULACEAE

***Ranunculus* L.**

e***R. aydogdui* Karaman, Tekşen & H. Duman in Nordic Journal of Botany 39(1): 2 (2021). [Karaman-Erkul, Tekşen, & Duman, 2021]

Type: Turkey. Aksaray: 3 km east of Eskil, salty steppe, 1000m a.s.l. 22 jun 2017, M. Tekşen & S. Karaman 3239 (holo-type: GAZI: isotype: AKSU).

e***R. solhanensis* Behçet & A. Sinan in Phytotaxa 497(2): 158 (2021). [Sinan, Behçet, & Yapar, 2021]

Type: B8 Bingöl, Solhan, Yaylım Mountain, rocky slopes, 38°80′267″N 40°98′814″E, 1713 m a.s.l., 08 April 2018, A.Sinan 1781 (holotype: Bingöl Univ. Biology Dept. Herbarium (BIN), isotypes Bingöl Univ. Biology Dept. Herbarium and ANK).

RUBIACEAE

***Asperula* L.**

e***A. cankiriense* B. Şahin & Sağıroğlu in Turk J Bot 45: 247 (2021). [Şahin, Sağıroğlu, & Başer, 2021]

Type: Turkey. A4 Çankırı: Süleymanlı village, Salt cave road, gypsum steppe, 600–700 m, 23.06.2016. B. Şahin 6526 & M. Sağıroğlu (holotype GAZI, isotypes: GAZI, HUB, ANK).

***Crucianella* L.**

e***C. turcica* Hamzaoğlu in Türler ve Habitatlar 2(2): 115 (2021). [Hamzaoğlu, 2021a]

Type: Turkey. Kayseri: Tomarza, Toklar ve Işıklar köyleri arası, Kızıldağ etekleri, 1600 m, taşlı bozkır, 4.7.2021, E.Hamzaoğlu 7877 (holotip: GAZI; izotipler: GAZI, ANK).

***Plocama* Aiton**

e***P. calabrica* (L.f.) M. Backlund & Thulin var. ***alba*** Göktürk, O. D. Düşen, B. Gürcan & U. Sarpkaya in Acta Bot. Croat. 78 (2), 143 (2019). [Göktürk, Düşen, Kaya, Gürcan, & Sarpkaya, 2019]

Type: Turkey. C2 Denizli: Çameli, between Akpınar and Yaylapınar villages, limestone slopes, 1166 m, 21 June 2017, O. D. Düşen (2584) & R. S. Göktürk (holotype PAMUH, isotypes Akdeniz Univ. Herb.).

ROSACEAE

***Pyrus* L.**

******P. pyraster* (L.) Burgsd. in Anl. Erzieh. Holzart. 2: 193 (1787). [Aydın Uğurlu & Dönmez, 2019]

Examined specimen: Turkey. Kırklareli: 5. Km from Dereköy to Şükrüpaşa, N 41°56′13″, E 027°25′ 09″, 452 m, 14.10.2012, ZUG 466-A.A. Dönmez (HUB).

SAXIFRAGACEAE

***Saxifraga* L.**

e***S. artvinensis* V. A. Matthews subsp. ***meryemii*** Terzioğlu & Coşkunç. in Turk J Bot 43: 689 (2019). [Terzioğlu, Çoşkunçelebi, & Güzel, 2019]

Type: Turkey. A7 Trabzon: Altındere Vadisi National Park, around the Sumela Monastery, 1126 m, 31.03.2013, Terzioğlu & Coşkunçelebi 1246 (KATO 9843), holo: KATO; iso: KTUB.

SCROPHULARIACEAE

***Scrophularia* L.**

******S. vernalis* L. in Sp. Pl. 2: 620 (1753) [Uzunhisarcıklı, Güner, Özbek, & Ekici, 2019]

Examined specimens: Turkey. Artvin: Borçka, Karagöl, on clearings of *Pinus sylvestris* forest, ca. 1400 m, 4 june 2015, M.E. Uzunhisarcıklı 2678 (GAZI).

***Verbascum* L.**

e***V.* × ***aytachii*** H. Duman & Uzunh. in GU J Sci, 34(4): 938 (2021). [Duman, Uzunhisarcıklı, & Özbek, 2021]

Type: Turkey. B2 Bursa: Orhaneli, SW of Çeki village, 800 m, 19.vii.2017, serpentine areas, M. E. Uzunhisarcıklı 2720a & H. Duman (holotype GAZI, isotypes ANK, HUB).

e***V. ekicii* H. Duman & Uzunh. in Botany Letters, 167 (4): 410 (2020). [Duman, Uzunhisarcıklı, & Özbek, 2020]

Type: Turkey. B2 Bursa: Orhaneli, SW of Çeki village, 800 m, 19 July 2017, serpentine areas, M.E. Uzunhisarcıklı 2720 & H. Duman (holotype GAZI, isotypes ANK, HUB).

e***V.* × ***malkaraense*** Demir, Cingay & Cabi in Bağbahçe Bilim Dergisi 8(3): 10 (2021). [Demir, Çıngay, & Cabi, 2021]

Type: Turkey. Tekirdağ: Malkara-Keşan yolu, Haliç-Danışment köyü arası, orman açıklığı, yol kenarı, 286 m, 3.vı.2019, O. Demir(1172a) ve F. Demir (holo.NGBB 009556; iso.CBB).

e***V. seydisehirense* Tugay & Ulukuş in Phytotaxa 450 (2): 230 (2020). [Ulukuş, Tugay, & Sağlam, 2020]

Type: Turkey. C4 Konya; Seydişehir, Kozlu Village, steppe, 1700 m alt., 37°38.356′N, 32°03.543′E, 26 August 2017, *O.Tugay 14915*, *C.Sağlam & D.Ulukuş* (holotype: KNYA, isotype: NGBB).

UMBELLIFERAE

***Astrodaucus* Drude**

A. orientalis** (L.) Drude var. ***eriocarpus (Boiss.) Woronow in Exsicc. (Fl. Cauc.): 289 (1927). [Sert & Aytaç, 2021]

Examined specimens: Turkey. A5 Çorum: Bayat, Çerşeş köyü yukarısı, orman açıklığı, taşlı yamaçlar, 1300 m, 03.07.2020, R. Sert 1692 (GAZI).

***Bunium* L.**

e***B. sancakense*** Yıld. & Kılıç in Ot Sistematik Botanik Dergisi, 26(1): 42 (2019). [Yıldırımlı & Kılıç, 2019h].

Type: B8 Bingöl, Sancak, Hasanova mezrası çevresi, bozkır, taşlı alanlar, y.1550-1600 m, 03.07.2018, Ö. Kılıç 7051 (holo. Yıldırımlı Otluk'u)

e***B. sivasicum*** M. Çelik & Y. Bağcı in Phytotaxa 416 (4): 266 (2019). [Çelik & Bağcı, 2019]

Type: Turkey. B6 Sivas: Şarkışla, Mescit village, Demirkaya hill, 13.08.2016, 2156 m, steppe, M. Çelik 476 (holotype: KNYA, isotypes: KNYA, HUB, ANK).

***Bupleurum* L.**

B. aequiradiatum (H. Wolff) Snogerup & B. Snogerup in Willdenowia 31(2): 302 (2001). [Raab-Straube & Raus, 2020]

Examined specimens: Turkey. Bithyn[ia], prope Brussam, in fruticetis, Jul 1874, *Pichler* (BP 274226, as *Bupleurum gerardi* Jacq. [non All.]).

***Ferula* L.**

e***F. pisidica*** Akalın & Miski in Plants 9, 740 (2020). [Akalın, Tuncay, Olcay, & Miski, 2020]

Type: Turkey. C4 Antalya: Near Beyreli village, 1550 m, 26 June 2015, 36°50′ 24.8″ N, 32°22′ 14.41″ E, .M. Miski, E. Akalın & S. Anıl. (holotype: ISTE 117051).

***Ferulago* W.D.J.Koch**

e***F. akpulatii*** Akalın & Gürdal in Phytotaxa 518 (2): 101 (2021). [Gürdal, Olcay, Tuncay, & Akalın, 2021]

Type: Turkey. Sivas Province: Sivas-Hafik yolu 10. km, jipsli alanlar, 1400 m elevation, 21 June 2018, E. Akalın, A. Akpulat, M. Miski, N. Tan & M. Eruçar (holotype: ISTE 115906).

***Peucedanum* L.**

e***P. akaliniae*** Akpulat, Gürdal & Tuncay in Phytotaxa 425 (3): 146 (2019). [Gürdal, Tuncay, & Akpulat, 2019]

Type: Turkey. Antalya: Elmalı, Öndibek Yaylası (Kohu Yaylası altları, 1654 m, 36°28′39″ N, 29°52′34″E, 30 June 2017, E. Akalın & U. Uruşak (holotype: ISTE 114900).

***Pimpinella* L.**

e***P. adiyamanensis*** Yıld. & Kılıç in Ot Sistematik Botanik Dergisi, 26(1): 45 (2019). [Yıldırımlı & Kılıç, 2019h].

Type: C7 Adıyaman, Koçali-Çelikhan arası, sulu vadi, meşe (*Quercus brandii*) ormanı, 1061-1120 m, 29.04.2018, Ş. Yıldırımlı 44185 & Ö. Kılıç (holo. Yıldırımlı Otluk'u (YO); iso. GAZI, HUB, YO).

P. kurdica Rech.f. & Riedl in Anz. Österr. Akad. Wiss., Math. -Naturwiss. Kl. xcviii. 249 (1961). [Fırat, 2019b]

Examined specimens: Turkey. C9 Hakkâri; Zap valley, rocky cliffs, 37°30′14″ N, 043°43′03″ E, 1215 m, 15.07.2014, *M. Fırat* 31182, VANF & private herb M. Fırat; ibid. 20.08.2015. *M. Fırat* 32626. VANF & private herb M. Fırat (in fruit).

***Prangos* Lindley**

e***P. aricakensis*** Behçet & Yapar in Phytotaxa 401(1): 56 (2019). [Behçet, Yapar, & Olgun, 219]

Type: Turkey. B8 Elazığ: Arıcak, Akdağ mountain, Cuber tableland, rocky slopes, 38°36′01″N 40°07′58″E, 2000–2050 m elevation, 25 August 2017, *Y. Yapar 3150* (holotype: Bingöl Univ. Biology Dept. Herbarium, isotypes: ANK).

***Rhabdosciadium* Boiss.**

e***R. hizanense*** Fırat & Güzel in Phytotaxa 395 (3): 1828 (2019). [Fırat & Güzel, 2019]

Type: Turkey. B9 Bitlis: Hizan Province, İhtiyarşahap Mountains (Lolan hill), Mêrga Mehîr plateau, rocky, stone, and calcareous areas, 2221 m, 38°21′38″N, 42°25′39″E, coll. 2 August 2015, M. Fırat 32618 (holotype: VANF, isotypes: HUB, ANK and Herb. M. Fırat).

***Seseli* L.**

e***S. salsugineum*** A. Duran & Lyskov in Phytotaxa 529 (1): 35 (2021). [Duran, Samıgullın, & Lyskov, 2021]

Type: Turkey. C4 Konya: Cihanbeyli, between Gölyazı-Tuz Gölü, 9th kilometer, 923m, salty marshes, 25 September 2011, A.Duran & et al. 9855 (holotype: HUB, isotypes: ANK, MW barcode MW0595744, MW0595746, MW0595747).

***Trigonosciadium* Boiss.**

e***T. solhanense*** Yıld. & Kılıç in Ot Sistematik Botanik Dergisi, 26(1): 48 (2019). [Yıldırımlı & Kılıç, 2019h].

Type: B8 Bingöl, Solhan, Hazarşah köyü mezrası, Aksakal göl mezrası, Yüzenada mevkisi, sulu dere boyunca ve üstündeki tepeler, volkanik taşlı yamaçlar, çayırlık, 1300-1400 m, 12.06.2015, Ş. Yıldırımlı 41492 & Ö. Kılıç (holo. Yıldırımlı Otluk'u).

MONOCOTYLEDONS

ARECACEAE

***Phoenix* L.**

e***P. theophrasti*** Greuter subsp. ***golkoyana*** Boydak in Forestist, 69(2): 141 (2019). [Boydak, 2019]

Type: Turkey. C1 Muğla: Bodrum, Gölköy (Göltürkbükü), female, latitude 37.1147° N, longitude 27.3981° E, Plain, 3 m., 27 August

2018, M. Boydak (holotype: ISTO 38308); ibid, (paratypes: (male) ISTO 38309, (female) ISTO 38310); ibid, 01 October 1990, M. Boydak (paratype: ISTO 27384).

ARACEAE

***Zantedeschia* K.Koch**

***Z. aethiopica** (L.) Spreng. in Syst. Veg., 3: 765 (1826). [Yıldırım & Erdem, 2019]

Examined specimen: Muğla: Ula, Akçapınar Mahallesi, Akçapınar Azmağı kenarı bataklık alanlar, 37° 01′ 39.9″ K, 28° 21′ 10.6″ D, 10 m, 26. Iii. 2019, H. Yıldırım 7551, B. Topçuoğlu (EGE-43189).

ASPARAGACEAE

***Chionodoxa* Boiss.**

eC. salbacus** Yıldırım in Bağbahçe Bilim Dergisi 8(1): 77 (2021). [Yıldırım & Altıoğlu, 2021]

Type: Turkey. Denizli: Babadağ, Karababa tepesi altı, kuzey yamaç, açık yamaçlar, şistli topraklar üzeri, 1569 m, 09 iv 2014, H. Yıldırım 2797, Y. Altıoğlu, R. Çetiner ve Z. Doğmaz (holotip: EGE, izotipler: EGE, NGBB, ANK)

■■ ***Gemicia*** Yıldırım in Bağbahçe Bilim Dergisi 8(1): 40 (2021). [Yıldırım, 2021]

Type species: *Gemicia vardariana* (Yıldırım & Y. Gemici) Yıldırım (≡ *Scilla vardaria* Yıldırım & Y. Gemici).

eG. vardariana** (Yıldırım & Y. Gemici) Yıldırım in Bağbahçe Bilim Dergisi 8(1): 41 (2021). [Yıldırım, 2021]

Type: Turkey. Rize: Çamlıhemşin, Kaçkar Mountain, 1520 m, openings in *Picea orientalis* forests, 28.iv.2010, H. Yıldırım 1675 (holotype EGE; isotypes K; EGE; HUB and Herb. Yıldırımlı).

***Leopoldia* Parl.**

eL. archibaldii** Rukšāns in International Rock Gardener 134 (2020) [Rukšāns, 2020b]

Type: Turkey. Erzincan Province, roadside after the Pülümür valley along the road from Tunceli to Erzincan, 2004, BATM-282, 39°35.970′N 39°51.949′E, alt. 1300 m. Holo: GB – ex culturae in horto Jānis Rukšāns.

***Loncomelos* Raf.**

eL. koprulense** Bogdanović, Brullo & Salmeri in PhytoKeys, 175, 35 (2021). [Bogdanović, Brullo, & Salmeri, 2021]

Type: Turkey. Antalya: District of Manavgat, Köprülü Kanyon National Park, Bozyaka road, cultivated specimen, 15 June 2010, Brullo s.n. (Holotype: CAT).

***Puschkinia* Adams**

eP. kurdistanica** Rukšāns in International Rock Gardener 116 (2019) [Rukšāns, 2019]

Type: Plants from E Turkey, S coast of Lake Van, 19 km after Tatvan along the Tatvan-Van rd., 38°28′ N and 42°29′ E; alt. apr. 1670 m., leg. Rukšāns, Seisums & Zetterlund 26th of May, 2004 as BATMAN-060. Holotype GB (Gothenburg) ex culturae in horto Jānis Rukšāns, 05-04-2019.

CYPERACEAE

***Eleocharis* R.Br.**

eE. divaricata** M.Keskin in Front Life Sci RT 2(1): 10 (2021). [Keskin & Merrick, 2021]

Type: Turkey, Antalya: Konyaaltı, Boğa çayı vicinity, 4.xii.2020, D.Merrick (Holo NGBB)

GRAMINAE

***Arrhenatherum* P. Beauv.**

A. elatius** (L.) P. Beauv. ex J. Presl. & C. Presl. subsp. ***bulbosum (Willd.) Schübl. & Martens in Fl. Vürtemberg 70 (1834). [Terzioğlu, 2020]

Examined specimen: A8 Rize - Fındıklı, in tea and hazelnut plantations, 35 m, 29.05.2019, UTM: 0680491, 4569884, KATO 23230

***Bromus* L.**

B. arvensis** L. subsp. ***parviflorus (Desf.) H. Scholz in Florist. Rundbr. 36: 35 (2002). [Raab-Straube & Raus, 2019]

Examined specimen: Turkey. W Anatolia, Bafa Gölü, W side, c. 1.5 km S of Serçin, 37°31′47″N, 27°23′11″E, 2 – 5 m, abandoned field near waterside, salt influenced, 9 May 2006, Ristow (herb. Ristow).

eB. orientalis** Behçet & Yapar in Nord J Bot 39(4)-e02959: 2 (2021). [Behçet & Yapar, 2021]

Type: Turkey. B8 Bingöl: Çapakçur Valley, Alıncak between Aşağıköy, moist forest clearances, 1577 m a.s.l., 38°51′55.14″N, 40°24′57.48″E, 13 Jul 2018, Behçet & Yapar 15773 (holotype: Bingöl Univ. Biology Dept. Herbarium, isotypes: Bingöl Univ. Biology Dept. Herbarium and ANK).

■***Leptatherum* Nees**

***L. boreale** (Ohwi) C.-H. Chen, C.-S. Kuoh & Veldk. in Blumea 54: 179 (2009). [Terzioğlu & Özkan, 2020]

Examined specimen: Trabzon, Sürmene, Çamburnu, in Scots pine forest including Çamburnu Nature Park, along the road, 10 m, 10.11.2019, UTM: 0602590 - 4531287, KATO 24445

IRIDACEAE

***Crocus* L.**

eC. akdagensis** Kerndorff & Pasche in Stapfia 103: 76 (2015) [Kerndorff, Pasche, & Harpke, 2015]

Type: Turkey. Pisidia, Burdur Province, Akkaya Tepesi, 1600-1700 m, 19.03.2002, HKEP 0211 (Gatersleben GAT 7128).

e***C. akkayaensis* Kerndorff & Pasche in Stapfia 103: 74 (2015) [Kerndorff et al., 2015]

Type: Turkey. Lycia, Antalya Province, eastern Ak Dağlar, 1500-1600 m, 20.02.2011, HKEP 9721 (Gatersleben GAT 7389).

e***C. asymmetricus* Erol in Phytotaxa 430: 74 (2020). [Ciftci, Harpke, Mollman, Yildirim, & Erol, 2020]

Type: Turkey. Kahramanmaraş: border of Maraş – Osmaniye, Ceyhan Valley 850–920m a.s.l., 6 March 2019, *Mehmet Çelik s.n.* (holotype ISTF 41370, isotype: EGE)

e***C. bowlesianus* Kerndorff & Pasche in Stapfia 103: 75 (2015) [Kerndorff et al., 2015]

Type: Turkey. Lycian Taurus, Antalya Province, Katrancik Dağlari, 1400-1500 m, 25.03.2000, HKEP 0009 (Gatersleben GAT 7377).

e***C. concinnus* Kerndorff & Pasche in Stapfia 101: 10 (2014) [Kerndorff, Pasche, & Harpke, 2014]

Type: Turkey. Central Taurus (Kuyucak Dağları), Antalya province, 1000-1800 m, 26.2.2000, HKEP 0007 (Gatersleben, GAT 23069)

e***C. filis-maculatis* Kerndorff & Pasche in Turk J Bot 38: 1195 (2014) [Harpke et al., 2014]

Type: Turkey. Anti-Taurus, Province Adana, hills north of Adana towards the Taurus mountains, 800– 1000 m, 17 March 2012, HKEP 1207, (GAT 25833).

e***C. gembosii* Rukšāns in International Rock Gardener 76 (2016) [Rukšāns, 2016]

Type: Turkey. Antalya Province, Gembos Yaila, 37 °15.855 N, 31 °27.646 E, alt. 1220m: 24-03-2014. Rukšāns, 14TUS-029. Holo: GAT.

e***C. harpkeae* Rukšāns in International Rock Gardener 125 (2020) [Rukšāns, 2020a]

Type: Turkey. Adana Province, yaila below Kaan Geçidi, 38013' N; 36014' E; JJVV-023, leg. J. Rukšāns, 7th of March, 2010. Holo: GAT; Isotype: GB – ex culturae in horto Jānis Rukšāns, 02-03-2016.

e***C. katrancensis* Kerndorff & Pasche in Stapfia 103: 75 (2015) [Kerndorff et al., 2015]

Type: Turkey. Pisidia, Antalya Province, Katranç dagı, 1600-1700 m, 13.03.2002, HKEP 0222 (Gatersleben GAT 7150).

e***C. keltepensis* Yüzb. in Phytotaxa 418 (2): 231 (2019). [Yüzbaşıoğlu, 2019]

Type: Turkey. Kocaeli: Keltepe, zirve, 1650 m, 29 April 1974, A. Baytop & E. Tuzlacı (holotype ISTE 26764; isotypes ISTE).

e***C. rechingeri* Kerndorff & Pasche in Stapfia 101: 9 (2014) [Kerndorff et al., 2014]

Type: Turkey. Lycian Taurus, Antalya province, Babadağ, 1200-1400 m, 15.3.2001, HKEP 0115 (Gatersleben, GAT 7246)

e***C. sakaltutanensis* Rukšāns in International Rock Gardener 76 (2016) [Rukšāns, 2016]

Type: Ex culturae in horto Jānis Rukšāns. (Plants from N.E. Turkey, Erzincan Province, Sakaltutan mountain pass 39.870523°N. 39.134231°E, alt. 2120m). Cultivated plants originally collected on 28-05-2009, long after blooming, with almost dry leaves (Rukšāns, JRRK-011). Holotype: GAT; Isotype: ISTF.

e***C. salurdagensis* Kerndorff & Pasche in Stapfia 103: 77 (2015) [Kerndorff et al., 2015]

Type: Turkey. Lycia, Antalya Province, Salur dağ, 1500-1600 m, 20.02.1997, HKEP 9701 (Gatersleben GAT).

e***C. stevensii* Rukšāns in International Rock Gardener 76 (2016) [Rukšāns, 2016]

Type: ex culturae in horto Jānis Rukšāns. (Plants from S.W. Turkey, Honaz Dağı, over vil. Honaz, alt.1650m J. Archibald.). Cultivated plants collected on 29.02.2016. Holotype: GAT.

e***C. terzioghluii* Erol in Phytotaxa 420(3): 225 (2019). [Ciftci, Harpke, & Erol, 2019]

Type: Turkey. Muğla: Marmaris, ca. 65 m a.s.l., 13 December 2018, O. Erol & A. Gemicioğlu (holotype, ISTF 41368)

e***C. xanthosus* Kerndorff & Pasche in Stapfia 103: 77 (2015) [Kerndorff et al., 2015]

Type: Turkey. Lycia, Antalya Province, Kofu dağ, 1400-1500 m 10.03.2001, HKEP 0124 (Gatersleben GAT 7126)

e***C. zetterlundii* Rukšāns in International Rock Gardener 64 (2015) [Rukšāns, 2015]

Type: Ex culturae in horto Jānis Rukšāns. (Plants from NW Turkey, Bolu province, near Bakırlı yaylasi, altitude 1360m). Collected on 04-06-2005 (LST-109). Holotype: GB.

***Freesia* Eckl. ex Klatt**

***F. leichtlinii* Klatt subsp. *alba* (G.L.Mey.) J.C.Manning & Goldblatt in Strelitzia 27: 70 (2010). [Eker & Tuzlacı, 2021]

Examined specimens: A2(A) İstanbul: Burgazada, orman içi açıklık, 91 m, 09 v 2017, H.İ. Tuzlacı 752 (AIBU); Burgazada, orman içi, 31 m, 23 iii 2017, H.İ. Tuzlacı 647 (AIBU); Heybeliada, orman içi açıklık, 15 m, 22 iii 2017, H.İ. Tuzlacı 634 (AIBU); Kınalıada, orman içi açıklık, 62 m, 09 iii 2016, H.İ. Tuzlacı 168 (AIBU); Kınalıada, makilik, 54 m, 22 iii 2016, H.İ. Tuzlacı 222 ve İ.Eker (AIBU); Kınalıada, orman içi açıklık, 72 m, 24 iv 2018, H.İ. Tuzlacı 964 (AIBU); ibid., H.İ. Tuzlacı 967 (AIBU); ibid., H.İ. Tuzlacı 968 (AIBU).

***Iris* L.**

e***I. yedisuensis* Yıld. & Kılıç in Ot Sistematik Botanik Dergisi, 26(2): 2 (2019). [Yıldırımlı & Kılıç, 2019b].

Type: B8 Bingöl, Karlıova'dan Yedisu'ya doğru, y. 30 km, yol kıyısı, karkal, bayır, 1740 m, 23.04.2019, Ş. Yıldırımlı 45132 & Ö. Kılıç (holo. Yıldırımlı Otluk'u (YO); iso. GAZI, YO).

***Gladiolus* L.**

e***G. aladagensis* Eker & Sağıroğlu in Phytotaxa 478 (1): 152 (2021). [Sağıroğlu & Eker, 2021]

Type: Turkey. A3 Bolu: Between Karacasu-Kıbrıscık, 34. km, along stream bed in the *Pinus sylvestris* forest openings, 1484 m of elevation, 07 July 2019, *İ. Eker 12727 & M. Sağıroğlu* (holotype AIBU; isotypes Herbarium of Sakarya University, GAZI, HUB).

e***G. hamzaoglui* H. Duman, Sağıroğlu & Tekşen in Phytotaxa 496 (3): 247 (2021). [Tekşen, Duman, & Sağıroğlu, 2021]

Type: Turkey. Erzincan: İliç, N of Çakmaktepe, 1290 m elevation, serpentine gravelly slopes and slightly moist meadows, 10 July 2020, H. Duman 10626 (holotype GAZI; isotypes HUB, ANK).

e***G. izzet-baysalii* Eker & Sağıroğlu in Phytotaxa 527 (2): 98 (2021). [Eker & Sağıroğlu, 2021]

Type: Turkey. C4 Karaman: Sarıveliler, above Göktepe, Saçak Mountain, meadow, dry creek bed, 1779 m of elevation, 26 May 2021, *İ. Eker 13199* (holotype AIBU; isotypes AIBU, SAKU).

LILIACEAE

***Allium* L.**

e***A. adiyamanense* Yıld. & Kılıç in Ot Sistematik Botanik Dergisi, 26(1): 34 (2019). [Yıldırımlı & Kılıç, 2019e].

Type: C7 Adıyaman, Koçali-Çelikhan arası, sulu vadi, meşe (*Quercus brandii*) ormanı, 1061-1120 m, 29.04.2018, Ş. Yıldırımlı 44252 & Ö. Kılıç (holo. Yıldırımlı Otluk'u (YO); iso. GAZI, YO).

e***A. dönmezii* Mutlu & Karakuş in Phytotaxa 411 (3): 199 (2019). [Karakuş & Mutlu, 2020]

Type: Turkey. Malatya, Akçadağ, Başyurt plateau, Kartalkaya Hill, 2400 m, 14 July 2012, Ş. Karakuş & B. Mutlu 3124 (holotype: INU12528-2013; isotype; INU 12528-2013).

e***A. mardinense* Balos, H. Akan & Yıldırım in Ann Bot Fenn 58(4-6): 342 (2021). [Balos, Akan, Yıldırım, & Geçit, 2021]

Type: Turkey. Mardin Province, Artuklu Region, Akreste Pass to Sultançayırı meadows, limestone slopes, 1100 m a.s.l., 29 June 2019 M. Balos 4354 (holotype HARRAN isotypes HARRAN, EGE, NGBB).

e***A. muratozelii* Armağan in Phytotaxa 498 (4): 256 (2021). [Armağan, 2021]

Type: Turkey. Tunceli: 26 km from Tunceli to Ovacık (Munzur valley), steppe, 1100m, 39° 15′41.0″ N 39°28′06.0″E, 30 April 2015, M.Özel & M.Armağan 6688 (holotype KNYA-26923; AEF, NGBB).

e***A. nerimaniae* Koçyiğit & E. Kaya in Phytotaxa 435 (1): 17 (2020). [Koçyiğit & Kaya, 2020]

Type: Turkey. B9 Van, Gürpınar, around Sapakonak village, elevation 2535 m, 7 July 2013, E. Kaya 4455 (holotype: ISTE; isotypes: NGBB, AEF).

e***A. shahinii* H. Duman & Ekşi in Phytotaxa 461 (3) : 196 (2020). [Ekşi & Duman, 2020]

Type: Turkey. Erzincan: 1. km from İliç to Çaltı road, dynamic and graveled steppe, 1270 m, 37 S 463852 E, 4367451 N, 20.vi.2018, H. Duman 10602 (GAZI holotype; AEF, ANK isotypes).

e***A. shinasii* Armağan in Nord J Bot 39 (10)-e03145: 2 (2021). [Armağan, 2021]

Type: Turkey. Tunceli: Nazımiye, 7 km NE of Büyükyurt (Hakis) village, 39°17′27.8″N, 39°51′10.2″E, oak forest openings, 2070 m a.s.l., 15 Jul 2020, Armağan 8361 (holotype ANK; isotypes AEF, KNYA, NGBB).

e***A. sultanae-ismailii* Yıldırım in Phytotaxa 403 (1) : 40 (2019). [Yıldırım, 2019]

Type: Turkey. Malatya: Darende, Akbabaçalı Mountain, open mountain slopes, calcareous rocky areas, 2150 m, 30.05.2012, H. Yıldırım 2400 (holotype EGE; isotypes EGE, NGBB).

e***A. yamadagensis* Yıldırım & Ekşi in Phytotaxa 400 (1): 031-036 (2019). [Ekşi & Yıldırım, 2019]

Type: Turkey. Malatya: Hekimhan, Yama Dağı, zirve, volkanik kırık kayalık yamaçlar, 2570 m, 27 July 2015, H. Yıldırım 3487 (holotype, EGE, isotypes, EGE, NGBB, ANK, HUB).

***Bellevalia* Lapeyr.**

e***B. bayburtensis* Sefalı & Yıldırım in Phytotaxa 441 (3): 286 (2020). [Yıldırım & Sefalı, 2020]

Type: Turkey. Bayburt: Kopdağı silsilesi, Bahtlı Dağı, kırık taşlık ve çarşak alanlar, 2604 m, 40o 02' N, 40o 30' E, 01 June 2019, *A.Sefalı 433* (holotype: EGE 43190; isotypes: ANK, EGE 43191, NGBB).

e***B. guneriana* Tugay & Armağan in Bağbahçe Bilim Dergisi 8 (3): 2 (2021). [Tugay, Armağan, & Ulukış, 2021]

Type: Turkey. Hatay: Belen, Amanos Dağları, Karlık Tepe, kireçtaşlı yamaçlar, *Quercus coccifera* maki açıklığı, 900 m, 24.iv.1957, P.H. Davis 27040 & Hedge (holotype / holotip: E00340644).

e***B. sasonii* Fidan in Phytotaxa 394 (2): 127 (2019). [Fidan, 2019]

Type: Turkey. B8 Batman: Sason country Karameşe village road 1. km, 38°17′48.29″N, 41°19′53.97″E, forest clearance, 733 m, 11April 2017, *M Fidan* 3012 (holotype SUFAF; isotype VANF).

e***B. turcica* Pınar & Eroğlu in Biologia 74: 449 (2019). [Pınar & Eroğlu, 2019a]

Type: Turkey. C5 Adana: Pozantı, above Hamidiye village, Çukuryurt place, 37°32′36″ N, 34°59′37″ E, *Juniperus* yards, 1515 m, 15.05.2015, M. Pınar 6502, H. Eroğlu (holotype VANF, isotypes GAZI, ANK).

***Fritillaria* Tourn. ex L.**

e***F. arsusiana* Yıldırım & Tekşen in Phytotaxa 502 (2): 150 (2021). [Yıldırım & Tekşen, 2021]

Type: Turkey. Hatay: İskenderun, Arsuz, Hacıahmetli Köyü'nden askeri radara çıkış yolu, Amanos dağları, zirve civarı düzlük, çam ve meşe açıklıkları, serpantin topraklar, 1540 m a.s.l., 23 April 2008, H. Yıldırım 1309 (holotype, EGE 43194; isotype, GAZI).

e***F. gencensis* Yıld., Kılıç & A.Demirpolat in Ot Sistematik Botanik Dergisi, 26(1): 2 (2019). [Yıldırımlı, Kılıç, & Demirpolat, 2019].

Type: B8 Bingöl, Genç ilçesi, Çevirme köyü, Şehittepe mevkisi, küçük kepez başı, 1650 m, 22.04.2019, Ş. Yıldırımlı 45138, Ö. Kılıç & A.Demirpolat (holo. Yıldırımlı Otluk'u (YO); iso. EGE, GAZI, HUB, YO).

***Galanthus* L.**

e***G. bursanus* Zubov, Konca & A.P.Davis in Kew Bulletin 74:18 (2019). [Zubov, Konca, & Davis, 2019]

Type: Turkey. Bursa Province, Marmara Sea region, limestone rocky outcrops near Bursa, c. 500 m, fl. 26 Nov. 2016, Zubov & Konca s.n. (holotype KWHA).

***Muscari* Mill.**

******M. commutatum* Guss. in Fl. Sic. Prodr. 1:426, t. 180 f. 4 (1827). [Uysal, Bozkurt, & Demirelma, 2021]

Examined specimens: Turkey. Antalya: Tahtalı Dağı, teleferik tesisinin kuzeydoğusu, taşlık kayalık sekiler, kaya oyukları, 735–745 m, 24.03.2019, *T.Uysal 3808* (KNYA); Kemer, Tahtalı Dağı teleferik civarı kalker kayalık alan, 720 m, 07.03.2020; *T.Uysal 3970 & H.Demirelma* (KNYA); Kumluca, Adrasan beldesi, denize bakan güney eğimli orman yamaçları ve açıklıkları, 5–60 m, 07.03.2020; *T.Uysal 3976 & H.Demirelma* (KNYA); Kuyucak Dağı, close to Altıkaya, around and above the remains of ancient Selge, 900–1200 m, 20.04.2006, *Staudinger 11743* (W); İzmir: Efes antik kenti, Bülbül Dağı, 25–200 m, 25.03.2002, *F.Speta s.n.* (W);

e***M. erzincanicum* Eker in Phytotaxa 487 (1): 44 (2021). [Eker, 2021]

Type: Turkey. B7 Erzincan: Between Erzincan to Refahiye, surroundings of Sakaltutan Pass, on fine serpentine stacks, 1531 m of elevation, 08 May 2020, İ. Eker 12931, A. Kandemir, E. Eker (holotype AIBU; isotypes AIBU, HUB).

e***M. fatmacereniae* Eker in Phytotaxa 397 (1): 102 (2019). [Eker, 2019a]

Type: Turkey. C5 Adana: Tufanbeyli, around Güzelim village, on streamside in openings of pine forest, 1436 m of elevation, 09 May 2018, I. Eker 12586 (holotype AIBU; isotypes AIBU, NGBB, HUB).

e***M. inundatum* Yıldırım & Eker in Phytotaxa 484 (2): 182 (2021). [Eker & Yıldırım, 2021]

Type: Turkey. C6 Hatay: Antakya, Amanos Mountain, above Kisecik Village, Yukarı Zorkun Upland, creek edges in pine forest openings, 1518 m a.s.l., 28 April 2020, İ. Eker 12808 (holotype AIBU; isotypes AIBU, EGE).

e***M. muglaensis* Eker, H. Duman & Yıldırım in Phytotaxa 475 (4): 268 (2020). [Eker, Duman, & Yıldırım, 2020]

Type: Turkey. C2 Muğla: Köyceğiz, Sandras Mountain, Lake Kartal surroundings, on ophiolite rock cracks and serpentine soils, 1952 m of elevation, 08 June 2020, İ. Eker 13030 (holotype AIBU; isotypes AIBU, EGE, GAZI).

e***M. nazimiyensis* Yıld. & Kılıç in Ot Sistematik Botanik Dergisi, 26(1): 14 (2019). [Yıldırımlı & Kılıç, 2019c].

Type: B7 Tunceli, Nazimiye, Büyükyurt (Hakis) köyü girişi, dere başo, karışık orman, 1500 m, 31.05.2015, Ş. Yıldırımlı 41190 & Ö. Kılıç (holo. Yıldırımlı Otluk'u; iso. HUB).

e***M. pamiryigidii* Eker in Phytotaxa 408(4): 256 (2019). [Eker, 2019b]

Type: Turkey. A3 Bolu: Mudurnu, Abant Mountains, around antenna tower, alpine and subalpine areas, meadow and stony places, 1756 m of elevation, 18 May 2012, *İ. Eker 2768* (holotype AIBU; isotypes AIBU, NGBB, HUB).

******M. pallens* (M.Bieb.) Fisch. in Cat. Jard. Gorenki, ed. 2: 9 (1812). [Eker, Yıldırım, & Armağan, 2019]

Examined specimens: Turkey. B9 Van: Gevaş, Deveboynu Yarımadası, göl kenarı, açık yamaçlar, 29 iv 2007, HY-1124 (EGE); Göründü-Altınsaç Köyleri arası, 4. km, bozkır, 1680 m, 03 v 2017, M.Armağan 7250 & 7251 (AIBU); Göründü-İnköy arası, 1700-1750 m, 03 v 1997, M.Koyuncu 11579 (AEF 25786); ibid., yamaçlar, 1750 m, 11 v 1997, M.Koyuncu 11622 (AEF 25778); Ağıllı Köyü, 1730 m, 03 v 1997, M.Koyuncu 11579 & N.Demirkuş 5111 (AEF 25781); ibid., taşlı yamaçlar, 1700 m, 11 v 1997, M.Koyuncu (AEF 25774); Göründü-Altınsaç köyleri arası, Ağıllı Köyü, yamaçlar, 1750 m, 19 iv 1998, N.Demirkuş (AEF 25092); ibid., 1750 m, 19 iv 1999, N.Demirkuş (AEF 25091); ibid., 1750 m, 19 iv 1998, N.Demirkuş (AEF 25093); Göründü-Altınsaç köyleri arası, step, yamaçlar, 1750 m, 06 v 2002, M.Koyuncu 14074 & N.Adıgüzel (AEF 25811); ibid., Van Gölü'nün güneyi, yamaçlar, 1700 m, 18 iv 2004, M.Koyuncu 14074 (AEF 25650).

e***M. sabihapinari* Eroğlu, Pinar & Fidan in Nordic Journal of Botany 37 (11): 2 (2019). [Eroğlu, Pınar, & Fidan, 2019]

Type: Turkey. B6 Adana: Tufanbeyli, Demiroluk village, Bey Mountain-Kartal Mountain, steppe, stony slopes, 38°14′12″N, 36°02′55″E, 2025 m, a.s.l., 18 May 2015, H. Eroğlu 1153 (holotype VANF; isotypes GAZI, ANK).

e***M. savranii* Uysal & Doğu in Phytotaxa 402 (3): 157 (2019). [Doğu & Uysal, 2019]

Type: Turkey. B5 Kayseri: Kocasinan, Oymaağaç Köyü üstleri, Jipsli yamaçlar, elev. 1150–1200 m, 26 April 2018, *S. Doğu 3051* (Holo KNYA).

e_M. tauricum_** S. Demirci, N. Özhatay & E. Kaya in Phytotaxa 399 (2): 112 (2019). [Demirci-Kayıran, Özhatay, & Kaya, 2019]

Type: Turkey. C4 Mersin: Tarsus-Çamlıyayla, 18 km of Çamlıyayla, wet rift valley, 1140 m of elevation, 14 April 2011, E. Kaya 1918 (holotype ISTE 94347).

***Ornithogalum* L.**

e_O. gulnariensis_** Demir. & Uysal in Kew Bulletin 75: 18 (2020). [Demirelma, 2020]

Type: Turkey. C4 İçel, Mut-İçel, Dağ Pazarı-Kilise road, 2 – 3 km, near Çivi village, *Pinus–Quercus* openings, 36°44.8′ 74″N, 33°23.9′71″(961)E, 860 m, 19 April 2013, T. Uysal 2840, O. Tugay&H. Demirelma (holotype: KNYA).

e_O. malatyanum_** Mutlu subsp. ***aricakense*** Behçet & Yapar in Nord J Bot 38(11)-e02837: 2 (2020). [Yapar & Behçet, 2020]

Type: Turkey. B8 Elazığ: Arıcak district, east of Karakaş village, rocky area, accumulated soil between the rocks, 1200 m a.s.l., 15 Jun 2019. Y.Yapar 3068. (holotype: Bingöl Univ. Biology Dept. Herbarium (BIN); isotypes: ANK, KNYA, BIN).

e_O. nitidum_** Yıld. & Kılıç in Ot Sistematik Botanik Dergisi, 26(2): 11 (2019). [Yıldırımlı & Kılıç, 2019f].

Type: B7 Tunceli, Nazimiye, Büyükyurt (Hakis) köyü üstü, Şirince çevresi, titrekkavak (*Populus tremula*) ve meşe (*Quercus*) orman altı, 1850 m, 31.05.2015, Ş. Yıldırımlı 41234 & Ö. Kılıç (holo. Yıldırımlı Otluk'u iso. GAZI).

e_O. plurifolium_** Yıld. & Kılıç in Ot Sistematik Botanik Dergisi, 26(2): 16 (2019). [Yıldırımlı & Kılıç, 2019f].

Type: B7 Tunceli, Ovacık, Yılan dağı, Cevizlidere köyünden İt yokuşu-Eşek meydanı-Dev boğazı, Tapik tepe-Deve boynu-Barasor deresi izleğiyle Karataş köyüne, bozkır, 1550-2250 m, 19.06.2015, Ş. Yıldırımlı 41600 & Ö. Kılıç (holo. Yıldırımlı Otluk'u iso. GAZI).

e_O. sancakense_** Yıld. & Kılıç in Ot Sistematik Botanik Dergisi, 26(2): 24 (2019). [Yıldırımlı & Kılıç, 2019f].

Type: B8 Bingöl, Bingöl-Sancak arası, bayır, yamaç, meşelik (*Quercus brandii*), yamaçlar, çayırlık, 1550-1600 m, 04.05.2018, Ş. Yıldırımlı 44506 & Ö. Kılıç (holo. Yıldırımlı Otluk'u (YO); iso. GAZI, YO).

e_O. yesilyurtense_** Yıld. & Kılıç in Ot Sistematik Botanik Dergisi, 26(2): 29 (2019). [Yıldırımlı & Kılıç, 2019f].

Type: B6 Malatya, Yeşilyurt-Çelikhan arası, kayalık ve taşlık, bayır, 1500-1700 m, 25.04.2019, Ş. Yıldırımlı 45168 & Ö. Kılıç (holo. Yıldırımlı Otluk'u).

***Scilla* L.**

e_S. hakkariensis_** Fırat & Yıldırım in Adansonia 42 (2): 90 (2020). [Fırat & Yıldırım, 2020]

Type: Turkey. Hakkari: Şemdinli district, Gelyaşlı region, on rock area and *Crataegus* bushes opening, 890 m, 37°4′45″ N, 44°25′46″ E, 7.ıv.2012, M. Fırat 28629 (holo-, VANP; iso.-, EGE, HUB, VANP, and in the personal herbarium of the collector Herb. Fırat).

ORCHIDACEAE

***Ophrys* L.**

eO. bingoelensis** Yıld. & Kılıç in Ot Sistematik Botanik Dergisi, 26(2): 40 (2019). [Yıldırımlı & Kılıç, 2019j].

Type: B8 Bingöl, Sancak-Uğurova arası, güney kesimleri, meşe altı, nemli yerler, 1450-1500 m, 24.05.2019, Ö. Kılıç 8979 (holo. Yıldırımlı Otluk'u).

***Anacamptis* L.**

***_A. × parvifolia_** (Chaub.) H. Kretzschmar, Eccarius & H.Dietr. in Orchid. Gen. Anacamptis, Orchis, Neotinea, ed. 2: 427 (2007). [Eker & Başaran, 2019]

Examined specimen: Turkey. A3 Bolu: Abant yolu, ıslak çayırlıklar, 813 m, 14.vi.2015, Başaran 87 & Eker (AIBU).

***Orchis* Tourn. ex L.**

e_O. bingoelensis_** Yıld. & Kılıç in Ot Sistematik Botanik Dergisi, 26(2): 44 (2019). [Yıldırımlı & Kılıç, 2019j].

Type: B8 Bingöl, Kiğı, Karakoçan-Sancak yol ayrımından sonra y. 6 km, mezarlık yakını, yol kıyısı, yamaç, 1450 m, 24.05.2017, Ş. Yıldırımlı 43375 & Ö. Kılıç (holo. Yıldırımlı Otluk'u iso. GAZI).

***Zeuxine* Lindl.**

***_Z. strateumatica_** (L.) Schltr. in Bot. Jahrb. Syst. 45(3): 394 (1911). [Bozkurt, Öz, & Yıldırım, 2021]

Examined specimen: Muğla: Ortaca, Sarıgerme mahallesi, deniz kenarı, açık çayır alanlar ve kumullar, 5 m, 23 ii 2021, N.Bozkurt, A.Öz ve H.Yıldırım (EGE 43528).

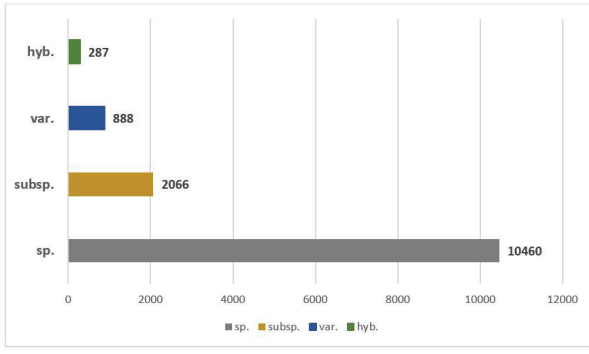

Figure 3. Current data about Turkish flora.

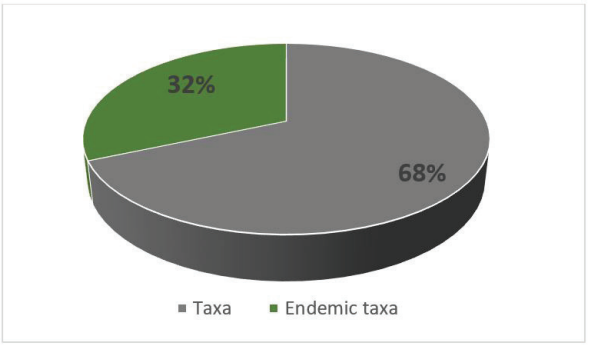

Figure 4. Endemism of the Turkish flora.

CONCLUSION

The diversity of vegetation and richness of Turkey's flora are legendary. Based on the data gathered from these recent studies, a new taxon has been added to the flora of Turkey every 4 days and 20 hours in recent years. Up to date, 10,460 species, 2,066 subspecies, 888 varieties, and 287 hybrids have been listed in Turkish flora (Figure 3). According to recent data, endemism is at 32% (Figure 4). As a result of native vascular plant species, a total of 4,319 endemics have been recorded within Turkey to date.

Turkey's flora is rich in endemic taxa. However, the number and status of taxa change on a regular basis for a variety of reasons, including taxa described as new to science, species thought to be endemic to Turkey being discovered elsewhere, and the taxonomy of several groups still being debated. This list must be updated. Current information indicates that the numbers given here belong to December 2021. It is useful to update the numbers at certain times, and these numbers can change.

Peer-review: Externally peer-reviewed.

Informed Consent: Written consent was obtained from the participants.

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

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REFERENCES

- Akalın, E., Tuncay, H.O., Olcay, B., & Miski, M. (2020). A new *Ferula* (Apiaceae) species from Southwest Anatolia: *Ferula pisidica* Akalın & Miski. *Plants, 9*, 740. doi: 10.3390/plants9060740
- Akçiçek, E. (2020). Taxonomic revision of *Stachys* sect. Olisia (Lamiaceae: Lamioideae) in Turkey. *Phytotaxa, 449* (2), 109-148. doi.org/10.11646/phytotaxa.449.2.2
- Akgül, G., & Kılıçkaya, N. (2019). A new variety for the flora of Turkey, *Globularia orientalis* L. var. *nevshehirensis* Akgül. *Ot Sistematik Botanik Dergisi*, *26* (2), 33-38.
- Aksoy, A., Çelik, J., Bozkurt, M., & Uysal, T. (2020). Türkiye Florası İçin Yeni Bir Tür Kaydı: *Scabiosa lucida* Vill. (Caprifoliaceae). *Bağbahçe Bilim Dergisi*, *7*, (3), 49-57. doi.org/ 10.35163/bagbahce.800995
- Aksoy, A., Özcan, T., Girişken, H., Çelik, J., & Dirmenci, T. (2020). A phylogenetic analysis and biogeographical distribution of *Teucrium* Sect. Teucrium (Lamiaceae) and taxonomic notes for a new species from southwest Turkey. *Turkish Journal of Botany*, *44* (3), 322-337.
- Arabacı, T., Dirmenci, T., & Yıldız, B. (2020). *Origanum* L. (Ballıbabagiller/ Lamiaceae) Cinsine Ait Yeni Bir Melez: *Origanum*× *malatyanum* Yıldız, Arabacı & Dirmenci. *Bağbahçe Bilim Dergisi*, *7* (1), 10-15.
- Arabaci, T., Çelenk, S., Özcan, T., Martin, E., Yazici, T., Açar, M., Üzel, D., & Dirmenci, T. (2021). Homoploid hybrids of *Origanum* (Lamiaceae) in Turkey: morphological and molecular evidence for a new hybrid. *Plant Biosystems*, *155* (3), 470-482.
- Armağan, M. (2021). *Allium muratozelii* (Amaryllidaceae), a new species from Turkey. *Phytotaxa*, *498* (4), 255-264.
- Armağan, M. (2021). *Allium shinasii* (Amaryllidaceae), a new species from Turkey. *Nordic Journal of Botany*, *39* (10), e03145. doi: 10.1111/njb.03145.
- Aydın-Uğurlu, Z., & Dönmez, A.A. (2019). Taxonomic and biogeographic notes on the genus *Pyrus* L. (Rosaceae): a new record and a new synonym, with data on seed morphology. *Plant & Fungal Research*, *2* (1), 2-8.
- Aykurt, C., Sümbül, H., Gülben, M., Sarı, D., & Konuralp, L.Y. (2021). *Omphalodes nedimeae* (Boraginaceae), a new species from the Mediterranean Region of Turkey. *Phytotaxa*, *498* (4), 242-254.
- Aytac, Z., Rabaute, P., & Coulot, P. (2020). *Onobrychis silvanensis* sp. nov., a new Fabaceae (sect. Hymenobrychis) taxon from Turkey. *Phytotaxa*, *477* (2), 253-260.
- Aytaç, Z., Çeçen, Ö., & Fişne, A. (2020). *Astragalus sertavulensis* (sect. Onobrychoidei/Fabaceae), a new species from Turkey. *Nordic Journal of Botany*, *38* (9), e02829.
- Aytaç, Z., Kaptaner-İğci, B., Körüklü, T.S. (2020). *Hedysarum nallihanse* (Fabaceae): A new species from Turkey. *Phytotaxa*, *471* (3), 276-282.
- Balos, M.M., Akan, H., Yıldırım, H., & Geçit, M. (2021). *Allium mardinense* (Amaryllidaceae), a new species from southeastern Turkey. *Annales Botanici Fennici*, *58* (4-6), 341-346.
- Başköse, İ., & Yaprak, A.E. (2019). A new species from Central Anatolia in Turkey, *Atriplex turcica* (Chenopodiaceae). *Phytotaxa*, *424* (4), 232-242.
- Başköse, İ., & Yaprak, A.E. (2021). A New *Suaeda* Record For Flora of Turkey: *Suaeda Aegyptiaca* (Hasselquist) Zohary (Chenopodiaceae/Amaranthaceae). *Trakya University Journal of Natural Sciences*, *22* (2), 179-185.
- Behçet, L., & Yapar, Y. (2020). *Lactuca anatolica* (Asteraceae: Lactucinae), a new species from eastern Anatolia (Turkey). *Phytotaxa*, *455* (4), 287-294.
- Behçet, L., & Yapar, Y. (2021). *Bromus orientalis* (Poaceae: B. sect. Bromopsis), a new species from Turkey. *Nordic Journal of Botany*, *39* (4), e02959. doi: 10.1111/njb.02959
- Behçet, L., Yapar, Y., & Olgun, Ş. (2019). *Prangos aricakensis* (Apiaceae), a new species from eastern Turkey. *Phytotaxa*, *401* (1), 55-63.
- Bogdanović, S., Brullo, S., & Salmeri, C. (2021). *Loncomelos koprulense* (Asparagaceae), a new species from southern Turkey. *PhytoKeys*, 175, 33-44.

- Boydak, M. (2019). A new subspecies of *Phoenix theophrasti* Greuter (*Phoenix theophrasti* Greuter subsp. *golkoyana* Boydak) from Turkey. *Forestist*, *69* (2), 133-144.
- Bozkurt, N., Öz, A., & Yıldırım, H. (2021). Türkiye Florası İçin Yeni Bir Bitki Türü Kayıdı: *Zeuxine strateumatica* (L.) Schltr. (Orchidaceae). *Bağbahçe Bilim Dergisi*, *8* (2), 1-6.
- Bozyel, D., Pelit, N.B., & Şenol, S.G. (2021). A new record from the mediterranean temporary wetland habitats, for Flora of Turkey: *Crassula vaillantii*. *Biyolojik Çeşitlilik ve Koruma, 14* (3), 359-364.
- Celep, F., Karaer, F., & Duman, H. (2021). Resurrection of *Lamium ponticum* (Lamiaceae) with a new subspecies, *Lamium ponticum* subsp. *anatolicum*, from Turkey. *Phytotaxa*, *511* (1), 71-76.
- Celep, F., Raders, E., & Drew, B. (2020). Two new hybrid species of *Salvia* (*S.× karamanensis* and *S.× doganii*) from Turkey: evidence from molecular and morphological studies. *Turkish Journal of Botany*, *44* (6), 647-660.
- Ciftci, A., Harpke, D., & Erol, O. (2019). A new autumn flowering *Crocus* (Iridaceae) from SW Turkey: C. terzioghluii. *Phytotaxa*, *420* (3), 224-232.
- Ciftci, A., Harpke, D., Mollman, R., Yildirim, H., & Erol, O. (2020). Notes on *Crocus* L. Series Flavi Mathew (Iridaceae) and a new species with unique corm structure. *Phytotaxa*, *430*, 65-79.
- Çeçen, Ö., & Özcan, T. (2021). A new *Teucrium* L. (Lamiaceae) Species from South Anatolia (Turkey). *Turkish Journal of Botany*, *45* (4), 353-370.
- Çelik, M., & Bağcı, Y. (2019). A new species of Bunium (Apiaceae: Apioideae) from Central Anatolia in Turkey: *Bunium sivasicum*. *Phytotaxa*, *416* (4), 263-277.
- Çilden, E., & Gençay Çelemli, Ö. (2020). *Minuartia alpuensis* (Caryophyllaceae), a new endemic species from inner Anatolia, Turkey. *Phytotaxa*, *443* (1), 79-91.
- Davis, P.H. (ed). (1965-1985). *Flora of Turkey and the East Aegean Islands, Vol. 1-9*. Edinburgh, UK: Edinburgh University Press.
- Davis, P.H., Tan, K., & Mill, R.R. (1988). *Flora of Turkey and the East Aegean Islands, Vol.10*. Edinburgh, UK: Edinburgh University Press.
- Demir, O., Çıngay, B., & Cabi, E. (2021). Tekirdağ'dan Yeni Bir *Verbascum* L. (Sığırkuyruğu) Melezi, *Verbascum x malkaraense* (Sırcaotugiller / Scrophulariaceae). *Bağbahçe Bilim Dergisi*, *8* (3), 9-20.
- Demirci-Kayıran, S., Özhatay, N., & Kaya, E. (2019*). Muscari tauricum* (Asparagaceae, Scilloideae), a new species from Turkey. *Phytotaxa*, *399* (2), 109-118.
- Demirelma, H. (2020). *Ornithogalum gulnariensis* (Asparagaceae), a new species from southern Anatolia, Turkey. *Kew Bulletin*, *75*(1), 18.
- Dinç, M., & Doğu, S. (2020). *Arenaria goekyigitii* (Caryophyllaceae), a new species from Turkey. *Phytotaxa*, *459* (1), 69-74.
- Dirmenci, T., Duman, H., & Arabaci, T. (2020). Türkiye'den yeni bir köygöçüren [*Cirsium* Mill.(Papatyagiller/Asteraceae)] türü ve cinsin şüpheli bir türünün yeniden keşfi. *Bağbahçe Bilim Dergisi*, *7* (3), 35-44.
- Dirmenci, T., Özcan, T., Arabacı, T., Çelenk, S., İsmayilova, G., & Yıldız, B. (2019). Hybridization among three *Cirsium* (Asteraceae) species and important evidence for three new hybrids from Turkey. *Turkish Journal of Botany*, *43* (3), 366-385.
- Dirmenci, T., & Robson, N.K. (2019). Two new taxa of *Hypericum* (Hypericaceae) from Turkey. *Kew Bulletin*, *74* (4), 1-9.
- Dirmenci, T., Yıldız, B., & Öztekin, M. (2019). Türkiye Florası için yeni bir tür kaydı: *Satureja metastasiantha* Rech.f. (Ballıbabagiller / Lamiaceae. *Bağbahçe Bilim Dergisi*, *6* (1), 54-58.
- Doğan, M., Akaydın, G., & Erdal, J. (2020). Phytogeography, ecology and conservation of the genus *Limonium* (Plumbaginaceae) in Turkey and a taxonomic revision. *Plant Systematics and Evolution*, *306* (6), 89.
- Doğu, S., & Bağcı, Y. (2021). *Stachys ahmet-savranii* Doğu and Bağcı (Lamiaceae), a New Species From South Anatolia, Turkey. *Bangladesh Journal of Botany*, *50* (2), 319-326.
- Doğu, S., & Uysal, T. (2019). Muscari savranii (Asparagaceae), a new species from Central Anatolia, Turkey. *Phytotaxa*, *402* (3), 155-164.
- Duman, H., Aytac, Z., & Ozbek, F. (2020). *Astragalus aybarsii* a new species of sect. Onobrychoidei DC.(Fabaceae) from Turkey. *Turkish Journal of Botany*, *44* (6), 661-669.
- Duman, H., & Çakır-Dindar, E.G. (2020). *Hypericum alacamdaglariense* (Hypericaceae), a new species from Turkey. *Phytotaxa*, *470* (2), 176-185.
- Duman, H., Uzunhisarcıklı, E., & Özbek, F. (2021). A new natural hybrid of *Verbascum* L.(Scrophulariacae) from Turkey. *Gazi University Journal of Science*, *34* (4), 937-946.
- Duman, H., Uzunhisarcıklı, M.E., & Bahadır, Y.N. (2021). A new species of *Centaurea* (Asteraceae) from northern Turkey. *Nordic Journal of Botany*, *39* (10), e03244. doi: 10.1111/njb.03244.
- Duman, H., Uzunhisarcıklı, M.E., & Özbek, F. (2020). A new species of *Verbascum* (Scrophulariacae) from western *Turkey Botany Letters*, *167* (4), 409-416. doi: 10.1080/23818107.2020.1796782
- Duran, A., Samıgullın, T., & Lyskov, D. (2021). *Seseli salsugineum* (Apiaceae), a new species from Central Anatolia, Turkey. *Phytotaxa*, *529* (1), 027-042.
- Eker, İ. (2019a). *Muscari fatmacereniae* (Asparagaceae, Scilloideae), a new species from southern Anatolia. *Phytotaxa*, *397* (1), 099-106.
- Eker, İ. (2019b). *Muscari pamiryigidii* (Asparagaceae, Scilloideae), a new species from northwestern Anatolia. *Phytotaxa*, *408* (4), 255-266.
- Eker, İ. (2021). What is *Muscari massayanum* and what is not? Second species born of confusion: *Muscari erzincanicum* (Asparagaceae, Scilloideae), a new species from Turkey. *Phytotaxa*, *487* (1), 41-55.
- Eker, İ., & Başaran, M. (2019). Türkiye Florası için yeni bir hibrit Orkide kaydı: *Anacamptis x parvifolia* (Chaub.) H. Kretzschmar, Eccarius & H.Dietr. (Salepgiller / Orchidaceae). *Bağbahçe Bilim Dergisi*, *6* (1), 33-39.
- Eker, İ., Duman, H., & Yıldırım, H. (2020). *Muscari muglaensis* (Asparagaceae, Scilloideae), a new species from southwestern Anatolia. *Phytotaxa*, *475* (4), 267-278.
- Eker, İ., & Sağıroğlu, M. (2021). *Gladiolus izzet-baysalii* (Iridaceae, Crocoideae), a new species from Turkey. *Phytotaxa*, *527* (2), 97-106.
- Eker, İ., & Tuzlacı, H.İ. (2021). Türkiye Florası İçin Doğallaşmış Yeni Bir Egzotik Bitki Kaydı: *Freesia leichtlinii* subsp. *alba* (Iridaceae). *Bağbahçe Bilim Dergisi*, *8* (1), 96-105.
- Eker, İ., & Yıldırım, H. (2021). *Muscari inundatum* (Asparagaceae, Scilloideae), a new species from southern Anatolia. *Phytotaxa*, *484* (2), 181–194.
- Eker, İ., Yıldırım, H., & Armağan, M. (2019). Türkiye Florası İçin Yeni Bir Müşkürüm Kaydı: *Muscari pallens* (M.Bieb.) Fisch. (Kuşkonmazgiller/ Asparagaceae). *Bağbahçe Bilim Dergisi*, *6* (1), 45-53.
- Ekşi, G., & Duman, H. (2020). *Allium shahinii*: a new species of A. sect. Scorodon (Amaryllidaceae) from Turkey. *Phytotaxa*, *461*(3), 195-203.
- Ekşi, G., & Yıldırım, H. (2019). *Allium yamadagensis* (Amaryllidaceae) a new species from Turkey. *Phytotaxa*, *400* (1), 031-036.
- Eroğlu, H. (2021). Türkiye Florası'na Yeni Bir Kayıt: *Onopordum blancheanum* (Eig) Danin (Asteraceae: Cardueae). *Bağbahçe Bilim Dergisi*, *8* (3), 21-29.
- Eroğlu, H., Pınar, S.M., & Fidan, M. (2019). *Muscari sabihapinari* sp. nov. (Asparagaceae) from Anatolia, Turkey. *Nordic Journal of Botany*, *37* (11), 1-8.
- Ertuğrul, K., Hamzaoğlu, E., Demirelma, H., Uysal, T., Bozkurt, M., Şirin, E., Yılmaz-Çtak, B., & Al-Shehbaz, I. (2021). *Aethionema aytachii* (Brassicaceae): A new species from central Anatolia, Turkey. *Turkish Journal of Botany*, *45* (6), 563-572.
- Fırat, M. (2019a). *Hirtellina kurdica* (Asteraceae); a species new to the flora of Turkey, with contributions to its taxonomy**.** *Iranian Journal of Botany*, *25* (1), 30-35.

- Fırat, M. (2019b). *Pimpinella kurdica* (Apiaceae), a new record for the flora of Turkey. *Iranian Journal of Botany*, *25* (2), 123-126.
- Fırat, M. (2021). *Stachys semsurensis* (Lamiaceae), a new species from Adıyaman province (Turkey) belonging to section Infrarosularis. *Phytotaxa*, *511*(3), 275-282.
- Fırat, M., & Güzel, Y. (2019). Contributions to the taxonomy of the Irano-Turanian genus *Rhabdosciadium* (Apiaceae): Nomenclatural notes, carpology, molecular phylogeny and the description of a new species from Bitlis (Turkey). *Phytotaxa*, *395* (3), 179–198.
- Fırat, M., & Yıldırım, H. (2020). *Scilla hakkariensis*, sp. nov. (Asparagaceae: Scilloideae): a new species of *Scilla* L. from Hakkari (eastern Anatolia). *Adansonia*, *42* (2), 89-94.
- Fidan, M. (2019). *Bellevalia sasonii* (Asparagaceae): a new species from Turkey. *Phytotaxa*, *394* (2), 126-132.
- Fidan, M., Pınar, S.M., & Eroğlu, H. (2019). *Cousinia gigantosphaera* Rech, f. (Asteraceae-Compositae); Türkiye'nin Güneydoğu Anadolu Bölgesi'nden Yeni Bir Bitki Türü Kaydı. *Türkiye Tarımsal Araştırmalar Dergisi*, *6* (3), 308-311.
- Firat, M., & Binzet, R. (2021). *Onosma satensis* sp. nov.(Boraginaceae: Lithospermeae), a new species from Hakkari (eastern Anatolia, Turkey). *Adansonia*, *43* (16), 185-195.
- Göktürk, R.S., Düşen, O., Kaya, E., Gürcan, B., & Sarpkaya, U. (2019). A new variety of *Plocama calabrica* (Rubiaceae) from Denizli (Turkey) onfirmed by morphological and molecular ISSR markers. *Acta Botanica Croatica*, *78* (2), 142-146.
- Güner, A., Aslan, S., Ekim, T., Vural, M., & Babaç, M.T. (2012). *Türkiye Bitkileri Listesi (Damarlı Bitkiler)*. İstanbul, Turkey: Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği Yayını (in Turkish).
- Güner, A., Özhatay, N., Ekim, T., & Başer, K.H.C. (2000). *Flora of Turkey and the East Aegean Islands, Vol. 11*. Edinburgh, UK: Edinburgh University Press.
- Güner, Ö., Akcicek, E., & Dirmenci, T. (2021). A new *Stachys* species from Turkey: *Stachys siirtensis* (Lamiaceae). *Phytotaxa*, *516* (3), 252-262.
- Güneş, F. (2019). A new species of *Lathyrus cirpicii* F.Güneş (Fabaceae) from Turkey. *Biodicon*, *12*(2), 161-168.
- Gürdal, B., Olcay, B., Tuncay, H.O., & Akalın, E. (2021). *Ferulago akpulatii* (Apiaceae): A new species from Central Anatolia, Turkey. *Phytotaxa*, *518* (2), 100–108.
- Gürdal, B., Tuncay, H.O., & Akpulat, H.A. (2019). *Peucedanum akaliniae* (Apiaceae), a new species from Southern Turkey. *Phytotaxa*, *425* (3), 145-153.
- Güzel, Y. (2021). A new *Dionysia* (Primulaceae) species from southern Turkey, the most western species of the Irano-Turanian genus. *Phytotaxa*, *525* (4), 281-294.
- Hamzaoğlu, E. (2020a). *Astragalus askaleensis* (sek. Adiaspastus, Fabaceae), Türkiye'den yeni bir tür. *Türler ve Habitatlar*, *1* (2), 114-123.
- Hamzaoğlu, E. (2020b). *Dianthus dumanii*: Orta Anadolu jipsli bozkırlarından yeni bir tür. *Türler ve Habitatlar*, *1* (1), 21-30.
- Hamzaoğlu, E. (2021a). *Crucianella turcica* (sek. Crucianella, Rubiaceae), Türkiye'den Yeni Bir Tür. *Türler ve Habitatlar*, *2* (2), 113-122.
- Hamzaoğlu, E. (2021b). Türkiye'den Yeni Bir Tür, *Asyneuma hasandaghense* (Campanulaceae). *Türler ve Habitatlar*, *2* (1), 68-76.
- Hamzaoğlu, E., Behçet, L., & Yapar, Y. (2020). A New Suffruticose Taxon of Dianthus (Caryophyllaceae) from Bingöl, Turkey. *KSU Journal of Agriculture and Nature*, *23* (6), 1529-1534. doi: 10.18016/ksutarimdoga.vi.714530.
- Hamzaoğlu, E., & Koç, M. (2019). Türkiye florası için yeni *Dianthus* (Caryophyllaceae) kayıtları. *Kahramanmaraş Sütçü İmam Üniversitesi Tarım ve Doğa Dergisi*, *22* (3), 381-388.
- Hamzaoğlu, E., & Koç, M. (2020). *Hedysarum turcicum* (Hedysareae, Fabaceae), a new species from Turkey. *Phytotaxa*, *428* (1), 1-6.
- Hamzaoğl, E., & Koç, M. (2020). Türkiye florası için yeni bir kayıt, *Centaurea gulissashwilii* (sek. Centaurea, Asteraceae). *Türler ve Habitatlar*, *1* (2), 106-113.
- Hamzaoğlu, E., Koç, M., & Büyük, İ. (2021). *Dianthus yilmazii* (Caryophyllaceae), a new species from central Turkey. *Kew Bulletin*, *76*, 523-530.
- Hamzaoğlu, E., Koç, M., & Topal, M.N. (2021). A new species, *Gypsophila malyerii* (Caryophyllaceae) from Turkey. *Kew Bulletin*, *76* (3), 531-538.
- Harpke, D., Peruzzi, L., Kerndorff, H., Karamplianis, T., Constantinidis, T., Randelovic, V. … Blattner, F.R. (2014). Phylogeny, geographic distribution, and new taxonomic circumscription of the *Crocus reticulatus* species group (Iridaceae). *Tukish Journal of Botany*, *38*, 1182-1198.
- Inceer, H., & Kalmuk, A.N. (2019). *Crepis setosa* subsp. *topaliana* (Asteraceae): a new record for the flora of Turkey. *Botanica Serbica*, *43* (1), 109-112.
- İlçim, A., & Karahan, F. (2020). *Rheum telianum* (Polygonaceae), a new species from Southeastern Anatolia (Turkey). *Phytotaxa*, *477* (1), 81-89.
- Kandemir, A., Aytaç, Z., & Fişne, A.Y. (2017). *Aethionema erzincanum* (Brassicaceae), a new species from Turkey. *Annales Botanici Fennici*, *54* (1–3), 1-5.
- Karaer, F., Terzioğlu, S., & Kutbay, H.G. (2020). A New Genus Record for the Flora of Turkey: *Reynoutria* (Polygonaceae). *Kahramanmaraş Sütçü İmam Üniversitesi Tarım ve Doğa Dergisi*, *23* (3), 606-610.
- Karaer, F., Terzioğlu, S., & Kutbay, H.G. (2020). A New Record for the Flora of Turkey: *Cerastium szowitsii* Boiss. (Caryophyllaceae). *Kastamonu University Journal of Forestry Faculty*, *20* (1), 58-61.
- Karakuş, Ş., & Mutlu, B. (2019). *Allium dönmezii*, a new species of *Allium* sect. Melanocrommyum (Amaryllidaceae) from Turkey: morphological and molecular evidence. *Phytotaxa*, *411* (3), 194-204.
- Karaman-Erkul, S., Tekşen, M., & Duman, H. (2021). *Ranunculus aydogdui* (Ranunculaceae) a new species. *Nordic Journal of Botany*, *39* (1), 1-8.
- Kerndorff, H., Pasche, E., & Harpke, D. (2014). *Crocus isauricus* Siehe ex Bowles (Liliiflorae, Iridaceae) and its relatives. *Stapfia*, *101*, 3–18
- Kerndorff, H., Pasche, E., & Harpke, D. (2015). *Crocus lyciotauricus* Kerndorff & Pasche (Liliiflorae, Iridaceae) and its relatives. *Stapfia*, *103*, 67–80
- Keskin, M. (2021a). Novelties in the genus *Cerastium* (Caryophyllaceae) in Turkey. *Frontiers in Life Sciences and Related Technologies*, *2* (2), 35-43.
- Keskin, M. (2021b). The genus *Moenchia* (Caryophyllacae) in Turkey. *Biodicon*, *14* (1), 119-131.
- Keskin, M., & Merrick, D. (2021). A new species from Turkey: *Eleocharis divaricata* (Cyperaceae) and a note for *E. atropurpurea*. *Frontiers in Life Sciences and Related Technologies*, *2* (1), 10-13.
- Keskin, M., & Severoğlu, Z. (2020). The genus *Persicaria* (Polygonaceae) in Turkey with a new taxon record. *EMU Journal of Pharmaceutical Sciences*, *3* (2), 97-105.
- Keskin, M., & Severoğlu, Z. (2021a). A new species as a member of the Flora of Turkey: *Persicaria hydropiperoides* (Polygonaceae). *Frontiers in Life Sciences and Related Technologies*, *2*(3), 85-91. doi: 10.51753/flsrt.951412
- Keskin, M., & Severoğlu, Z. (2021b). Türkiye Florası için Yeni bir *Persicaria* L. Taksonu ve İstanbul'un Polygonaceae Taksonlarının Taksonomik Durumu. *Afyon Kocatepe Üniversitesi Fen ve Mühendislik Bilimleri Dergisi*, *21* (4), 764-775.
- Koc, M. (2020). Morphological and molecular evidence for a new species of *Dianthus* (Caryophyllaceae) from Turkey. *Phytotaxa*, *439* (1), 56-66.
- Koc, M., Hamzaoğlu, E., & Aksoy, A. (2019). Morphological and molecular evidence of Turkish *Minuartiella* species (Caryophyllaceae), with a desciption of a new species and a proposal for a new combination. *Phytotaxa*, *409* (3), 161-171.

- Koç, M., Hamzaoğlu, E., & Aksoy, A. (2019). A new species and five new combinations of Sabulina (Caryophyllaceae) from Turkey. *Folia Geobotanica*, *54*, 279–289.
- Koç, M., Hamzaoğlu, E., & Aksoy, A. (2019). Morphological and molecular evidence of some *Minuartia* species (Caryophyllaceae) from Turkey, with a description of a new species. *Phytotaxa*, *391* (2), 122-130.
- Koç, M., Hamzaoğlu, E., & Aksoy, A. (2020). Contributions to the genus *Minuartia* (Caryophyllaceae) in Turkey. *Türler ve Habitatlar*, *1* (1), 14-20.
- Koç, M., Hamzaoğlu, E., & Aksoy, A. (2021). Molecular and morphological evidences of *Sabulina nerimanae* sp. nov. (Caryophyllaceae). *Phytotaxa*, *498* (4), 281-289.
- Koç, M., Hamzaoğlu, E., & Büyük, I. (2019). Morphological and molecular evidence for a new species of Bolanthus (Caryophyllaceae) from Turkey. *Systematic Botany*, *44* (1), 189-196.
- Koçyiğit, M., & Kaya, E. (2020). *Allium nerimaniae* (Amaryllidaceae), a new species of A. sect. Codonoprasum from Turkey. *Phytotaxa*, *435* (1), 016-024.
- Menemen, Y., Yalçınkaya, R., & Erden, A. (2021). *Lepyrodiclis alinihatii* (Caryophyllaceae), a new species from Turkey. *Nordic Journal of Botany*, *39* (3), e03037. doi: 10.1111/njb.03037
- Orcan, N., & Binzet, R. (2003). *Fumaria pugsleyana* (Maire ex Pugsley) Liden: A new record for the Flora of Turkey. *Bangladesh Journal of Botany*, *32* (1), 43-45.
- Ozdeniz, E. (2019). *Bufonia ali-nihaii* (Caryophyllaceae), A New Species From Turkey. *Fresenius Environmental Bulletin*, *28* (12), 9176-9180.
- Özbek, M.U. (2021). *Centaurea kirikkalensis* (Asteraceae), a new species from Turkey. *Nordic Journal of Botany*, *39* (7), e03235. doi: 10.1111/njb.03235
- Özbek, M.U., Duman, H,. Özbek, F., & Aytaç, Z. (2021). *Anthemis ekicii* (Asteraceae), a new species from Turkey. *Turkish Journal of Botany*, *45* (1), 59-68.
- Özbek, M.U., Koç, M., & Hamzaoğlu, E. (2019). Contributions to the *Hypericum* L. section Oligostema (Boiss.) Stef. (Hypericaceae), and *Hypericum turcicum* sp. nov. as a new species from Turkey. *Turkish Journal of Botany*, *43*, 694-702.
- Özbek, M.U., & Onaylı, H. (2020). A new variety of the *Tripleurospermum* (Asteraceae) from Turkey. *Biodicon*, *13* (2), 136-143.
- Özbek, M.U., & Uzunhisarcıklı, M.E. (2019). A new species of *Silene* (Caryophyllaceae) from Turkey. *Phytotaxa*, *397* (1), 074–082.
- Özçandır, A., Aykurt, C., & Özüdoğru, B. (2019). *Ricotia candiriana* (Brassicaceae), a new species from southern Anatolia, Turkey. *Phytotaxa*, *388* (4), 287-294.
- Özgişi, K. (2020). A new species of *Noccaea* (Brassicaceae) from central Anatolia. *Phytotaxa*, *432* (1), 95-103.
- Özgişi, K., & Ocak, A. (2021). A new taxon of *Hypericum* (Hypericaceae) from southeastern Turkey. *Phytotaxa*, *479* (1), 129-136.
- Özhatay, F.N., Kültür, Ş., & Gürdal, M.B. (2011). Check-list of additional taxa to the Supplement Flora of Turkey V. *Turkish Journal of Botany*, *35*, 589-624.
- Özhatay, N., & Kültür, Ş. (2006). Check-list of additional taxa to the Supplement Flora of Turkey III. *Turkish Journal of Botany*, *30*, 281-316.
- Özhatay, N., Kültür, Ş., & Aslan, S. (2009). Check-list of additional taxa to the Supplement Flora of Turkey IV. *Turkish Journal of Botany*, *33*, 191-226.
- Özhatay, N., Kültür, Ş., & Gürdal, B. (2013). Check-list of additional taxa to the Supplement Flora of Turkey VI. *Journal of Faculty of Pharmacy Istanbul University*, *43*, 33-82.
- Özhatay, N., Kültür, Ş., & Gürdal, B. (2015). Check-list of additional taxa to the Supplement Flora of Turkey VII. *Journal of Faculty of Pharmacy Istanbul University*, *45*, 61-86.
- Özhatay, N., Kültür, Ş., & Gürdal, B. (2017). Check-list of additional taxa to the supplement flora of Turkey VIII. *Istanbul Journal of Pharmacy*, *47*, 31-46.
- Özhatay, N., Kültür, Ş., & Gürdal, B. (2019). Check-list of additional taxa to the supplement flora of Turkey IX. *Istanbul Journal of Pharmacy*, *49* (2), 105-120.
- Özüdoğru, B., & Yıldırım, H. (2019). Doğu Anadolu'dan yeni bir topaçhardalı alttürü: *Heldreichia bupleurifolia* subsp. *malatyana* (Brassicaceae). *Bağbahçe Bilim Dergisi*, *6* (3), 1-8.
- Pınar, S.M., & Eroğlu, H. (2019a). *Bellevalia turcica* sp. nov. (Asparagaceae): a new species from South Anatolia, Turkey. *Biologia*, *74*, 447-454.
- Pınar, S.M., & Eroğlu, H. (2019b). *Onopordum nezaketianum* sp. nov. (Asteraceae: Cardueae): a new species from Central Anatolia, Turkey. *Turkish Journal of Botany*, *43*, 126-134.
- Raab-Straube, E., von, & Raus, Th. (ed.) (2019). Euro+Med-Checklist Notulae, 10. *Willdenowia*, *49*, 95-115. doi: https://doi.org/10.3372/wi.49.49111
- Raab-Straube, E., von, & Raus, Th. (ed.) (2020). Euro+Med-Checklist Notulae, 12. *Willdenowia*, *50*, 305-341. doi: https://doi.org/10.3372/wi.50.50214
- Raab-Straube, E., von, & Raus, Th. (ed.) (2021). Euro+Med-Checklist Notulae, 14. *Willdenowia*, *51*, 355–369. https://doi.org/10.3372/wi.51.51304
- Rukšāns, J. (2015). Some New *Crocus* Taxa (Iridaceae) from Western Turkey and East Aegean Islands. *International Rock Gardener*, *64*.
- Rukšāns, J. (2016). Some new *Crocus* species. *International Rock Gardener*, *76*.
- Rukšāns, J. (2019). *Puschkinia kurdistanica* (Asparagaceae) – a new species from the shores of Lake Van in Turkey. *International Rock Gardener*, *116*.
- Rukšāns, J. (2020a). *Crocus harpkeae* Rukšāns – A new Crocus species (*Liliiflorae, Iridaceae*) separated from the large complex of the so-called *Crocus chrysanthus* group. *International Rock Gardener*, *125*.
- Rukšāns, J. (2020b). *Leopoldia archibaldii* sp. nova (Asparagaceae): a new species of genus. *Leopoldia* Parl. from Eastern Anatolia, Turkey. *International Rock Gardener*, *134*.
- Sağıroğlu, M., & Eker, I. (2021). *Gladiolus aladagensis* (Iridaceae), a new species from Turkey. *Phytotaxa*, *478* (1), 151-161.
- Sefali, A. (2021). *Androsace azizsancarii* sp. nov.(Primulaceae): a new species from northeastern Anatolia, Turkey. *Nordic Journal of Botany*, *39* (7), e03208. doi: 10.1111/njb.03208
- Semiz, G., Şenol, S.G., Günal, B., Cicek, M., & Eroğlu, V. (2021). *Helichrysum× kani-isikii* (Asteraceae), a new nothospecies from Turkey. *Phytotaxa*, *507* (4), 283-292.
- Sert, R., & Aytaç, Z. (2021). Türkiye Florası için Yeni bir Kayıt: *Astrodaucus orientalis* (L.) Drude var. *eriocarpus* (Boiss.) Woron. (Apiaceae) ve Tiplendirme. *Bağbahçe Bilim Dergisi*, *8* (3), 41-46.
- Sezer, O. (2021). *Onosma onur-koyuncui* sp. nov.(Boraginaceae), A new species from Kütahya, Turkey. *Pakistan Journal of Botany*, *53* (4), 1315-1323.
- Sinan, A., Behçet, L., & Yapar, Y. (2021). *Ranunculus solhanensis* (Ranunculaceae), a new species from eastern Turkey. *Phytotaxa*, *497* (2), 157-164.
- Şahin, B., Sağıroğlu, M., & Başer, B. (2021). A new *Asperula* L. (Rubiaceae) species from gypsum steppes of Çankırı province in Turkey. *Turkish Journal of Botany*, *45* (3), 243-252.
- Şen Gökmen, F., & Duman, H. (2021). *Cistus x florentinus* Lam. (Cistaceae): Türkiye Florası için yeni bir Melez Kaydı. *Bağbahçe Bilim Dergisi, 8* (3), 30-40.
- Şirin, E., Çeçen, Ö., Bozkurt, M., & Ertuğrul, K. (2019). *Centaurea uysalii* (Cyanus/Asteraceae), a new species from Turkey. *Turkish Journal of Botany, 43*, 809-816.

- Şirin, E., Uysal, T., Bozkurt, M., & Ertuğrul, K. (2020). *Centaurea akcadaghensis* and *C. ermenekensis* (Asteraceae), two new species from Turkey. *Mediterranean Botany*, *41*(2), 173-179.
- Şirin, E., Yildirim, H., Uysal, T., & Ertuğrul, K. (2021). A new species of *Centaurea* L. subgen. Cyanus Mill.(Asteraceae) from Turkey. *Botanica Serbica*, *45* (1), 13-22.
- Tekşen, M., Duman, H., & Sağıroğlu, M. (2021). A new *Gladiolus* (Iridaceae) species from East Anatolia (Turkey): *Gladiolus hamzaoglui*. *Phytotaxa*, 496 (3), 245-260.
- Terzioğlu, S. (2020). *Arrhenatherum elatius* subsp. *bulbosum* (Poaceae): A New Alien Record for the Flora of Turkey. *Kahramanmaraş Sütçü İmam Üniversitesi Tarım ve Doğa Dergisi*, *23* (4), 924-927.
- Terzioğlu, S., Çoşkunçelebi, K., & Güzel, M.E. (2019). A new subspecies of *Saxifraga artvinensis* VA Matthews (Saxifragaceae) from Northeastern Anatolia. *Turkish Journal of Botany*, *43* (5), 687-693.
- Terzioğlu, S., Özkan, K. (2020). *Leptatherum* (Poaceae): A New Genus Record for the Flora of Turkey. *Kastamonu University Journal of Forestry Faculty*, *20* (2), 175-180.
- Tugay, O., Armağan, M., & Ulukış, D. (2021). Güney Anadolu'dan Yeni bir Tür: *Bellevalia guneriana* Tugay & Armağan (Kuşkonmazgiller / Asparagaceae). *Bağbahçe Bilim Dergisi*, *8* (3), 1-8.
- Tugay, O., Ertuğrul, K., Aslan, S., & Ulukuş, D. (2019). Türkiye Florası için yeni bir Akhatmi kaydı: *Kitaibela vitifolia* Willd. (Ebegümecigiller / Malvaceae). *Bağbahçe Bilim Dergisi*, *6* (3), 35-40.
- Tugay, O., & Ulukuş, D. (2019) *Linum aksehirense* (sect. *Dasylinum*, Linaceae), a new species from Central Anatolia (Turkey). *PhytoKeys*, *136*, 23–34. doi: 10.3897/phytokeys.136.46477
- Tugay, O., Ulukuş, D., Ertuğrul, K., Uysal, T., Demirelma, H., & Dural, H. (2019). A new species of *Cousinia* (sect. Cousinia, Asteraceae) from the Ağrı Mountain (eastern Turkey): evidence from morphology, karyology and anatomy. *Phytotaxa*, *427* (4), 259-269.
- Tunçkol, B., Aksoy, N., & Yaşacak, H. (2021). *Knautia arvensis* (Caprifoliaceae), a new record for the flora of Turkey. *Botanikai Közlemények*, *108* (1), 85-90.
- Tunçkol, B., Aytaç, Z., Aksoy, N., & Fişne, A. (2020). *Astragalus bartinense* (Fabaceae), a new species from Turkey. *Acta Botanica Croatica*, *79* (2), 131–136.
- Tunçkol, B., Yaşayacak, H., Liang, Z.L., Aksoy, N., & Zhang, L.B. (2020). *Polystichum asiae-minoris* (Dryopteridaceae), a new fern from Kastamonu, Turkey. *Phytotaxa*, *447* (4), 296–300.
- Uğurlu-Aydın, Z., Koch, M.A., & Dönmez, A.A. (2020). A new variety of *Dianthus anatolicus* (Caryophyllaceae) from Turkey as evidenced by morphology and AFLP data. *Annales Botanici Fennici*, *58* (1-3), 19-26.
- Ulukuş, D., Tugay, O., & Sağlam, C. (2020). *Verbascum seydisehirense* (Scrophulariaceae), a new species from Konya, Central Turkey. *Phytotaxa*, *450* (2), 229-236.
- Uygur, S., Tetik, Ö., & Doğru-Koca, A. (2021). Türkiye İçin Yeni Bir Yabancı Ot Türü: *Amaranthus crassipes* Schltdl. (Horozibiğigiller/ Amaranthaceae Juss.). *Bağbahçe Bilim Dergisi*, *8* (2), 7-11.
- Uysal, T., Bozkurt, M., & Demirelma, H. (2021). Türkiye Florası İçin Yeni Bir Kayıt, *Muscari commutatum* (Asparagaceae). *Türler ve Habitatlar*, *2* (2), 123-133.
- Uzun, A., Aytac, Z., & Tülücü, F. (2021). *Astragalus nurhakdagensis* (sect. Hololeuce Bunge/Fabaceae), a new species from Turkey. *Turkish Journal of Botany*, *45* (6), 573-586.
- Uzunhisarcıklı, M.E., Güner, E., Özbek, F., & Ekici, M. (2019). *Scrophularia vernalis*: a new species record for Turkey, and its comparison with *S. chrysantha* (Scrophulariaceae). *Phytotaxa*, *397* (1), 91-98.
- Vladimirov, V., Aybeke, M., & Tan, K. (2020). New floristic records in the Balkans: 42. *Phytologia Balcanica: International Journal of Balkan Flora and Vegetation*, *26* (2), 187-213.
- Vladimirov, V., Aybeke, M., & Tan, K. (2021). New floristic records in the Balkans: 44. *Phytologia Balcanica: International Journal of Balkan Flora and Vegetation, 27* (1), 127-150.
- Yapar, Y., & Behçet, L. (2020). A new subspecies of *Ornithogalum malatyanum* (Asparagaceae: Scilloideae) from eastern Anatolia, Turkey. *Nordic Journal of Botany*, *38* (11), e02837.
- Yıldırım, H. (2019). A new *Rindera* (Boraginaceae) species from Western Anatolia, Turkey. *Phytotaxa*, *427* (4), 249-258.
- Yıldırım, H. (2019). *Allium sultanae-ismailii* (Amaryllidaceae), a new species from eastern Turkey. *Phytotaxa*, *403* (1), 39-46.
- Yıldırım, H. (2021). *Gemicia* Yıldırım, Kuşkonmazgiller (Asparagaceae)›den, Kuzeydoğu Anadolu/Türkiye›den Monotipik Yeni Bir Cins. *Bağbahçe Bilim Dergisi*, *8* (1), 38-50.
- Yıldırım, H., Altıoğlu, Y. (2021). *Chionodoxa* Boiss.(Asparagaceae) Cinsinin Taxonomik Statüsünün Sürdürülmesi, Türkiye'de Yayılış Gösteren Taksonlarının Morfolojik Revizyonu ve Yeni Bir Türü. *Bağbahçe Bilim Dergisi*, *8* (1), 51-95.
- Yıldırım, H., Çiçek, M., Akbaş, K., & Şeker, E. (2021). *Scutellaria topcuoglui* (Lamiaceae), a new serpentinomorph species from southwestern Anatolia, Turkey. *Phytotaxa*, *528* (3), 180–190.
- Yıldırım, H., & Erdem, S. (2019). Türkiye Florası için yeni bir bitki takson kaydı: *Zantedeschia aethiopica* (L.) Spreng. (Araceae). *Bağbahçe Bilim Dergisi*, *6* (3), 17-21.
- Yıldırım, H., Özdöl, T., & Yaşayacak, H. (2019). An alien species of *Bidens* (Asteraceae): *Bidens pilosa* L. new to the Turkish flora. *Acta Biologica Turcica*, *32* (1), 61-64.
- Yıldırım, H., Özdöl, T., & Yaşayacak, H. (2019). Türkiye için yeni bir istilacı tür kaydı: *Malvastrum coromandelianum* (L.) Gracke (Ebegümecigiller / Malvaceae Juss.). *Bağbahçe Bilim Dergisi*, *6* (1), 40-44.
- Yıldırım, H., & Sefalı, A. (2020). *Bellevalia bayburtensis* (Asparagaceae): a new species from northeastern Anatolia, Turkey. *Phytotaxa*, *441* (3), 285-293.
- Yıldırım, H., Şentürk, O., Özdöl, T., & Pirhan, A.F. (2019). A new bellflower, *Campanula phitosiana* sp. nov.(Campanulaceae) from Western Anatolia, Turkey. *Phytotaxa*, *399* (1), 25-36.
- Yıldırım, H., & Tekşen, M. (2021). *Fritillaria arsusiana* (Lilieae, Liliaceae), a new species from southern Anatolia. *Phytotaxa*, *502* (2), 133-159.
- Yıldırımlı, Ş. (2019b). Two new species of Brassicaceae, *Descurainia tugayi* and *Lepidium tuzgoeluense* from Konya, Turkey. *Ot Sistematik Botanik Dergisi*, *26* (1), 93-101.
- Yıldırımlı, Ş. (2019c). Two new species of *Cephalaria* (Dipsacaceae/ Caprifoliaceae) from Turkey. *Ot Sistematik Botanik Dergisi*, *26* (2), 105-111.
- Yıldırımlı, Ş. (2019a). A new section and four new species of *Scabiosa* L. (Dipsacaceae/Caprifoliaceae) from Turkey. *Ot Sistematik Botanik Dergisi*, *26* (1), 63-77.
- Yıldırımlı, Ş. (2020a). A new genus, *Aytacia* Yıld. and a new species, *A. turkica* Yıld. (Asteraceae) based on *Scorzonera* s.l. from Turkey. *Ot Sistematik Botanik Dergisi*, *27* (1-2), 15-31.
- Yıldırımlı, Ş. (2021b). A new species, *Crepis vuralii* Yıld. (Asteraceae) from Turkey. *Ot Sistematik Botanik Dergisi*, *28* (1-2), 149-156.
- Yıldırımlı, Ş. (2021c). New infrageneric taxa, species, combinations and synonymus of *Podospermum* DC. (Asteraceae) and their current species and related genera keys from Turkey. *Ot Sistematik Botanik Dergisi, 27* (1-2), 51-94.
- Yıldırımlı, Ş., & Kılıç, Ö. (2019a). A new *Aethionema* (Brassicaceae), *A. sancakense* p.p. and a new description of *A. adiyamanense* from Turkey. *Ot Sistematik Botanik Dergisi*, *26* (2), 49-58.
- Yıldırımlı, Ş., & Kılıç, Ö. (2019b). A new enigmatic species of *Iris* L. (Iridaceae), *Iris yedisuensis* from Yedisu, Bingöl, Turkey. *Ot Sistematik Botanik Dergisi*, *26* (2), 1-7.
- Yıldırımlı, Ş., & Kılıç, Ö. (2019c). A new species of *Muscari* Mill. (Asparagaceae/Liliaceae), *M. nazimiyensis* from Tunceli, Turkey. *Ot Sistematik Botanik Dergisi*, *26* (1), 13-16.

- Yıldırımlı, Ş., & Kılıç, Ö. (2019d). A new species of *Rosularia* (DC.) Stapf (Crassulaceae), *R. paluensis* from Elazığ, Turkey. *Ot Sistematik Botanik Dergisi*, *26* (2), 97-104.
- Yıldırımlı, Ş., & Kılıç, Ö. (2019e). A new species, *Allium adiyamanense* (Amaryllidaceae/Alliaceae/Liliaceae), from Turkey. *Ot Sistematik Botanik Dergisi*, *26* (1), 33-39.
- Yıldırımlı, Ş., & Kılıç, Ö. (2019f). A new subgenus and four new species of *Ornithogalum* L. (Asparagaceae/Liliaceae), *O. nitidum*, *O. plurifolium*, *O. sancakense* and *O. yesilyurtense* from Turkey. *Ot Sistematik Botanik Dergisi*, *26* (2), 9-31.
- Yıldırımlı, Ş., & Kılıç, Ö. (2019g). A remarkable new species, *Achillea kirschneri* (Asteraceae) from Karakoçan-Elazığ-Turkey. *Ot Sistematik Botanik Dergisi*, *26* (1), 79-84.
- Yıldırımlı, Ş., & Kılıç, Ö. (2019h). Three new species of Apiaceae family from Turkey, *Bunium sancakense, Pimpinella adiyamanensis, Trigonosciadium solhanense*. *Ot Sistematik Botanik Dergisi, 26* (1), 41-52.
- Yıldırımlı, Ş., & Kılıç, Ö. (2019i). Three new species of Caryophyllaceae, *Dianthus demirkushii*, *Silene bocquetiana* and *Silene karakotchanensis* from Turkey. *Ot Sistematik Botanik Dergisi*, *26* (2), 59-72.
- Yıldırımlı, Ş., & Kılıç, Ö. (2019j). Two new species of Orchidaceae, *Ophrys bingoelensis* and *Orchis bingoelensis* from Sancak, Bingöl, Turkey. *Ot Sistematik Botanik Dergisi*, *26* (2), 39-48.
- Yıldırımlı, Ş., Kılıç, Ö., & Demirpolat, A. (2019). A new unexpected species of *Fritillaria*, *F. gencensis* (Liliaceae) from Genç, Bingöl, Turkey. *Ot Sistematik Botanik Dergisi*, *26* (1), 1-11.
- Yılmaz, H. (2021). *Gamochaeta coarctata*: A new allien species for Turkey. *Turkish Journal of Biodiversity*, *4* (2), 77-81.
- Yüzbaşıoğlu, S. (2019). *Crocus keltepensis* (Iridaceae): a new *Crocus* species from the highest part of the Samanlı Mountains, NW Turkey. *Phytotaxa*, *418* (2), 230-236.
- Zubov, D.A., Konca, Y., & Davis, A.P. (2019). *Galanthus bursanus* (Amaryllidaceae): a new species of snowdrop from the Marmara Sea region, NW Turkey. *Kew Bulletin*, *74*, 18.