ACTA PHARMACEUTICA SCIENCIA

International Journal in Pharmaceutical Sciences, Published Quarterly

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Founded in 1953 by Kasım Cemal GÜVEN

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Contents

Aims and Scope of Acta Pharmaceutica Sciencia

Acta Pharmaceutica Sciencia is a continuation of the former "Eczacılık Bülteni" which was first published in 1953 by Prof. Dr. Kasım Cemal GÜVEN's editorship. At that time, "Eczacılık Bülteni" hosted scientific papers from the School of Medicine-Pharmacy at İstanbul University, Türkiye.

In 1984, the name of the journal was changed to "Acta Pharmaceutica Turcica" and it became a journal for national and international manuscripts, in all fields of pharmaceutical sciences in both English and Turkish. (1984-1995, edited by Prof. Dr. Kasım Cemal GÜVEN, 1995-2001, edited by Prof. Dr. Erden GÜLER, 2002-2011, edited by Prof. Dr. Kasım Cemal GÜVEN)

Since 2006, the journal has been published only in English with the name, "Acta Pharmaceutica Sciencia" which represents internationally accepted high-level scientific standards. The journal has been published quarterly except for an interval from 2002 to 2009 in which its issues were released at intervals of four months. The publication was also temporarily discontinued at the end of 2011 but since 2016, Acta Pharmaceutica Sciencia has continued publication with the reestablished Editorial Board and also with the support of you as precious scientists.

Yours Faithfully

Prof. Dr. Gülden Zehra OMURTAG
Editor

INSTRUCTIONS FOR AUTHORS

Manuscripts must be prepared using the manuscript template

Manuscripts should contain the following elements in the following order :

Title Page

Abstract

Keywords

Introduction (Without author names and affiliations)

Methodology

Results and Discussion

Statement of Ethics

Conflict of interest Statement

Author Contributions

Funding Sources (optional)

Acknowledgments (optional)

References

It is best to use the Times New Roman' font, 11 font size, and all kinds of articles must be 1.5 spaced including text, references, tables, and legends.

The title should be concise and informative. Avoid abbreviations and formulae, where possible. The title page should include full title, author names and affiliations, present addresses, corresponding author, and ORCID numbers for every author Also, the full manuscript should include a full title page

Abstracts should not be separated into categories, it should be written in a paragraph format. Keywords: Max. 5

Graphics may be included with both in the text and uploaded as separate files

Sections: (Capital letters should be used in) Introduction, Methodology, Results and Discussion, Statement of Ethics, Conflict of Interest Statement, Author Contributions, Funding Sources (optional), Acknowledgement (optional).

Table and figure titles should not be abbreviated exp. fig. is not acceptable. It should be written as; Table 1. .... Figure 1. .....

Figure captions: A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves

to a minimum but explain all symbols and abbreviations used. Figure captions should be written on the bottom.

Titles: Number tables consecutively by their appearance in the text and place any table notes below the table body. Table captions should be written on the top.

References in the text should be identified using Arabic numerals. Years of the references should not be written boldly. More than one reference from the same author(s) in the same year must be identified by the letters “a”, “b”, “c”, etc., placed after the year of publication. References should conform to Vancouver style and be numbered consecutively in the order in which they are cited in the text.

*Obligatory files are manuscript main document, title page and copyright form for submission. If exist, supplementary files should also be added.

1. Scope and Editorial Policy

1.1. Scope of the Journal

Acta Pharmaceutica Sciencia (Acta Pharm. Sci.), formerly known as Bulletin of Pharmacy and Acta Pharmaceutica Turcica is a peer-reviewed scientific journal publishing current research and reviews covering all fields of pharmaceutical sciences since 1953.

The original studies accepted for publication must be unpublished work and should contain data that have not been published elsewhere as a whole or a part. The reviews must provide critical evaluation of the state of knowledge related with the subject.

All manuscripts has to be written in clear and concise English.

Starting from 2016, the journal will be issued quarterly both in paper and online formates also publish special issues for national or international scientific meetings and activities in the coverage field.

1.2. Manuscript Categories

Manuscripts can be submitted as Research Articles. Review articles will not be accepted.

Research Articles are definitive accounts of significant, original studies. They are expected to present important new data or provide a fresh approach to an established subject.

1.3. Prior Publication

Authors should submit only original work that has not been previously published and is not under consideration for publication elsewhere. Academic theses, including those on the Web or at a college Web site, are not considered to be prior publication.

1.4. Patents and Intellectual Property

Authors need to resolve all patent and intellectual property issues. Acceptance and publication will not be delayed for pending or unresolved issues of this type. Note that Accepted manuscripts and online manuscripts are considered as published documents.

1.5. Professional Ethics

Editors, reviewers, and authors are expected to adhere to internationally accepted criteria's for scientific publishing. Helsinki declaration is applied and accepted for the ethical standards of the journal.

World Medical Association. (2001). World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects.. Bulletin of the World Health Organization, 79 (4), 373- 374.

1.5.1 Author Consent

Submitting authors are reminded that consent of all coauthors must be obtained prior to submission of manuscripts. If an author is removed after submission, the submitting author must have the removed author consent to the change by e-mail or faxed letter to the assigned Editor.

1.5.2. Plagiarism

Manuscripts must be original with respect to concept, content, and writing. It is not appropriate for an author to reuse wording from other publications, including one's own previous publications, whether or not that publication is cited. Suspected plagiarism should be reported immediately to the editorial office. Report should specifically indicate the plagiarized material within the manuscripts. Acta Pharmaceutica Sciencia uses iThenticate or Turnitin software to screen submitted manuscripts for similarity to published material. Note that your manuscript may be screened during the submission process.

1.5.3. Use of Human or Animal Subjects

For research involving biological samples obtained from animals or human subjects, editors reserve the right to request additional information from au-

thors. Studies submitted for publication approval must present evidence that the described experimental activities have undergone local institutional review assessing safety and humane usage of study subject animals. In the case of human subjects authors must also provide a statement that study samples were obtained through the informed consent of the donors, or in lieu of that evidence, by the authority of the institutional board that licensed the use of such material. Authors are requested to declare the identification or case number of institution approval as well as the name of the licensing committee in a statement placed in the section describing the studies' Material and Methods.

World Medical Association. (2001). World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects.. Bulletin of the World Health Organization, 79 (4), 373- 374.

1.6 Issue Frequency

The Journal publishes 4 issues per year.

2. Preparing the Manuscript

2.1. General Considerations

Manuscripts should be kept to a minimum length. Authors should write in clear, concise English, employing an editing service if necessary. For professional assistance with improving the English, figures, or formatting in the manuscript before submission please contact to editorial office by e-mail for suggestions. Authors are required to subject their manuscript for

The responsibility for all aspects of manuscript preparation rests with the authors. Extensive changes or rewriting of the manuscript will not be undertaken by the Editors. A standard list of Abbreviations, Acronyms and Symbols is in section 5.

It is best to use the font "Times New Roman". Other fonts, particularly those that do not come bundled with the system software, may not translate properly. Ensure that all special characters (e.g., Greek characters, math symbols) are present in the body of the text as characters and not as graphic representations. Be sure that all characters are correctly represented throughout the manuscript—e.g., 1 (one) and l (letter l), 0 (zero) and O (letter o).

All text (including the title page, abstract, all sections of the body of the paper, figure captions, scheme or chart titles, and footnotes and references) and tables should be in one file. Graphics may be included with the text or uploaded as separate files. Manuscripts that do not adhere to the guidelines may be returned to authors for correction.

2.1.1 Articles of all kind

Use page size A4. Vertically orient all pages. Articles of all kind must be double-spaced including text, references, tables, and legends. This applies to figures, schemes, and tables as well as text. They do not have page limitations but should be kept to a minimum length. The experimental procedures for all of experimental steps must be clearly and fully included in the experimental section of the manuscripts.

2.1.2 Nomenclature

It is the responsibility of the authors to provide correct nomenclature. It is acceptable to use semisynthetic or generic names for certain specialized classes of compounds, such as steroids, peptides, carbohydrates, etc. In such a case, the name should conform to the generally accepted nomenclature conventions for the compound class. Chemical names for drugs are preferred. If these are not practical, generic names, or names approved by the World Health Organization, may be used.

Authors may find the following sources useful for recommended nomenclature:

· The ACS Style Guide; Coghill, A. M., Garson, L. R., Eds.; American Chemical Society: Washington DC, 2006.

· Enzyme Nomenclature; Webb, E. C., Ed.; Academic Press: Orlando, 1992.

· IUPHAR database of receptors and ion channels (http://www.guidetopharmacology.org/).

2.1.3 Compound Code Numbers

Code numbers (including peptides) assigned to a compound may be used as follows:

· Once in the manuscript title, when placed in parentheses AFTER the chemical or descriptive name.

· Once in the abstract.

· Once in the text (includes legends) and once to label a structure. Code numbers in the text must correspond to structures or, if used only once, the chemical name must be provided before the parenthesized code number, e.g., “chemical name (JEM-398).” If appearing a second time in the text, a bold Arabic number must be assigned on first usage, followed by the parenthesized code number, e.g., “1 (JEM-398).” Subsequently, only the bold Arabic number may be used. All code numbers in the text must have a citation to a publication or a

patent on first appearance.

Compounds widely employed as research tools and recognized primarily by code numbers may be designated in the manuscript by code numbers without the above restrictions. Their chemical name or structure should be provided as above. Editors have the discretion of determining which code numbers are considered widely employed.

2.1.4 Trademark Names

Trademark names for reagents or drugs must be used only in the experimental section. Do not use trademark or service mark symbols.

2.1.5 Interference Compounds

Active compounds from any source must be examined for known classes of assay interference compounds and this analysis must be provided in the General Experimental section. Many of these compounds have been classified as Pan Assay Interference Compounds (PAINS; see Baell & Holloway, J. Med. Chem. 2010, 53, 2719-2740). These compounds shown to display misleading assay readouts by a variety of mechanisms by forming reactive compounds. Provide firm experimental evidence in at least two different assays that reported compounds with potential PAINS liability are specifically active and their apparent activity is not an artifact.

2.2 Manuscript Organization

2.2.1 Title Page. Title

The title of the manuscript should reflect the purposes and findings of the work in order to provide maximum information in a computerized title search. Minimal use of nonfunctional words is encouraged. Only commonly employed abbreviations (e.g., DNA, RNA, ATP) are acceptable. Code numbers for compounds may be used in a manuscript title when placed in parentheses AFTER the chemical or descriptive name.

Authors' Names and Affiliations: The authors' full first names, middle initials, last names, and affiliations with addresses at time of work completion should be listed below the title. The name of the corresponding author should be marked with an asterisk (*).

2.2.2 Abstract and keywords

Articles of all types must have an abstract following the title page. The maximum length of the Abstract should be 200 words, organized in a findings-oriented format in which the most important results and conclusions are sum-

marized. Code numbers may be used once in the abstract. After the abstract, a section of Keywords not more than five has to be given. Be aware that the keywords, chosen according to the general concept, are very significant during searching and indexing of the manuscripts.

Keywords: instructions for authors, template, journal

2.2.3 Introduction

The Introduction should argue the case for the study, outlining only essential background, and should not include the findings or the conclusions. It should not be a review of the subject area, but should finish with a clear statement of the question being addressed. Authors should use this template when preparing a manuscript for submission to the ACTA Pharmaceutica Sciencia.

2.2.4. Methodology

Materials, synthetic, biological, demographic, statistical or experimental methods of the research should be given detailed in this section. The authors are free to subdivide this section in the logical flow of the study. For the experimental sections, authors should be as concise as possible in experimental descriptions. General reaction, isolation, preparation conditions should be given only once. The title of an experiment should include the chemical name and a bold Arabic identifier number; subsequently, only the bold Arabic number should be used. Experiments should be listed in numerical order. Molar equivalents of all reactants and percentage yields of products should be included. A general introductory section should include general procedures, standard techniques, and instruments employed (e.g., determination of purity, chromatography, NMR spectra, mass spectra, names of equipment) in the synthesis and characterization of compounds, isolates and preparations described subsequently in this section. Special attention should be called to hazardous reactions or toxic compounds. Provide analysis for known classes of assay interference compounds.

The preferred forms for some of the more commonly used abbrevations are mp, bp, ^{o}C, K, min, h, mL, μL, g, mg, μg, cm, mm, nm, mol, mmol, μmol, ppm, TLC, GC, NMR, UV, and IR. Units are abbreviated in table column heads and when used with numbers, not otherwise. (See section 4 for more abbreviations)

2.2.5 Results and Discussion

This section could include preparation, isolation, synthetic schemes and tables of biological and statistical data. The discussions should be descriptive. Authors should discuss the analysis of the data together with the significance of results and conclusions. An optional conclusions section is not required.

2.2.6 Ancillary Information

Include pertinent information in the order listed immediately before the references.

PDB ID Codes: Include the PDB ID codes with assigned compound Arabic number. Include the statement "Authors will release the atomic coordinates and experimental data upon article publication."

Homology Models: Include the PDB ID codes with assigned compound Arabic number. Include the statement "Authors will release the atomic coordinates upon article publication."

Corresponding Author Information: Provide telephone numbers and email addresses for each of the designated corresponding authors.

Present/Current Author Addresses: Provide information for authors whose affiliations or addresses have changed.

Author Contributions: Include statement such as "These authors contributed equally."

Acknowledgment: Authors may acknowledge people, organizations, and financial supporters in this section.

Abbreviations Used: Provide a list of nonstandard abbreviations and acronyms used in the paper, e.g., YFP, yellow fluorescent protein. Do not include compound code numbers in this list. It is not necessary to include abbreviations and acronyms from the Standard Abbreviations and Acronyms listed in section 4.

2.2.7 References and Notes

Vancouver style is used in the reference list and citations. List manuscripts as "in press" only accepted for publication. Manuscripts available on Web with a DOI number are considered published. For manuscripts not accepted, use "unpublished work" after the names of authors. Incorporate notes in the correct numerical sequence with the references. Footnotes are not used. List submitted manuscripts as "in press" only if formally accepted for publication. Manuscripts available on the Web with a DOI number are considered published. For manuscripts not accepted, use "unpublished results" after the names of authors. Incorporate notes in the correct numerical sequence with the references. Footnotes are not used. In-text citations should be given superscript numbers (see examples) according to order in the manuscript.

References

Please check with your faculty for any specific referencing or formatting requirements

• References are listed in numerical order, and in the same order in which they are cited in text. The reference list appears at the end of the paper.

• Begin your reference list on a new page and title it 'References'.

• The reference list should include all and only those references you have cited in the text. (However, do not include unpublished items such as correspondence.)

• Use Arabic numerals (1, 2, 3, 4, 5, 6, 7, 8, 9) as a superscripts.

• Abbreviate journal titles in the style used in the NLM Catalog.

• Check the reference details against the actual source - you are indicating that you have read a source when you cite it.

• Use of doi URL at the end of reference is strongly advised.

Examples

For printed articles

• Article with 1-6 authors:

Author AA, Author BB, Author CC, Author DD. Title of article. Abbreviated title of journal. Date of publication YYYY;volume number(issue number):page numbers.

Sahin Z, Ertas M, Berk B, Biltekin SN, Yurttas L, Demirayak S. Studies on non-steroidal inhibitors of aromatase enzyme; 4-(aryl/heteroaryl)-2-(pyrimidin-2-yl)thiazole derivatives. Bioorg Med Chem, 2018; 26(8): 1986–1995. https://doi.org/10.1016/j.bmc.2018.02.048.

• Article with more than 6 authors:

Author AA, Author BB, Author CC, Author DD, Author EE, Author FF, et al. Title of article. Abbreviated title of journal. Date of publication YYYY Mon DD;volume number(issue number):page numbers.

Electronic journal article:

Author AA, Author BB. Title of article. Abbreviated title of Journal [Internet]. Date of publication YYYY MM [cited YYYY Mon DD];volume number(issue number):page numbers. Available from: URL

Electronic journal article with DOI:

Author AA, Author BB, Author CC, Author DD, Author EE, Author FF. Title of article. Abbreviated title of Journal [Internet]. Year of publication [cited YYYY Mon DD];volume number(issue number):page numbers. Available from: URL DOI

Books and book chapters

Book :a.) Print book OR b.) Electronic book

a.) Author AA. Title of book. # edition [if not first]. Place of Publication: Publisher; Year of publication. Pagination.

b.) Author AA. Title of web page [Internet]. Place of Publication: Sponsor of Website/Publisher; Year published [cited YYYY Mon DD]. Number of pages. Available from: URL DOI: (if available)

2.2.8 Tables

Tabulation of experimental results is encouraged when this leads to more effective presentation or to more economical use of space. Tables should be numbered consecutively in order of citation in the text with Arabic numerals. Footnotes in tables should be given italic lowercase letter designations and cited in the tables as superscripts. The sequence of letters should proceed by row rather than by column. If a reference is cited in both table and text, insert a lettered footnote in the table to refer to the numbered reference in the text. Each table must be provided with a descriptive title that, together with column headings, should make the table self-explanatory. Titles and footnotes should be on the same page as the table. Tables may be created using a word processor's text mode or table format feature. The table format feature is preferred. Ensure each data entry is in its own table cell. If the text mode is used, separate columns with a single tab and use a return at the end of each row. Tables may be inserted in the text where first mentioned or may be grouped after the references.

2.2.9 Figures, Schemes/Structures, and Charts

The use of illustrations to convey or clarify information is encouraged. Structures should be produced with the use of a drawing program such as ChemDraw. Authors using other drawing packages should, in as far as possible, modify their program's parameters so that they conform to ChemDraw preferences. Remove all color from illustrations, except for those you would like published in color. Illustrations may be inserted into the text where mentioned or may be consolidated at the end of the manuscript. If consolidated, legends should be grouped on a separate page(s). Include as part of the manuscript file.

To facilitate the publication process, please submit manuscript graphics using the following guidelines:

1. The preferred submission procedure is to embed graphic files in a Word document. It may help to print the manuscript on a laser printer to ensure all artwork is clear and legible.

2. Additional acceptable file formats are: TIFF, PDF, EPS (vector artwork) or CDX (ChemDraw file). If submitting individual graphic files in addition to them being embedded in a Word document, ensure the files are named based on graphic function (i.e. Scheme 1, Figure 2, Chart 3), not the scientific name. Labeling of all figure parts should be present and the parts should be assembled into a single graphic.

EPS files: Ensure that all fonts are converted to outlines or embedded in the graphic file. The document settings should be in RGB mode. NOTE: While EPS files are accepted, the vector-based graphics will be rasterized for production. Please see below for TIFF file production resolutions.

3. TIFF files (either embedded in a Word doc or submitted as individual files) should have the following resolution requirements:

- Black & White line art: 1200 dpi

- Grayscale art (a monochromatic image containing shades of gray): 600 dpi

- Color art (RGB color mode): 300 dpi

· The RGB and resolution requirements are essential for producing high-quality graphics within the published manuscript. Graphics submitted in CMYK or at lower resolutions may be used; however, the colors may not be consistent and graphics of poor quality may not be able to be improved.

· Most graphic programs provide an option for changing the resolution when you are saving the image. Best practice is to save the graphic file at the final resolution and size using the program used to create the graphic.

4. Graphics should be sized at the final production size when possible. Single column graphics are preferred and can be sized up to 240 points wide (8.38 cm.). Double column graphics must be sized between 300 and 504 points (10.584 and 17.78 cm's). All graphics have a maximum depth of 660 points (23.28 cm.) including the caption (please allow 12 points for each line of caption text).

Consistently sizing letters and labels in graphics throughout your manuscript will help ensure consistent graphic presentation for publication.

2.2.10 Image Manipulation

Images should be free from misleading manipulation. Images included in an account of research performed or in the data collection as part of the research require an accurate description of how the images were generated and produced. Apply digital processing uniformly to images, with both samples and controls. Cropping must be reported in the figure legend. For gels and blots, use of positive and negative controls is highly recommended. Avoid high contrast settings to avoid overexposure of gels and blots. For microscopy, apply color adjustment to entire image and note in the legend. When necessary, authors should include a section on equipment and settings to describe all image acquisition tools, techniques and settings, and software used. All final images must have resolutions of 300 dpi or higher. Authors should retain unprocessed data in the event that the Editors request them.

2.3 Specialized Data

2.3.1 Biological Data

Quantitative biological data are required for all tested compounds. Biological test methods must be referenced or described in sufficient detail to permit the experiments to be repeated by others. Detailed descriptions of biological methods should be placed in the experimental section. Standard compounds or established drugs should be tested in the same system for comparison. Data may be presented as numerical expressions or in graphical form; biological data for extensive series of compounds should be presented in tabular form.

Active compounds obtained from combinatorial syntheses should be resynthesized and retested to verify that the biology conforms to the initial observation. Statistical limits (statistical significance) for the biological data are usually required. If statistical limits cannot be provided, the number of determinations and some indication of the variability and reliability of the results should be given. References to statistical methods of calculation should be included.

Doses and concentrations should be expressed as molar quantities (e.g., mol/kg, μmol/kg, M, mM). The routes of administration of test compounds and vehicles used should be indicated, and any salt forms used (hydrochlorides, sulfates, etc.) should be noted. The physical state of the compound dosed (crystalline, amorphous; solution, suspension) and the formulation for dosing (micronized, jet-milled, nanoparticles) should be indicated. For those compounds found to be inactive, the highest concentration *(in vitro)* or dose level *(in vivo)* tested should be indicated.

If human cell lines are used, authors are strongly encouraged to include the following information in their manuscript:

· the cell line source, including when and from where it was obtained;

· whether the cell line has recently been authenticated and by what method;

· whether the cell line has recently been tested for mycoplasma contamination.

2.3.2 Purity of Tested Compounds

Methods: All scientifically established methods of establishing purity are acceptable. If the target compounds are solvated, the quantity of solvent should be included in the compound formulas. No documentation is required unless asked by the editors.

Purity Percentage: All tested compounds, whether synthesized or purchased, should possess a purity of at least 95%. Target compounds must have a purity of at least 95%. In exceptional cases, authors can request a waiver when compounds are less than 95% pure. For solids, the melting point or melting point range should be reported as an indicator of purity.

Elemental analysis: Found values for carbon, hydrogen, and nitrogen (if present) should be within 0.4% of the calculated values for the proposed formula.

2.3.3 Confirmation of Structure

Adequate evidence to establish structural identity must accompany all new compounds that appear in the experimental section. Sufficient spectral data should be presented in the experimental section to allow for the identification of the same compound by comparison. Generally, a listing of 1H or 13C NMR peaks is sufficient. However, when the NMR data are used as a basis of structural identification, the peaks must be assigned.

List only infrared absorptions that are diagnostic for key functional groups. If a series contains very closely related compounds, it may be appropriate merely to list the spectral data for a single representative member when they share a common major structural component that has identical or very similar spectral features.

3. Submitting the Manuscript

3.1. Communication and log in to Author's Module

All submissions to Acta Pharmaceutica Sciencia should be made by using e-Collittera (Online Article Acceptance and Evaluation) system on the journal main page (www. actapharmsci.com)

3.2. Registration to System

It is required to register into the e-Collittera system for the first time while entering by clicking "Create Account" button on the registration screen and the fill the opening form with real information. Some of the information required in form is absolutely necessary and the registration will not work if these fields are not completely filled.

After the registration, a "Welcome" mail is sent to the user by the system automatically reminding user name and password. Authors are expected to return to the entry screen and log on with their user name and password for the submission. Please use only English characters while determining your username and password.

If you already registered into the e-Collittera system and forget your password, you should click on "Forgot My Password" button and your user name and password will be mailed to your e-mail in a short while.

3.3 Submitting A New Article

The main page of author module consists of various parts showing the situation of manuscripts in process. By clicking the New Manuscript button, authors create the beginning of new submission, a process with a total of 9 consecutive levels. In first 7 levels, information such as the article's kind, institutions, authors, title, summary, keywords etc. are asked respectively as entered. Authors can move back and forth while the information is saved automatically. If the is transaction is discontinued, the system move the new submission to "Partially Submitted Manuscripts" part and the transaction can be continued from here.

3.1.1. Sort of Article Authors should first select the type of article from the drop down menu.

Warning. If "Return to Main Page" button is clicked after this level, the article automatically assined as "Partially Submitted Manuscripts".

3.2.2. Institutions Authors should give their institutional information during submission.

3.2.3. Authors The authors' surnames, names, institutional information appear as entered order in the previous page. Filling all e-mail adresses are required. Institutional information is available in Manuscript Details table at the top of the screen. After filling all required fields, you may click the Continue button.

3.2.4 Title should be English, explaining the significance of the study. If the title includes some special characters such as alpha, beta, pi or gamma, they

can easily be added by using the Title window. You may add the character by clicking the relevant button and the system will automatically add the required character to the text.

Warning. No additions to cornered parenthesis are allowed. Otherwise the system will not be able to show the special characters.

3.2.5. Abstract The summary of the article should be entered to Abstract window at this level. There must be an English summary for all articles and the quantity of words must be not more than 200. If special characters such as alpha, beta, pi or gamma are used in summary, they can be added by Abstract window. You may add the character by clicking the relevant button and the system will automatically add the required character to the text. The abstract of the articles are accessible for arbitrators; so you should not add any information related to the institutions and authors in this summary part. Otherwise the article will returned without evaluation. Authors will be required to comply with the rules.

Warning. No additions to cornered parenthesis are allowed. Otherwise the system will not be able to show the special characters.

3.2.6. Keywords There must be five words to define the article at the keywords window, which will diverged with commas. Authors should pay attention to use words, which are appropriate for "Medical Subjects Headings" list by National Library of Medicine (NLM).

3.2.7. Cover Letter If the submitting article was published as thesis and/or presented in a congress or elsewhere, all information of thesis, presented congress or elsewhere should be delivered to the editor and must be mentioned by the "Cover Letter" field.

3.3.1. Adding Article This process consists four different steps beginning with the loading of the article in to system. Browse button is used to reach the article file, under the Choose a file to upload tab. After finding the article you may click to Choose File and file will be attached.

Second step is to select the file category. Options are: Main Document, Black and White Figure, Color Figure and Video.

The explanation of the files (E.g.. Figure 1, Full Text Word File, supplements etc.) should be added on third step and the last step is submitting the prepared article into the system. Therefore, Download button under the Send your file by clicking on download button tab is clicked.

Reminder If the prepared article includes more than one file (such as main document, black and white figure, video), the transaction will be continued by starting from the first step. The image files must be in previously defined format. After all required files were added, Continue button should be clicked. All details and features of the article might be reached from the Article Information page.

This page is the last step of the transaction which ensures that entered information is controlled.

3.3.2. Your Files After adding the article you may find all information related to article under Your Files window.

File Information This window includes file names, sizes, forming dates, categories, order numbers and explanations of files. The details about the files can be reached by clicking on Information button.

If you click on Name of File, the file download window will be opened to reach the copy of the file in system.

File Download This window submits two alternatives, one of them is to ensure the file to be opened in valid site and the second one is to ensure to download submitted file into the computer.

Opening the Category part on fourth column can change the category of the file.

Opening the Order column on fifth column can change the order of file.

The file can be deleted by clicking on Delete button on the last column. Before deleting, system will ask the user again if it's appropriate or not.

3.3.3 Sending Article Last level is submitting the article and the files into the system. Before continuing the transaction, Article Information window must be controlled where it is possible to return back; by using Previous button and required corrections can be made. If not, clicking the Send the Article button completes transaction.

3.3.4. Page to Follow The Article The Main Page of Author ensures possibility to follow the article. This page consists three different parts; some information and bridges related to the sent articles, revision required articles and the articles that are not completed to be sent.

3.3.4.1. Articles Not Completed to be Sent After the sending transaction was started, if article is not able to continue until the ninth step or could not be sent due to technical problems shown at this part. Here you can find the

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3.3.4.2. Articles That Require Revision Articles, which were evaluated by the referee and accepted by the editor with revision, continues to Waiting for Revision table.

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3.3.5. Submitted Manuscripts Information related to articles can be followed through the Submitted Manuscripts line. Here you can find the information such as the article's number assigned by system, title, sending date and transaction situation. The Manuscript Details and summary files can be reached by clicking the title of the article and the Processing Status part makes it possible to follow the evaluation process of the article.

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ORIGINAL ARTICLES

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Hypertension-related knowledge, attitudes, and behavior among hypertensive patients in a community pharmacy in Sivas, Türkiye: A regional descriptive study

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ABSTRACT

This study scrutinized hypertension-related knowledge, attitudes, and behavior among patients with hypertension. It was performed with 88 hypertensive patients in a community pharmacy in Sivas for one month. Suitably designed and pretested questionnaire was used. Chi-square test was used and $p<0.05$ was accepted as significant. More than half of the participants correctly explained that people have a family history of hypertension, diabetes, and overweight people. Among preventive factors, only being physically active, reduced alcohol intake, and smoking cessation had significance between males and females. When examined attitude, participants opined that hypertension was a chronic disease that needs to be treated and can be controlled for life long and reduction of alcohol intake, and smoking cessation can prevent hypertension. Behavioral parameters such as regular monitoring of blood preessure, not smoking and reduction of alcohol intake showed significance between males and females. Participants had good knowledge, while they had poor attitudes and behaviors.

Keywords: Hypertension, knowledge, attitudes, behavior

INTRODUCTION

Hypertension is a well-known preventable illness and is related with smoking, sedentary lifestyle, and alcohol intake like extrinsic factors [1]. It is a main risk factor for, and increases the prevalence of atherosclerosis, cerebrovascular ac-

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cidents, hearth and kidney failure, myocardial infarction, and stroke that leads to millions of deaths in the world [2]. It is considered to be related with large amount of illness and economic problems to the communities, especially in countries with low and middle incomes.

Globally, it is estimated that the rate of hypertension will increase over 29% by 2025. In addition, over 80% of deaths will be caused from hypertension and related to cardiovascular illnesses occur in countries with low and middle income [3,4]. Recent research of nationwide data in Türkiye reported that 40% of hypertensive patient were aware of their condition 31% of patients using drug and 40.3% of patients reached sufficient blood pressure control [5,6]. The main cause of high mortality and disability rates is insufficient control of hypertension [7]. In a study performed in 17 countries, it was stated that 53% of those with hypertension were not aware of it [8].

Various risk factors like socio-demographic factors, including gender [9], age and physical activity [10], urban residence and higher body mass index (BMI) have been found to be associated to hypertension [11]. They are regarded as main factors of the given the professional and social roles in health promotion, hypertension-related knowledge, attitudes and behavior (KAB) of patients became worth investigation. Some studies evaluated the KAB or improvements in KAB after the investigation. In a previous study, it was stated that younger hypertension patients are more knowledgeable about hypertension than the elderly[12]. When comparing general health behaviors, women do better than men [13].

There have been only restricted studies that focused on the effect of gender on knowledge, attitude, and behavior in the rural areas of Türkiye. Due to the lack of sufficient information on this topic, there is a need to bridge the information gap. The objective of this study was to assess the hypertension-related knowledge, attitude and the behavior of hypertensive participants.

METHODOLOGY

A regional descriptive study, using the suitable designed and pretested questionnaire was conducted to participants (age>18) living in Sivas city, Türkiye. The study was performed in a community pharmacy from September to December 2019. Those under 18-year-old, not giving consent were excluded. Incomplete questionnaires missing information were excluded, too. A priori power analysis was conducted using G *Power version 3.1.9.4. to determine the minimum sample size required to test the study hypothesis. Results indicates the required sample size to achieve 95% power for detecting a medium effect, at a significance criterion of a α= 0.05, was N=88 for chi square test. The re-

sults were expressed as mean ± SD. Analysis of data was performed by GraphPad Prism v. 5.04 program (GraphPad Software Inc., La Jolla, CA). Data was analyzed by univariate analysis and bivariate analysis (Chi-square and Fisher's exact test). $P<0.05$ was regarded as statistically significant. Structured questionnaire has three parts except sociodemographic characteristics. These were hypertension-related knowledge, attitudes towards hypertension and hypertension-related behavior of participants. First, the knowledge section includes general knowledge for its non-communicable illness, normal values of blood pressure, sign and symptoms, diagnosis and treatment, risk factors and preventive measures. Second, the attitude section explains about susceptibility, benefits of prevention and severity of risk factors towards hypertension. Third, behavior section includes preventive practices of hypertension such as regular physical activity, consuming less salty foods, eating less food contain high fat, sufficient consumption of vegetables and fruits, monitoring BP regularly, not smoking cigarette, no or less alcohol intake, controlling body weight and reducing stress.

Those who answered at least 11 knowledge, at least 7 attitude, and more at least 6 behavioral questions correctly were considered to have good knowledge, good attitude and good behavior, respectively.

RESULTS AND DISCUSSION

Socio-demographic characteristics of respondents

A total of 104 respondents participated in this study. After excluding questionnaires missing essential data concerning hypertension knowledge, attitudes and behavior, only 88 (55 female and 33 male) subjects remained for analysis. Demographic characteristics and other factors of the participants were shown in Table 1.

Table 1. Sex specific socio-demographic characteristics and other factors of participants related to hypertension

Variables	Male		Female		Total	
	n	(%)	n	(%)	n	(%)
Age						
30-49	5	(15.2)	2	(3.64)	7	(7.95)
50-59	6	(18.2)	16	(29.09)	22	(25)
60-69	11	(33.3)	20	(36.36)	31	(35.23)
70-89	10	(30.30)	16	(29.09)	26	(29.54)
90-	1	(3.0)	1	(1.82)	2	(2.28)
Marital status						
Single	1	(3.0)	2	(3.64)	3	(3.4)
Married	26	(78.8)	37	(67.27)	63	(71.6)
Widowed	3	(9.1)	10	(18.18)	14	(14.8)
Divorced	3	(9.1)	6	(10.90)	9	(10.2)
Education levels						
None	3	(9.1)	8	(14.55)	11	(12.5)
Primary	5	(15.2)	9	(16.36)	14	(15.91)
Secondary	6	(18.2)	13	(23.64)	19	(21.59)
High school	11	(33.3)	15	(27.27)	26	(29.55)
University	8	(24.2)	10	(18.18)	18	(20.45)
Job description						
Unemployed	8	(24.2)	32	(58.18)	40	(45.45)
Employed	25	(75.8)	23	(41.82)	48	(54.55)
Monthly income ($)						
<100	4	(12.12)	6	(10.91)	10	(11.36)
100-200	16	(48.48)	27	(49.09)	43	(48.8)
200-300	4	(12.12)	10	(18.18)	14	(15.90)
>300	3	(9.1)	4	(7.27)	7	(7.95)
Not declared	6	(18.18)	8	(14.55)	14	(15.90)
Physical activity						
Sedentary	27	(81.82)	49	(89.1)	76	(86.37)
Mildly active	3	(9.09)	4	(7.27)	7	(7.95)
Moderately active	2	(6.06)	0	(0.0)	2	(2.28)
Extremely active	1	(3.03)	2	(3.63)	3	(3.40)
Body mass index (kg/m2)						
Normal (18.5-24.9)	3	(9.1)	4	(7.27)	7	(7.95)
Overweight (25-29.9)	16	(48.48)	30	(54.55)	46	(52.28)

Obese (30≤)	14	(42.42)	21	(38.18)	35	(39.77)
Smoking						
No	22	(66.7)	54	(98.18)	76	(86.36)
Yes	11	(33.3)	1	(1.82)	12	(13.64)
Alcohol intake						
No	11	(33.3)	48	(87.27)	59	(67.05)
Yes	22	(66.7)	7	(12.73)	29	(32.95)
BP control						
No	15	(45.45)	38	(69.09)	53	(60.23)
Yes	18	(54.55)	17	(30.91)	35	(39.77)

When the age distribution of the participants by gender was examined, 60-69 age interval was found most common for male (33.3%) and female (36.36%). Most of the males (78.8%) and females (67.27%) were married. Graduation from high school was the most common among participants with rate of 33.3% in males and 27.27% in females. Employed participants were found 75.8% and 44.82% in males and in females, respectively. Monthly income was 100-200$ and approximately similar in male (48.48%) and female (49.09%) participants. While 81.82% of males lived sedentary, this rate increased to 89.1% in females. Most of the participants were found as overweight and obese 48.48% and 42.42% in males and 54.55% and 38.18% in females, respectively. Not smoking, not drinking alcohol and blood pressure monitoring were found more in females than males.

Hypertension related knowledge

The results indicated that the rates of correct answers regarding knowledge of hypertension ranged between 44.31 and 89.77% (Table 2).

Table 2. Hypertension-related knowledge of participants

Statements	Positive Response						p value
	Male		Female		Total		
	n	(%)	n	(%)	n	(%)	
Hypertension is not a communicable illness	21	(63.64)	51	(92.72)	72	(81.81)	0.0012*
Normal systolic/diastolic blood pressure is 120/80 mmHg	23	(69.68)	30	(54.55)	53	(60.22)	0.1832
Sign and symptoms							
People with hypertension can't always feel symptoms	18	(54.55)	27	(49.09)	45	(51.13)	0.6643
Blurred vision and headache can be signs of very high blood pressure	23	(69.68)	36	(65.46)	59	(67.04)	0.8157
Diagnosis and treatment							
People are diagnosed with hypertension when their systolic/diastolic pressure equals to or is higher than 140/90 mmHg in two separate measurements	21	(63.64)	47	(85.45)	68	(77.27)	0.0336*
Hypertension is not treated by drugs only	22	(66.67)	45	(81.82)	67	(76.13)	0.1262
Patients with hypertension need to use drugs life-long	27	(81.82)	52	(94.56)	79	(89.77)	0.0745
Following people are at risk of hypertension							
Diabetic patients	17	(51.52)	45	(81.82)	62	(70.45)	0.0037*
Overweight people	16	(48.49)	41	(74.56)	57	(64.77)	0.0206*
People have family history of hypertension	17	(51.52)	49	(89.09)	66	(75)	0.0002*
Those who don't exercise regularly	12	(36.36)	27	(49.09)	39	(44.31)	0.2746
Hypertension can be prevented by							
Being physically active	25	(75.76)	52	(94.55)	77	(87.5)	0.0171*
Less consumption of salty and high fatty food	21	(63.64)	44	(80)	65	(73.86)	0.1321
Vegetable and fruit consumption	24	(72.78)	37	(67.27)	61	(69.31)	0.6402
Reduced alcohol intake	22	(66.67)	48	(87.28)	70	(79.54)	0.0289*
Cessation of smoking	23	(69.69)	49	(89.09)	72	(81.81)	0.0429*
Reducing stress	24	(72.78)	38	(69.09)	62	(70.45)	0.8116

Statistical analysis performed by Chi-square (and Fisher's exact) test, showing the difference between male and female for each statement. $p< 0.05$ is considered statistically significant

Most of the patients had known that hypertension was not a communicable disease ($p<0.05$). In addition, results showed that risk factors of hypertension (obesity, overweight and family history) were known among participants ($p<0.05$). Participants stated positive response only diagnosis criteria of hypertension in the diagnosis and treatment part ($p<0.05$). On the other hand, while cessation of smoking, reduced alcohol intake, and regular physical activity were found significantly relevant to low blood pressure ($p<0.05$), approximately one-third of the participants did not acknowledge that reducing stress and eating less salty and fatty meal were related to low BP. Moreover, there was no significance between males and females regarding knowledge of hypertension in sign and symptoms criteria ($p>0.05$).

Attitudes towards hypertension

The rates of positive responses regarding attitudes towards hypertension ranged between 46.59 and 80.68% (Table 3).

Table 3. Summary of participants' attitudes towards hypertension

Items	Positive Response N (%)						p value
	Male		Female		Total		
	n	(%)	n	(%)	N	(%)	
If parents are hypertensive, children will have high risk of having disease	18	(54.54)	23	(41.81)	41	(46.59)	0.2761
If people consume salty and high fatty food, they become susceptible to hypertension	25	(75.75)	36	(65.45)	61	(69.31)	0.3488
I think that hypertension is a chronic disease, and it must be treated and controlled life long	20	(60.60)	45	(81.81)	65	(73.86)	0.044*
High blood pressure can cause serious complications	21	(63.63)	42	(76.36)	63	(71.59)	0.2283
People with hypertension should monitor their BP regularly	31	(93.93)	40	(72.72)	71	(80,68)	0.0235*
Smoking cessation can help prevent hypertension	17	(51.51)	50	(90.90)	67	(76.13)	0.0001*
Reducing alcohol intake can prevent hypertension	29	(87.87)	12	(21.81)	41	(46.59)	0.0001*
Controlling body weight can help prevent hypertension	28	(84.84)	43	(78.18)	71	(80.68)	0.5801
Regular physical exercise can help prevent hypertension	25	(75.75)	27	(49.09)	52	(59.09)	0.0153*
Reducing stress is effective for preventing hypertension	23	(69.69)	36	(65.45)	59	(67.04)	0.8157

Statistical analysis performed by Chi-square (and Fisher's exact) test, showing the difference between male and female for each statement. $p< 0.05$ is considered statistically significant; BP: blood pressure

The results showed that the participants knew that hypertension is a chronic disease that needs to be treated and can be controlled for life long ($p<0.044$). Among the items, "If parents are hypertensive, children will have high risk of having disease" and "Reducing alcohol intake can prevent hypertension" were the least beliefs (46.59%). Although alcohol intake was one the least believed preventive factors, it was found significantly different ($p<0.0001$). Smoking cessation was found statistically significant between males and females ($p<0.0001$).

Behavior of participants

Table 4 indicated the behaviors of participants. Almost none of women smoked (98.18%) while this rate was found 66.66% in males (p<0.0001). It was significantly found that more than two-third of the participants either did not drink alcohol at all or consumed very little alcohol (p<0.0001). Only 39.77% of participants were engaging with regular BP monitoring (p<0.0424). Contrary to expectation, most of the participants did not properly control fat intake, consumption of vegetables and fruits, and control body weight, reducing stress, about half of the participants did not control salt intake (p>0.05).

Table 4. Hypertension-related behaviors of participants

Items	Positive Response N (%)						p value
	Male		Female		Total		
	n	(%)	n	(%)	n	(%)	
Regular physical activity (mild, moderate, and extreme)	6	(18.18)	6	(10.90)	12	(13.63)	0.3540
Consume less salty foods	19	(57.57)	27	(49.09)	46	(52.27)	0.5114
Eating less food contain high fat (margarine, butter, and meat)	5	(15.15)	7	(12.72)	12	(13.63)	0.7574
Sufficient consumption of vegetables and fruits	4	(12.12)	11	(20)	15	(17.04)	0.3954
Monitoring BP regularly	18	(59.40)	17	(30.90)	35	(39.77)	0.0424*
Not smoke cigarette	22	(66.66)	54	(98.18)	76	(86.36)	0.0001*
No or less alcohol intake	11	(33.33)	48	(87.27)	59	(67.04)	0.0001*
Controlling body weight	3	(9.09)	4	(7.27)	7	(7.95)	1.000
Reducing stress	13	(39.39)	14	(25.45)	27	(30.68)	0.2327

Statistical analysis performed by Chi-square (and Fisher's exact) test, showing the difference between male and female for each statement. $p< 0.05$ is considered statistically significant; BP: blood pressure

To sum up, the study population generally had good knowledge; however, their attitudes and behaviors of hypertension prevention needed great improvement (Table 5).

Table 5. Summary of the proportion of the population that met the criteria for a positive response regarding knowledge, attitudes, and behaviors related to hypertension

Item group	Criteria for Positive Response n	Correct items (%)	
Knowledge	≥11 out of 17	11	64.70
Attitudes	≥7 out of 10	5	50.0
Behaviors	≥6 out of 9	1	11.11

Current study indicated that hypertension related knowledge and behavior were significantly associated with awareness in rural area. However, most patients enrolled in this study had relatively good hypertension knowledge. Hypertension is a chronic and non-communicable cardiovascular disease. In a study, performed by San and Plianbangchang (2018), three fifth of the participants gave correct answer about the non-communicable nature of the hypertension like our result [14]. In addition, the number of women who gave positive responses to this question was more than those of men, in our study.

This study indicated that most of the patients significantly knew the top and bottom levels for diagnosis. In agreement to result of our study, Anowie and Darkwa stated that 63.8% of respondents correctly explained normal values for tension and bottom and top values for hypertension diagnosis [15]. Contrary to our study, Li et al. stated that nearly 30% of patients were able to correctly identify and were aware of both systolic and diastolic BP measures as diagnostic criteria [7].

Hypertension is a disease that has little or no signs and symptoms, especially in the early stages. This is why many people go undiagnosed [16]. In a study, only 7% of participants correctly remarked that hypertension was asymptomatic disease [17]. Similar to previous study, only 39.2% knew the symptoms of the hypertension in another study [18]. Consistent with previous studies, only just over half of the participants gave correct answer in our study. Our investigation showed that the majority of patients were overweight and diabetic and also, they were physically inactive. There was a noteworthy result that participants knew that diabetic, overweight and people with family history of hypertension were associated with hypertension. Consistent to our results, different previous studies performed on hypertension stated same risk factors and they confirmed this relationship [19,20]. This finding may be associated with physical conditions and lifestyles of participants in our study. Most of them were overweight and obese who have sedentary lifestyle. In addition, the number of women who answered this question positively in our study was higher than that of men. It has been well known that regular exercise was helpful for reducing blood pressure.

Furthermore, our study revealed that enrolled participants knew whether hypertension was a preventable disease if risk factors were eliminated. In literature, regular exercise is one of the most well-known proven preventive factors to reduce high blood pressure and to maintain it under control [21]. Egan (2017) stated that replacing of sedentary lifestyle with regular physical activity can reduce incident diabetes, decrease high blood pressure. Physical exercise approximately halves heart diseases and related other metabolic diseases [22]. The other important preventive factor is reduction of alcohol intake. In a study by Sefah et al. (2021), hypertension can be prevented by regular physical activity [23]. As is well known, one of the other factors in the development of hypertension is alcohol intake and previous study has emphasized that alcohol consumption clearly contributed to hypertension [24]. Overwhelming evidence supported that cigarette smoking led to various cardiovascular diseases, including hypertension. In a study, the harm of smoking was also highlighted, and researchers stated that hypertensive smokers can develop severe forms of hypertension more than patients who are nonsmokers in this study [25]. Consistent with these studies, our study also reported that reduced/no alcohol intake and not smoking were also effective factor to decrease blood pressure. Our study did not find an association between fruit and vegetable, salt intake and high fatty food and hypertension.

The respondents in this study were found to have poor attitudes towards preventing hypertension as they opined that smoking cessation and reducing alcohol intake can prevent hypertension. This result is supported by a previous study [26]. In addition, almost all of them also agreed that hypertension was chronic disease, and it must be treated and controlled lifelong. In current study, smoking ratios were 33.3% and 1.82% in males and females, respectively; consistent with a different study [27]. In our study, the ratio of nonsmokers and nondrinkers were 86.36% and 67.05%, respectively. These results were supported by another study [28].

In our study, monitoring BP regularly, not smoke cigarette and no/less alcohol intake were the behaviors that had differences between males and females. Usually, the behaviors in females were better than those in males in our study. Consistent with another study performed before, males had a lower proportion of non-smokers and nondrinkers when compared with females [26]. This study also reported that, 76.9% of participants opined that monitoring of BP regularly was important. Although the rate of giving the correct answer was high, there was no significance between males and females. On the other hand, unlike this study, obtained findings in our study showed that less than half of participants hadn't monitored their BP regularly. However, males had a higher ratio when compared with females, 59.40% and 30.90%, respectively.

Study limitations

The most limitation of this study was study population. Secondly, because current study was performed in one city and one pharmacy of Türkiye and the findings could be dependent on the health education of participants, the outcomes can primarily be applicable for one district in Sivas-Türkiye. In future studies, study population should be increased.

In conclusion, enrolled participants in the study had good knowledge while they had poor attitudes and behaviors on hypertension. Intervention program based on the findings of current study may also be supported, to improve attitudes and to formation of healthy behaviors of community. This study also showed that gender had important effects on hypertension knowledge, attitudes, and behaviors that females clearly had high level of knowledge and behaviors than those of males.

STATEMENTS OF ETHICS

Ethic approval of this study was obtained from Istanbul Medipol University Local Ethical Committee at 01/08/2019 (2019/593).

CONFLICT OF INTEREST

We wish to confirm that there were no known conflicts of interest associated with this publication.

AUTHOR CONTRIBUTIONS

The authors contributed equally.

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The antiproliferative, antimigratory and anticlonogenic effects of *Croton membranaceus* Müll. Arg. (Euphorbiaceae) hydroethanolic root extract in human 22Rv1 castration-resistant prostate cancer cells

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ABSTRACT

This study employed the 22Rv1 *in vitro* model of CRPC to investigate the anticancer effect of the hydroethanolic root extract of *Croton membranaceus* (CMERE). The effects of CMERE on proliferation, migration and colony forming ability of 22Rv1 cells were studied. Isobologram analysis of combined effect of CMERE and enzalutamide, an androgen receptor blocker, on 22Rv1 proliferation, was also investigated. Lastly, selective cytotoxicity of CMERE was investigated using 22Rv1, BPH1 and THP-1 cells. We could show that CMERE inhibited testosterone-dependent and independent 22Rv1 cell proliferation. Moreover, drug combination studies showed that CMERE and enzalutamide may inhibit 22Rv1 cell proliferation via different mechanisms. Additionally, CMERE at all doses significantly inhibited the migration and colony formation of 22Rv1. Lastly, CMERE was selectively toxic to 22Rv1 and BPH1 cells but not to the non-prostate derived cell line THP-1 monocyte-like cells. Thus, CMERE possesses anticancer activity against 22Rv1 CRPC model.

Keywords: 22Rv1, castration-resistant prostate cancer, *Croton membranaceus*, isobologram, selective cytotoxicity

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INTRODUCTION

Prostate cancer (PCa) is a biologically and clinically heterogeneous malignant condition that originates from the prostate, a small gland below the bladder and anterior to the rectum [1]. It is the second most diagnosed cancer in men globally, with an alarming 1.2 million new cases and 350,000 PCa-related deaths recorded annually [2]. PCa thus affects millions, chiefly in regions of high human development index [2]. Nonetheless, low-income nations such as countries in sub-Saharan Africa, Central Asia and South Asia, have the highest rates of annual increase in PCa incidence and deaths despite the lowest documented incidence of the disease, as the disease is believed to be underestimated due to lack of screening, poor access to healthcare and environmental factors [3-5]. This illustrates the nature of PCa disease burden on global healthcare, highlighting the need to understand the disease and design effective treatment modalities.

Given the above, the androgen receptor has been identified as the most important molecular driver of prostate cancer progression [6,7]. Thus, inhibiting androgen receptor using androgen deprivation therapy is the mainstay of advanced prostate cancer therapy [8,9]. Despite initial positive response, nearly all patients fail to respond to androgen ablation following 2–3 years of ADT, and progress to a stage of the disease known as castration-resistant prostate cancer [7]. Castration-resistant prostate cancer remains incurable as the newer agents such as enzalutamide, flutamide, apalutamide and darolutamide are less effective [10]. Furthermore, apalutamide and darolutamide are significantly toxic and do not provide a lasting solution despite initial improvement in metastasis-free survival [11,12]. For this reason, the need for newer, highly effective and less toxic therapies in the treatment of castration-resistant prostate cancer is clearly highlighted.

Accordingly, plants and their products have proven beneficial for centuries as important sources of drug leads despite the increasing popularity of combinatorial chemistry [13]. In fact, it is estimated that 25.1% of approved anti-cancer drugs originated from natural products and their derivatives [14]. These products of natural origin may produce cost-effective promising results and fewer untoward effects [15]. One such important plant source is *Croton membranaceus* Müll. Arg. (Euphorbiaceae) which has been safely used as a root decoction in the management of benign prostatic hyperplasia (BPH) and other prostate diseases in various Ghanaian communities [16]. In addition, the cytotoxic effect of the methanolic extract has been demonstrated in human MCF-7 breast carcinoma, DLD-1 colon carcinoma, and PC3 prostate carcinoma cells [17]. However, little is known about the effect of the plant on other aspects of cancer development such as cancer cell migration and metastasis. To the best of the

authors' knowledge, no study has reported on the effect of the plant on CRPC (Castration-resistant prostate cancer). This study therefore sought to investigate the effect of the hydroethanolic root extract of *Croton membranaceus* on castration-resistant prostate cancer.

METHODOLOGY

Plant collection and preparation of hydroethanolic root extract

The roots of *Croton membranaceus* Müll. Arg., locally known as bokum by the Krobos of Ghana, were collected from Kwahu-Asakraka in the Eastern Region of Ghana on the 3rd of January, 2021 and were authenticated by Dr. George H. Sam of the Department of Herbal Medicine, KNUST. A voucher specimen (KNUST/HM1/2021/R005) was deposited at the Herbarium of the Department of Pharmacognosy, KNUST. Subsequently, the fresh roots of *C. membranaceus* were washed thoroughly, dried in shade and pulverized into a coarse powder. Thereafter, *C. membranaceus* roots were extracted as previously described by Sarkodie *et al.* [18] and Asare *et al.*[16], with slight modifications. Briefly, the hydroethanolic root extract (CMERE) was prepared by dissolving 1 kg of root into 4 L of 70% v/v ethanol. The roots were cold macerated in the solvent for 96 hours after which root materials were removed by decantation and the residues were further dissolved in 2.5 L of 70% ethanol for an additional 72 hours. Subsequently, the supernatant was decanted and filtered using a Whatman No.1 filter paper. The hydroethanolic root extract was concentrated in a rotary evaporator (Buchi Labotechnik Rotavap R-210) at 50°C and further dried in an oven (Gallenkamp OMT Oven, SANYO, Japan) at 50°C. The resulting soft mass was then placed in a desiccator to remove the residual moisture. Afterwards, the extract was stored at 4°C until further use. Percentage yield of 3.8%.was obtained for the hydroethanolic root extract.

Gas chromatography-mass spectrometry analysis of extract

Gas chromatography-mass spectrometry (GC-MS) fingerprinting was performed to identify the possible presence of compounds that may be responsible for the biological effects of the extract. Thus, GC-MS analyses of the hydroethanolic root extract was carried out using a PerkinElmer GC Clarus 580 Gas Chromatograph that is interfaced to a Mass Spectrometer PerkinElmer (Clarus SQ 8 S) and equipped with Elite-5MS (5% diphenyl/95% dimethyl poly siloxane) fused to a capillary column (30 × 0.25 μm ID × 0.25 μm DF). The temperature of the oven was serially set from 80°C with stepwise increments of 10°C/min to 250°C, then 5°C/min to 280°C and holding for 15 mins at 280°C. For GC-MS detection, an electron ionization system was operated in electron impact mode

with ionization energy of 70 eV. As a carrier gas, high purity helium gas was used and programmed at a constant flow rate of 1 mL/min, with an injection volume of 1µl. The temperature of the injector and the ion-source were kept at 250°C and 150 °C, respectively. Subsequently, the mass spectrum of the extract was generated at 70 eV with a scan interval of 0.1 s and fragments from 45 to 450Da were analyzed. The solvent delay and the total GC/MS running time were 0 to 2 mins, and 38 mins, respectively. The mass-detector and the software employed to handle mass spectra and chromatogram used in this analysis were Turbo-Mass and Turbo-Mass ver-6.1.0, respectively. Interpretation of the mass-spectrum GC-MS was conducted using the database of National Institute of Standard and Technology (NIST), which contains over 62,000 patterns [19].

Cell culture

Human prostate carcinoma cell line 22Rv1 and benign neoplastic cell line (BPH1) were obtained as a gift from the Steroid Hormone Lab of Prof. Dr. Andrew Cato of the Institute of Biological and Chemical Systems (IBCS), Karlsruhe Institute of Technology (KIT), Germany, and routinely cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) plus 1 mM sodium pyruvate, 100 units/mL penicillin and 100 mg/L streptomycin (Sigma, Germany), hereto referred to as complete growth medium. THP-1 monocyte-like cells acquired as a kind donation from Mutocheluh Lab (KNUST), were routinely cultured in complete growth medium supplemented with 2-mercaptoethanol. All cells were grown in sterile T-Flasks (Greiner Bio-One, Germany) of different kinds depending on the experimental conditions. At 80 - 90% confluence, culture media were removed, and adhered cells were washed once with pre-warmed PBS 1X to remove remnant media. The washed cells were then incubated with trypsin/EDTA in an incubator for approximately 5 minutes at 37 °C. Afterwards, the added trypsin was inactivated by the addition of fresh medium containing 10% FBS, and the cell suspension was transferred into sterile Falcon tubes and subsequently spined down at 500 rpm for 5 minutes at room temperature to pellet the cells. THP-1 suspension cells, were not trypsinized but spined directly to pellet the cells. Subsequently, the supernatants were pipetted off and the cells re-suspended in fresh complete medium. Afterwards, cells were either incubated in the appropriate T-flasks or counted and seeded into cell culture plates, depending on the type of assay to be carried out. All cell cultures were maintained at 37°C, 5% of CO_2 and 95% of humidity in an incubator (Herasafe KS Class II) [20].

Cytotoxic effects of *Croton membranaceus* extract and IC_{50} determination

Briefly, cells were seeded into tissue culture-treated 96-well plates at a seeding density of 5 x 10^3 cells/well. The cells were allowed to attach overnight and normal growth medium was replaced with treatment medium containing various concentrations of the hydroethanolic root extract (1, 3, 5, 10, 30, 50 and 100 mg/ml). The experiment was repeated three times. The cells were incubated for 72 hours, after which 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) was added to each well and the plate incubated in the dark at 37°C, in 5% CO_2 and 95% humidity for 4 hours. Subsequently, the media with MTT were removed from the wells, and the formed formazan crystals were dissolved by adding 120 µL of isopropanol to each well. Afterwards, the plate was allowed to stand for 30 minutes following which absorbance was read at 595 nm using microplate reader (iMark™, Bio-Rad). Finally, percentage change in absorbance was calculated as shown below and obtained values were fitted to non-linear regression to obtain the IC_{50}s [21].

$$\%\ Change\ in\ Absorbance = \frac{A_0 - A}{A_0} x\ 100 \quad Equation\ (1)$$

Where A_o is the absorbance at the least concentration, and A is the measured absorbance

Antiproliferative effects of CMERE in androgen-dependent and independent 22Rv1 cell proliferation

To investigate the effect of CMERE on testosterone-dependent and independent 22Rv1 cell proliferation, the cell culture medium was charcoal-stripped (hereto referred to as starvation medium) prior to the assay. This was done to deplete the medium off androgens and other steroid hormones that may serve as potential agonists or antagonists on the AR, or may serve as precursors to the production of androgens. Afterwards, 22Rv1 cells were cultured in normal complete growth medium and cells were seeded at a density of 5 x 10^3 cells/well into tissue culture-treated 96-well plates. Cells were allowed to adhere to the plate overnight after which complete growth medium was replaced with charcoal-stripped medium, and the cells maintained in it for an additional 48 hours. After 48 hours, cells were exposed to either 100 µL of starvation medium, 10 µM enzalutamide (positive control) or crude extract (1, 3 and 10 mg/mL) for 72 hours.

For androgen-dependent growth, the starvation medium was supplemented with 10 nM testosterone. After 72 hours, cell proliferation was determined using MTT assay as described previously. Conversely, the starvation medium was not supplemented with testosterone in the assessment of testosterone-independent 22Rv1 cell proliferation [22]. All experiments were repeated 3 times.

Isobologram analysis of a combination of enzalutamide and CMERE

The individual dose effect curves for the hydroethanolic extract and enzalutamide in inhibiting 22Rv1 cell proliferation, were established in an MTT assay and the respective IC_{50}s determined. The two drugs were combined in various ratios (1:1, ½ : ½, ¼ : ¼, $^1/_{16}$: $^1/_{16}$ and $^1/_{32}$: $^1/_{32}$) of their IC_{50}s and the dose effect curve and median-effect plot for the combination were determined using the software CompuSyn (1.0, Chou-Martin). The potency $(D_m)_{1,2}$ value, the shape $(m)_{1,2}$ value and $(r)_{1,2}$ values for the drug mixture were also obtained using the Compusyn-generated automated median-effect plot (the Chou plot). The r value signifies conformity of the data to the mass-action law. Subsequently, the Chou-Talalay method for drug combination, based on the median-effect equation [23,24], was used to determine the combination index (CI) and define additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in CMERE-enzalutamide combinations. These values were used to generate the fractional inhibition – combination index (Fa-CI) plot, dose-reduction index (Fa-DRI) plot and the classic and normalized isobologram. The following equations are the algorithms for the computerized simulation used to obtain the CI values:

$$\frac{f_a}{f_u} = \left[\frac{D}{D_m}\right]^m,$$

$$\mathrm{Log}\left[\frac{D}{D_m}\right] = m\log(D) - m\log(D_m),$$

$$\mathrm{CI} = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j},$$

Where D = Dose, D_m = median effect dose, D_x = dose of each drug that produces X% inhibition, fa = fraction inhibited, fu = fraction uninhibited, m = slope

The inhibitory effect of CMERE on migration of 22Rv1 cells

Here, 2 x 10^5 22Rv1 cells were seeded into 12-well plates. After 24 hours, complete media was replaced with starvation medium and the cells were maintained in it for an additional 48 hours. At 75-90% confluence, monolayers were scratched with a 10 µL pipette tip. The scratched monolayers were washed twice with phosphate buffered saline (PBS), and starvation medium containing respective treatments as described earlier, was added to respective wells. The experiment was repeated 3 times. Wounds were immediately photographed with a camera attached to a microscope (Nikon, USA) and the cells were afterwards incubated at 37°C, in 5% CO_2 and 95% humidity for 72 hours. Wounds were again photographed at 24, 48 and 72 hours after scratching [20]. The migrated distances by

the cells across the wounds were determined using ImageJ 1.46r software (NIH, USA) and percentage changes in wound area were calculated as shown below:

$$\%\ Change\ in\ wound\ area = \frac{A_0 - A_t}{A_0} x\ 100$$

Where A_o is the wound area at 0 hour, and A_t is the wound area at time, t

Effect of CMERE on colony formation of 22Rv1 cells

Cells were seeded into tissue culture-treated 6-well plates at a seeding density of 5 x 10^2 cells/well. The cells were allowed to attach overnight, after which complete growth medium was replaced with starvation medium and the cells maintained in it as described in earlier. Subsequently the medium was replaced with starvation medium containing respective treatments as described earlier. Following initial treatments, the cells were incubated for 2 weeks at 37°C, in 5% CO_2 and 95% humidity with the careful change of treatment-containing medium every 96 hours. After 2 weeks, growth medium was taken off and the formed colonies were fixed with ice cold methanol (-20°C for 10 minutes) and subsequently, stained with 0.5%$^{w}/_{v}$ crystal violet solution. The crystal violet solution was then carefully rinsed off by submerging the wells in a container full of water until the dye stopped coming off. Afterwards, the colonies were allowed to dry at room temperature and images were obtained. Subsequently, the colonies were counted using ImageJ 1.46r software (NIH, USA) and percentage colony formation rates were calculated as described by Rice *et al.* [25].

$$\%\ Colony\ formation\ rate = \frac{n}{N} x\ 100$$

Where n is the number of colonies counted, and is N is the seeding density

Selective cytotoxicity of CMERE in neoplastic prostate cells

The selective cytotoxicity of the hydroethanolic extract was determined using MTT assay. Briefly, human neoplastic 22Rv1 and BPH1 cells, and human THP1 monocyte-like cells were seeded into tissue culture-treated 96-well plates at a seeding density of 5 x 10^3 cells/well. The cells were allowed to attach overnight and normal growth medium was changed to treatment medium containing various concentrations of the hydroethanolic root extract (1, 3, 5, 10, 30, 50 and 100 mg/ml). The cells were treated for 72 hours. Absorbances were measured and IC_{50}s were determined as described in earlier. Subsequently, selective cytotoxicity of the extract was calculated as shown below [26]:

$$Selectivity\ Index = \frac{IC_{50}\ in\ normal\ cell}{IC_{50}\ in\ neoplastic\ cell}$$

Statistical analysis

Data are presented as Mean ± SEM. The time-course curves were subjected to two-way repeated measures analysis of variance (ANOVA) with Tukey's multiple comparisons test. One-way ANOVA and students t tests were also used, depending on the type of data. Graphs were plotted using GraphPad Prism for Windows Version 8.01 (GraphPad, San Diego, CA, USA). Analysis of isobolograms were performed with the program Compusyn (version 1.0, Chou & Martin).

RESULTS and DISCUSSION

Previous chemical analysis of *C. membranaceus* revealed the presence of some compounds which have been shown to possess antiproliferative or cytotoxic activities against cancer cells. Crotomembranafuran, a furano-clerodane isolated from the plant has been shown to exhibit antiproliferative activity against human prostate cancer (PC-3) cells [17]. In addition, the plant has been shown to contain scopoletin and β-sitosterol which are known antiproliferative agents and may account in part for the antiproliferative effect of *C. membranaceus* extract against cancer cells [17].

Gas chromatography mass spectrometry

In this study, GC-MS analysis revealed the presence of n-hexadecanoic acid which has been shown by previous studies to possess anticancer effects (Figure 1 and Table 1) [27]. In addition to fatty acids, GC-MS analysis of the hydroethanolic extract also showed the presence of 9, 10-Secocholeasta, astaxanthin and prednisolone acetate, all of which have been shown by previous studies to possess anticancer activity [28].

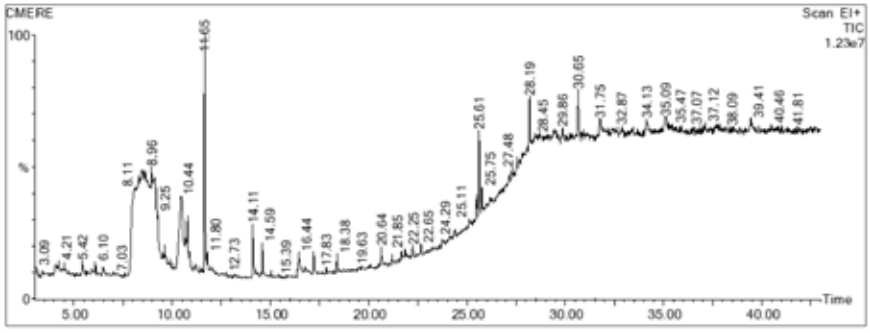

Figure 1. Gas chromatogram of *Croton membranaceus* hydroethanolic root extract

Table 1. GC-MS analysis for *Croton membranaceus* hydroethanolic root extract

#	RT	Area %	Norm %	Name
1	8.462	28.042	100.00	5-Hydroxypipecolic acid
2	8.957	11.588	41.32	Phenol, 3, 5-bis(1, 1-dimethylethyl)-
3	10.479	5.568	19.85	2-Octenoic acid, 5, 5, 7-trihyroxy
4	10.791	1.894	6.75	9, 10-Secocholeasta-5,7,10(19)-tiene-1,3-diol, 25-[(trimethylsilyl)oxy]-
5	11.653	3.274	11.67	Phenol, 4-(3-hydroxy-1-propenyl)-2-methoxy-
6	14.110	1.089	3.88	n-Hexadecanoic acid
7	25.606	1.331	4.75	Benzene, 1,2,4,5-tetrakis(1-methylethyl)-
8	30.648	1.387	4.95	Prednisolone acetate
9	31.748	1.147	4.09	1H, 4H-Pyrazolo[3,4-b]pyran-5-carbonitrile, 6-amino-4-(2, 4, 5-trimethoxyphenyl)-3-methyl-
10	35.085	1.130	4.03	Astaxanthin

GC-MS, gas chromatography mass spectrometry

CMERE is cytotoxic to human 22Rv1 prostate cancer cells

As GC-MS chromatograms showed the possible presence of cytotoxic compounds in the extract, it was hypothesized that the extract may inhibit the proliferation of human 22Rv1 CRPC cells. To investigate this effect, 22Rv1 cells were treated with different concentrations of either extract or the standard drug enzalutamide and cell proliferation was investigated using MTT assay. From the results obtained, enzalutamide was cytotoxic to 22Rv1 cells with an IC_{50} of 8.528 μM (Figure 2A). Interestingly, the hydroethanolic extract also showed cytotoxic effect against the 22Rv1 cells with IC_{50} values of 3.809 mg/ml (Figure 2B). These findings indicate that enzalutamide and CMERE inhibit the proliferation of human 22Rv1 PCa cells. The inhibition of proliferation of 22Rv1 CRPC cells may be partly attributed to the anticancer principles shown to be present in CMERE by GC-MS analysis.

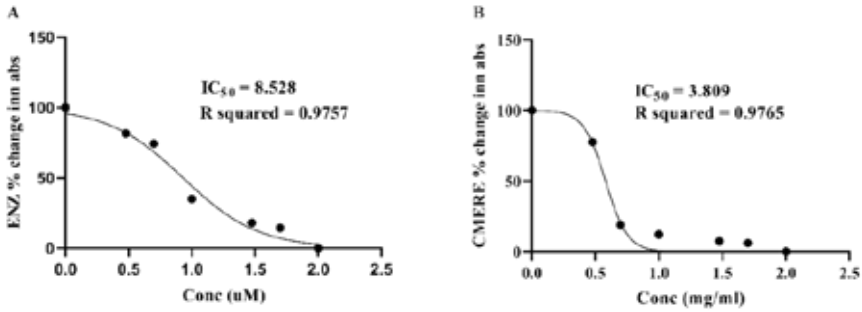

Figure 2. *Croton membranaceus* root extract is cytotoxic to human 22Rv1 prostate cancer cells. 22Rv1 cells were treated with varying concentrations of either enzalutamide or CMERE and cells were incubated for 72 hours. Cell viability was determined by MTT assay and absorbances were measured at 595 nm. All treatments were done in triplicates. (A) Enzalutamide-treated; (B) CMERE-treated.

CMERE inhibits testosterone-dependent and independent 22Rv1 cell proliferation

Having shown that *Croton membranaceus* root extract is cytotoxic to 22Rv1 cells, further investigations were conducted to determine if the inhibition was androgen-dependent or androgen-independent. Thus, the effects of the root extract were investigated in both testosterone-deprived and testosterone-supplemented environments. To investigate the effect of the extract on androgen-dependent cell proliferation, the growth medium was charcoal-stripped off steroid hormones to prevent interference with testosterone-dependent responses. Subsequently, the charcoal-stripped medium was supplemented with testosterone. Although supplementation of the growth medium with testosterone only resulted in a 0.3-fold increase in cell proliferation, the difference was still statistically significant when compared to the basal level cell proliferation (Figure 3).

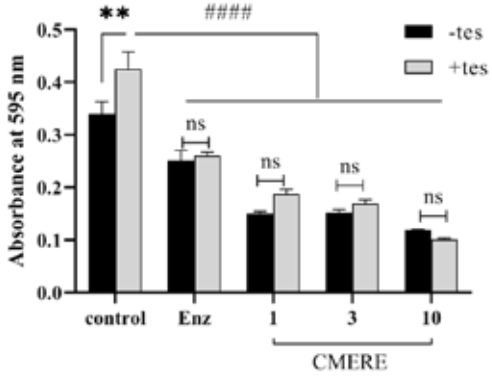

Figure 3. *Croton membranaceus* root extract inhibits testosterone-dependent and independent 22Rv1 cell proliferation. To investigate the effect of C. membranaceus extract on cell proliferation, 22Rv1 cells were treated over 72 hours with various concentrations of the hydroethanolic root extract. Cell proliferation was determined by MTT assay. Total inhibition of cell proliferation was calculated as AUC. Data is presented as mean ± SEM. **p < 0.01, hormone-independent control compared to hormone-treated control; ####p < 0.0001, extracts compared to hormone control; nsp > 0.05, extract in the absence of hormone compared to extract in the presence of hormone. Enz, enzalutamide.

Expectedly, treatment with the standard drug enzalutamide, significantly (p < 0.01) inhibited the proliferation of 22Rv1 cells compared to the untreated control (Figure 3) treatment with CMERE also significantly (p < 0.0001) inhibited testosterone-dependent proliferation of 22Rv1 cells compared to the solvent control (Figure 3). In addition, the extract significantly (p < 0.001) inhibited testosterone-independent proliferation of 22Rv1 cells, with less reductions in inhibitory effect compared to the inhibition of testosterone-dependent proliferation (Figure 3). However, there was no statistically significant difference between the effect of the extract on androgen-dependent and independent proliferation.

Combination of enzalutamide and CMERE synergistically inhibit 22Rv1 cell proliferation

It has been hypothesized that, drugs that act via similar mechanisms to achieve a common effect may likely produce additive effects when combined, whereas drugs that act through different mechanisms in achieving similar effects may interact synergistically [29]. Both enzalutamide and CMERE have been shown, in this study, to achieve similar effects i.e., inhibition of 22Rv1 cell growth. In addition, combination of the two drugs inhibited 22Rv1 cell proliferation (Figure 4A). Thus, interaction between the two drugs was investigated using isobologram analysis. Isobologram analysis of the combination of CMERE and enzalutamide showed synergistic inhibition of 22Rv1 cell proliferation with CI values of

0.17, 0.26, 0.44, 0.70 and 0.99 for 50, 75, 90, 95 and 97% dose effect levels respectively (Figure 4B). From the results, *r* value of 0.977 obtained, indicated the conformity of the data to mass-action law. Most importantly, a normalized isobologram constructed at 90% fractional inhibition indicated synergy between the effect of enzalutamide and CMERE (Figure 4C). Thus, combination of the two drugs was synergistic in producing more than 90% cell kill effect in 22Rv1 cells, an effect very relevant in anticancer therapy. The results also showed favorable dose reduction index for the selected combinations, indicating a reduction in the probability of occurrence of an adverse effect when enzalutamide and CMERE are used as combination therapy (Figure 4D). in summary, results from isobologram analysis suggest the two drugs act via different mechanisms to achieve similar pharmacological effect. Thus, the effect of CMERE on 22Rv1 may largely be mediated via pathway(s) other than the androgen receptor-dependent pathways.

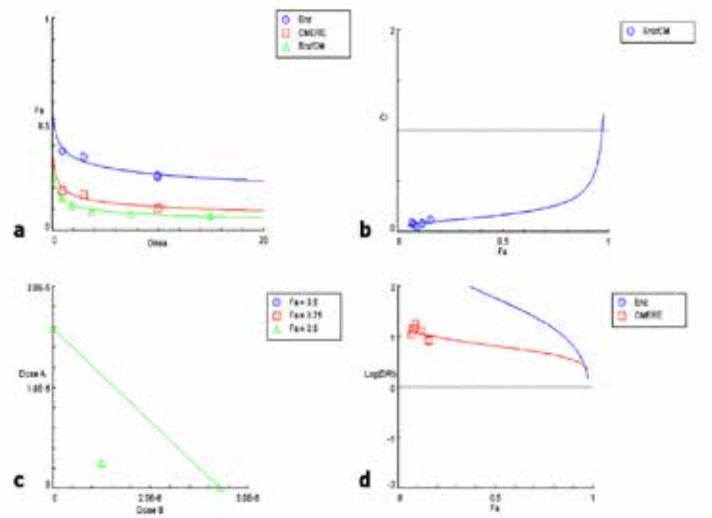

Figure 4. Combination of enzalutamide and CMERE synergistically inhibit 22Rv1 cell proliferation. MTT assay was used to obtain the dose effect curves. 22Rv1 cells were treated over 72 hours with various combinations of the EC50s of enzalutamide and CMERE. Additive effect (CI) = 1), synergism (CI < 1), and antagonism (CI > 1). (A) dose-effect curves; (B) combination index plot; (C) normalized isobologram at 90% dose-effect level; (D) Fractional inhibition-dose reduction index (DRI) plot.

Moreover, n-hexadecanoic acid shown by GC-MS analysis to be present in the hydroethanolic root extract is a known inducer of oxidative stress and apoptosis in cancer cells [30]. In addition, the extract was shown by GC-MS to contain prednisolone acetate, which has been shown to inhibit androgen receptor-bypass mechanisms and also exert direct cytotoxic activity through the induction or suppression of specific cytokines. Thus, the extract may have inhibited 22Rv1

cell proliferation via restoration of apoptotic signals and modulation of glucocorticoid receptor-signaling which is an androgen-receptor bypass mechanism [31].

CMERE inhibits migration of human 22Rv1 prostate cancer cells

Cancer cell migration is a crucial step in the development of metastasis [32]. Following the establishment of the antiproliferative effect of CMERE on 22Rv1 cells, the effect on collective cell migration was probed using the wound-healing assay. As shown in Figure 5, treatment with either enzalutamide or the extract reduced migration of 22Rv1 cells across the wounds created. Interestingly, treatment with all doses of the extract completely suppressed 22Rv1 cell migration within the first 24 hours of treatment (Figure 6A). Data from the different time points (Figure 6A) were used to calculate for total inhibition of cell migration by each treatment. From the results, CMERE significantly ($p < 0.0001$) inhibited the migration of 22Rv1 cells across the wound area (Figure 6B). Most notably, 10 mg/ml of the hydroethanolic suppressed migration of 22Rv1 cells across the wound area with cumulative percentage of 0.74 ± 0.12% compared to 84.19 ± 1.79% migration of the solvent control, representing 99.12 ± 0.14% suppression of cell migration (Figure 6B). This implies that the extract may inhibit migration of CRPC cells, which is a crucial step that precedes metastasis.

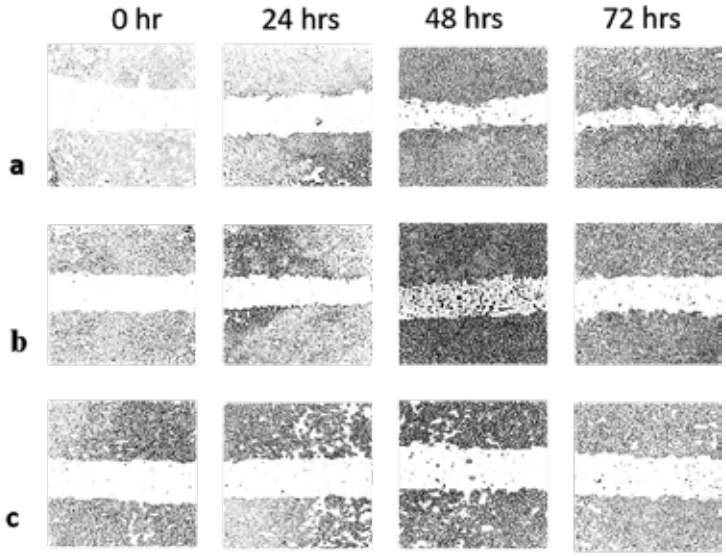

Figure 5. *Croton membranaceus* root extract inhibits human 22Rv1 cell migration. Human 22Rv1 cells were treated over 72 hours with various concentrations of either enzalutamide or CMERE. At confluence, monolayers were scratched with a 200 µL pipette tip and photographed at 0, 24, 48 and 72 hours. (A) disease control; (B) enzalutamide-treated (10µM); (C) CMERE (10 mg/ml); CMERE, *Croton membranaceus* hydroethanolic root extract.

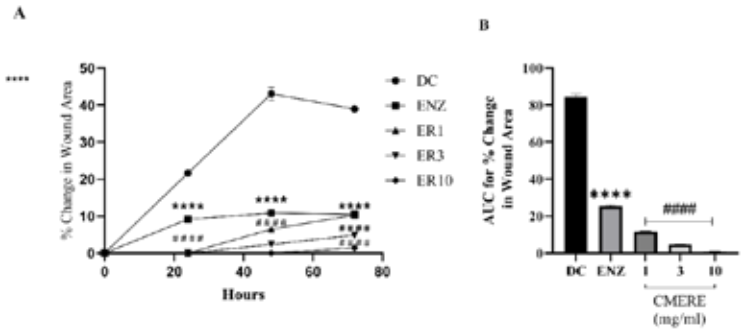

Figure 6. *Croton membranaceus* root extract suppresses overall migration of human 22Rv1 prostate cancer cells. To investigate the effect of *C. membranaceus* extracts on cell migration, 22Rv1 cells were treated over 72 hours with various concentrations of the hydroethanolic root extract. Cell motility was determined by wound healing assay. (A) CMERE time-course curve; (B) AUC for CMERE. Percentage wound closure was calculated as AUC. Data is presented as mean ± SEM. ***p < 0.001, ****p < 0.0001. DC, disease control; ENZ, enzalutamide.

CMERE inhibit colony forming ability of human 22Rv1 prostate cancer cells

Furthermore, as shown in Figure 7, CMERE was identified in this study to inhibit the ability of human 22Rv1 PCa cells to form colonies. This implies that, the extract may inhibit the complex multiple stages of intra- and intercellular remodeling that cancer cells go through to stay alive and establish colonies [30,33]. Metastasis remains a challenge to current treatment modalities and is the leading cause of cancer-related mortality [31]. Specifically, treatment with CMERE significantly ($p < 0.0001$) decreased the ability of 22Rv1 cells to form colonies with 10 mg/ml of the extract producing colony formation rate of 0.67 ± 0.133% compared to 43.00 ± 1.31% of the solvent control (Figure 8). This represents 98.44 ± 0.31% reduction in colony formation rate by the hydroethanolic extract, compared to the untreated control. In this regard, the inhibition of 22Rv1 colony formation by *C. membranaceus* root extracts suggests the ability of the extracts to inhibit the establishment of colonies post migration.

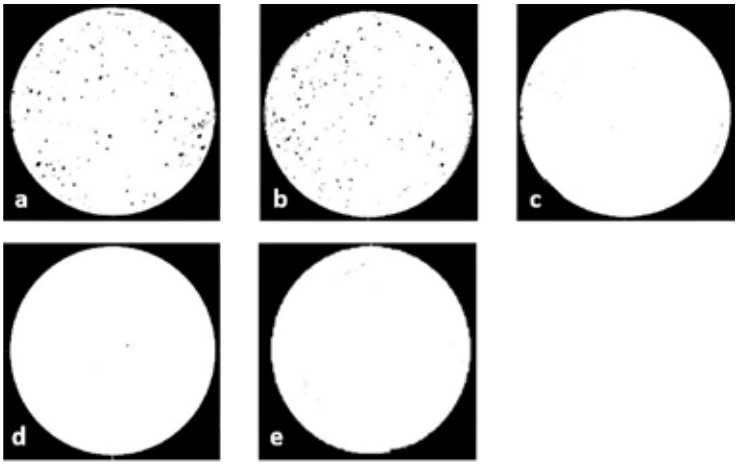

Figure 7. Colony-formation ability of human 22Rv1 prostate cancer cells is inhibited by CMERE. 22Rv1 cells (5 x 102 cells/well) were cultured for 14 days. Afterwards, colonies were fixed in ice cold methanol and stained with 0.5% w/v crystal violet solution. The wells were scanned and resulting images were analyzed with ImageJ software. (A) Disease control; (B) enzalutamide-treated (10μM); (C, D, E) CMERE (1, 3, 10 mg/ml).

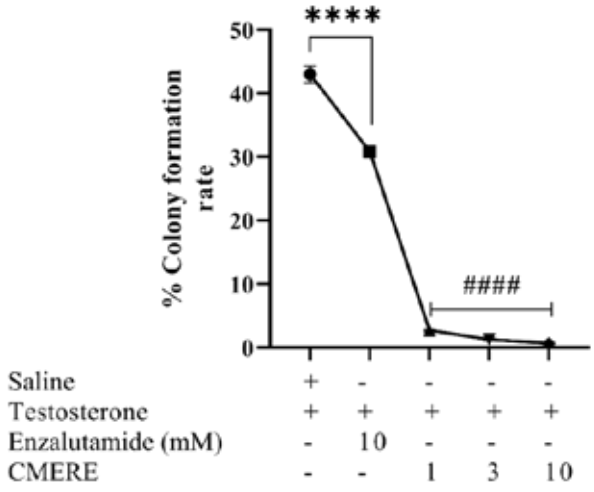

Figure 8. Colony-formation ability of human 22Rv1 prostate cancer cells is inhibited by *C. membranaceus* extract. 22Rv1 cells (5 x 102 cells/well) were cultured for 14 days. Afterwards, colonies were fixed in ice cold methanol and stained with 0.5% w/v crystal violet solution. Colonies were counted and graphed as percent colony formation over number of cells seeded. All treatments were done in triplicates. Total inhibition of colony formation was calculated as AUC. Data is presented as mean ± SEM. ****$p < 0.0001$, enzalutamide compared to nontreated control; ####$p < 0.0001$, extract compared to untreated control.

CMERE is selectively cytotoxic to human neoplastic prostate cells

Finally, to show that CMERE selectively inhibits human neoplastic 22Rv1 CRPC and BPH1 cells, but not normal human THP1 monocytes, comparative proliferation assay was carried out. As shown in Figure 9A and B, CMERE inhibited 22Rv1 and BPH1 cell proliferation with IC_{50}s of 3.809 mg/mL and 4.04 mg/mL, respectively. Conversely, CMERE did not inhibit the proliferation of THP 1 cells (Figure 9C). Suggesting that, the extract selectively inhibits the growth of neoplastic cells of prostate origin.

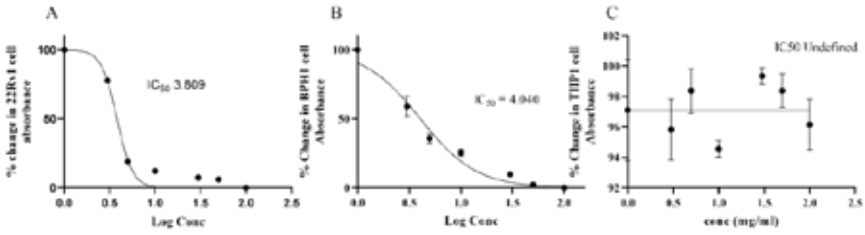

Figure 9. CMERE selectively inhibits human 22Rv1 prostate cancer and human BPH1 cells but not human THP1 monocyte-like cells. Human 22Rv1, BPH1 and THP1 cells were treated with varying concentrations of CMERE and subsequently cells incubated for 72 hours. Cell viability was determined by MTT assay. All treatments were done in triplicates. (A) 22Rv1; (B) BPH1; (C)THP1.

The lack of cytotoxic effect on human THP1 monocyte cells suggests that CMERE is selectively cytotoxic and treatment with it might not cause off-target effects. Accordingly, these findings support evidence from previous studies by Afriyie *et al.* [34] and Sarkodie *et al.* [18] that reported safety of *C. membranaceus* hydroethanolic and aqueous extracts in rodent models. This differential cytotoxicity to human neoplastic cells makes *C. membranaceus* extracts particularly interesting as potential sources of safer drug leads in the management of CRPC.

In conclusion, this study has, for the first time, provided *in vitro* evidence that the hydroethanolic root extract of *Croton membranaceus* is effective in the inhibition of castration-resistant prostate cancer cell growth. Furthermore, combination of CMERE and enzalutamide synergistically inhibits more than 90% proliferation of 22Rv1 cells and holds the potential for the effective treatment of CRPC with less off-target effects. The findings from this study, however, cannot be directly extrapolated to humans. Further studies involving 3D cell cultures and xenograft models, which assess the role of the tumour microenvironment in cancer development and progression are therefore required to improve upon the acquired knowledge.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

KOY: Design, acquisition of data, analysis of data, drafting of manuscript, statistical analysis. ME: Design, acquisition of data. EAN: Design, critical review of manuscript, supervision. GHS: Design, Plant collection and authentication, GA: Design, critical review of manuscript, supervision.

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Bioautography for evaluation of several *Lavandula* L. and *Origanum* species antimicrobial and antioxidant activity

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ABSTRACT

In the search of bioactive natural compounds, bioautography of plant extracts were associated in an antioxidant screening. Due to containing variety of phenolic compounds *Lavandula* and *Origanum* species are important medicinal plants. The antioxidant and free radical scavenging activities of *Lavandula angustifolia, L. stoechas, L. heterophylla, Origanum majorana, O. onites, O. vulgare, O. minituflorum,* and their main phenolic compounds linalool and carvacrol was carried out by TLC-bioautography method based on the $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ assays to compare essential oils and known main active constituents. The antimicrobial activity of the materials was tested using the in vitro broth microdilution assay towards two different microorganisms. Methicillin-resistant *Staphylococcus aureus* and *Streptococcus mutans* were used for the study. As a result of our studies, it is determined that *O. vulgare* showed the highest activity against *S. mutans* and *O. onites* and *O. vulgare* showed the highest activity against MRSA. compared to the tested antibiotic.

Keywords: *Lavandula, Origanum*, essential oil, bioautography, antioxidant

INTRODUCTION

In Türkiye the extensive use of aromatic and medicinal plant species as primary remedies of the local culture, covering a considerable area with different

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environmental conditions, is quite common [1]. The Lamiaceae family, incorporates rich source of plants containing wide variety of phenolic acids and terpenes which has medical applications in the Mediterranean region [2,3]. *Lavandula L.* species are widely used in folk medicine and industrial fields[4]. Over the last decades, phenolic compounds and essential oils (EO) from these species is expanding for cosmetic and pharmaceutical uses [5,6]. Recent phytochemical investigations on *Lavandula* species oils revealed that monoterpenes are major components of the fractions with high contents of linalool, linalyl acetate 1,8-cineole, camphor, carvacrol and fenchone [7,8]. Lately, due their economic values many researchers were investigated the phytochemical and pharmacological aspects of Lavandula species. Previous studies shown that the essential oils or extracts of lavender display a broad spectrum of bioactivities such as anti-bacterial, anti-fungal, antioxidant, anti-inflammatory, insecticide, sedative, and anti-cancer activies [8,9]. Additionally, based on latest literature, linalool has anxiolytic, anti-cholesterol, and antibacterial activity [10]. Previously, it is determined that *L. angustifolia,* and *L. stoechas* have significant antioxidant activity, however, lavender species generally possess low antioxidant activities relative to *Origanum* species [8]. The *Origanum L.* (Lamiaceae) genus include 38 species distributed around the Mediterranean region, although most of them are confined to the eastern Mediterranean area. *Origanum* species are defined by a wide variety of terpenic molecules and by the presence of chemical differences in essential oil composition [11,12]. *Origanum* sp. EO includes carvacrol, thymol, and γ-terpinene as major constituents [11,12]. Besides the ethnobotanical usages there are many studies shown various biological effects such as antimicrobial, cytotoxic, antifungal, insecticidal, antioxidant, anti-spasmodic, antitumoral, and analgesic activities of *Origanum* species were reported [13,14]. *Origanum* essential oils were found to be amongst the most effective antioxidant natural agents [15]. Antioxidant compounds play a crucial role in essential oils biological activities, which is justified by the involvement of oxidative stress in pathology. These attributes are because of the inherent ability of particularly phenols, and specifically carvacrol and thymol, to inhibit the aerobic oxidation of organic matter [15,16]. The use of bioautography combined with thin-layer chromatography (TLC) is a allows the detection of active components screening for the investigation of the antioxidant effect. This study aims to obtain the antioxidant activity of *Lavandula angustifolia*, *L. stoechas*, *L. heterophylla* (synonym *L. hybrida), Origanum majorana, O.onites, O. vulgare,* and *O. minituflorum* essential oils with TLC bioautography method based on the $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ assays, and to evaluate their antimicrobial activities via *in vitro* broth microdilution assay towards two different microorganisms.

METHODOLOGY

For the experiment, analytically approved commercial essential oils were kindly provided by a Turkish company, Doalinn. The GC–MS analyses were performed in our previously studies, analyzed by GC-FID and GC/MS Analysis of the Agilent 6890N GC and Agilent 5975 GC-MSD systems [17,18].

TLC–Fingerprinting and DPPH Bioautography

Chromatographic separation was carried out on silica gel 60 F254 chromatographic plates (20 cm × 10 cm) using 7:3 Hxn: EtOAc to develop TLC plates. The essential oils were dissolved in ethanol and spotted on the chromatographic plates, then developed using different mixtures for *Origanum* and *Lavandula* EOs. Plates were prepared as duplicates and one of the chromatographic plates was derivatized with Anisaldehyde reagent and then heated at 105°C, one was dipped into DPPH (0.2%), and one of the plates was dipped into ABTS reagent [19].

Antioxidant activities of the essential oils were evaluated with the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and 2,2-Azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid (ABTS) bioautography methods. The plates examined in daylight after 30 min. $DPPH^{\cdot}$ solutions were displayed in the form of yellow fluorescent bands with purple background, and ABTS solutions were displayed in the form of colorless or pink spots with a green background, which were easy to be identified and were of high sensitivity Additionally, the intensity of the colors can be measured with a chromameter [20,21].

The antibacterial potential was determined using the *in vitro* broth microdilution assay against methicillin-resistant *Staphylococcus aureus* and *Streptococcus mutans*. According to our knowledge TLC-fingerprinting of different *Origanum* species was studied before [19]. However, this is the first time that it researches four different *Origanum* species from Turkish flora and compares the activity-chemotype relationship between them.

Antimicrobial Activity

The *in vitro* antimicrobial activity was determined using the broth microdilution assay following the methods according to the Clinical and Laboratory Standards Institute to determine the minimum inhibitory concentrations (MIC) [22]. Methicillin-resistant *Staphylococcus aureus* (Clinical isolate) and *Streptococcus mutans* (ATCC 25175) strains were grown in Mueller Hinton Broth (MHB, Merck, Germany) in aerobic conditions at 37 °C for 24 h. Microorganisms were adjusted to 1×10^8 CFU/mL using McFarland No: 0.5 in sterile saline (0.85%)

solution. Stock solutions and serial dilutions of the test samples were prepared in dimethyl sulfoxide (DMSO). Final DMSO concentration was 1% in each well. The minimum non-reproductive concentration was reported as minimum inhibitory concentration (MIC, as μg/mL). Essential oils were studied with serial dilution starting from 1 mg/mL concentration and MIC values were calculated. 1 mg/mL used as the initial concentration. Amoxicillin serial dilution starting from 1 μg/mL and tetracycline serial dilution starting from 0.1 μg/mL concentration and MIC values were calculated. The MIC was calculated and reported as the mean of three repetitions compared to positive standards as shown in Table 1 and Table 2.

RESULTS AND DISCUSSION

GC/MS and GC-FID analyses

The essential oil compositions of the tested *Lavandula* sp. and *Origanum* sp. are showed in previous studies. Essential oils major compounds of *L. angustifolia and L. x heterophylla* were identified as linalool, linalyl acetate, camphor, 1,8-cineole, and borneol. Camphor, α-fenchone, bornyl acetate, 1,8- cineole, and camphene were characterized and confirmed as major components of *L. stoechas* essential oil. In tested four different *Origanum* sp. EOs carvacrol was identified as the major component [17,18].

Bioautography analyses

The fingerprinting of EOs obtained from four *Origanum* and three *Lavandula* specimens was done by thin-layer chromatography. Visualization of the volatile components present in all EOs was performed by derivatization with Anisaldehyde reagent. The bioautography was performed for EOs with DPPH and ABTS methods. Clear zones at a same R_f value presented in Figures 1 and 2. The TLC–fingerprint analysis revealed that EO hydro-distilled from the aerial parts of *O. vulgare* (B) is the most abundant in chemical constituents. Carvacrol and linalool which main compounds of EOs were also visible in the EO obtained from the flowers (G); however, their abundance was different [19].

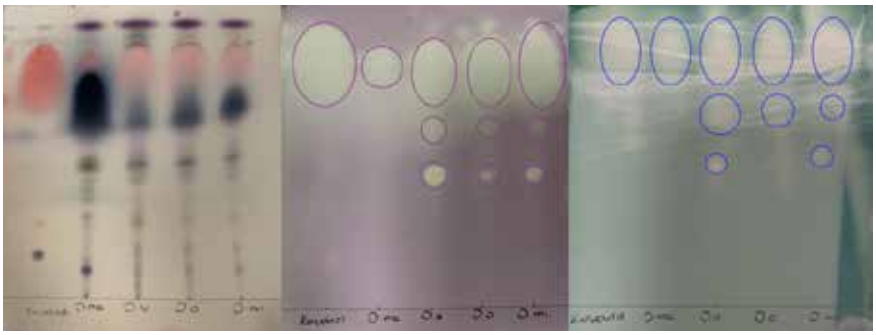

Figure 1. TLC-fingerprint, DPPH, and ABTS bioautography of studied material.
(From left to right: Carvacrol, *O. majorana, O. vulgare, O. onites, O. minutiflorum,* respectively)

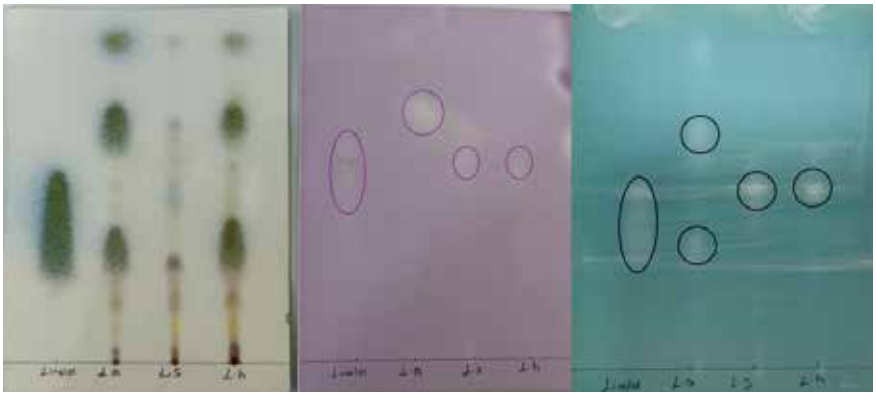

Figure 2. TLC-fingerprint, DPPH, and ABTS bioautography of studied material.
(From left to right: Linalool, *L. angustifolia, L. stoechas, L. x heterophylla,* respectively)

As shown in Figures 1 and 2, we can determine the major compounds of lavender and *Origanum* EOs are mostly responsible for antioxidant activity. By looking at the Rf values, we can indicate that carvacrol and linalool have significant antioxidant capacity and these compounds can be found in the tested EOs. However, in this assay, we can only understand the bioactivity of the compound that we've added to the TLC plate. In this case, we don't know the details about other active components. Further information on active components may be determined by different isolation techniques may be used such as PTLC, HPTLC, and GC-MS.

There are numerous studies have shown significant antioxidant activity results for different oregano and lavender species in the literature. Various essential oils such as clove oil, thyme oil, oregano oil, lavender oil, eucalyptus oil, pep-

permint oil, etc. play an important role in the inhibition of pathogenic microbial growth and food preservation [23–25]. Moreover, it is reported that twenty-one phenolic compounds isolated from *O. vulgare* ethanolic extract evaluated *in vitro* antioxidant activity using DPPH radical-scavenging and ferric-reducing antioxidant power (FRAP) assays[26]. Ethyl acetate, *n*-butanol, and water extracts of *O. vulgare* possessed a strong, and *O. majorana* showed moderate antioxidant activity in accordance with its phenolic compounds [27,28]. The importance of major compounds of *Origanum* essential oils is also indicated in various studies. Thymol and carvacrol showed high antioxidant activity according to different methods [29,30].

Studies indicates that linalool, camphor, and 1,8-cineole are the major constituent for *Lavandula* EOs. It is stated that, various *Lavandula* species has antioxidant activity due to their chemical composition [31–33]. Furthermore, linalool as itself showed antioxidant activity. The antioxidant activity from different cultivars affects environmental conditions, thus, it is expected to see different results in different samples. Some EOs of *Lavandula* plants were reported to have antioxidant properties, while some have none[33]**.** The bioautography results showed that there is antioxidant activity in *L. stoechas* and *L x heterophylla* due to linalool. However, in *L. angustifolia*, there are two different spots that we couldn't determine, has a significant antioxidant capacity as well.

Antimicrobial studies

Due to the development of antibiotic resistant microorganism and urge to find new antibacterial agents, essential oils are being evaluated as excellent resources to inhibit the resistant microorganisms. Therefore, lavender and *Origanum* essential oils were evaluated for their antibacterial activities (Table 1 and 2) by determining MIC.

Table 1. MIC values of amoxicillin, tetracycline and *Origanum* essential oils in µg/mL by broth microdilution assay

Material	*O. majorana*	*O. vulgare*	*O. onites*	*O. minutiflorum*	Amoxicillin
Methicillin Resistant *Staphylococcus aureus* (MRSA)	125	62.5	62.5	125	> 1000
Streptococcus mutans	15	7	3.5	1.75	125

Table 2. MIC values of amoxicillin, tetracycline and *Lavandula* essential oils in µg/mL by broth microdilution assay

Material	*L. angustifolia*	*L. stoechas*	*L. x heterophylla*	Amoxicillin
Methicillin Resistant *Staphylococcus aureus* (MRSA)	125	250	125	> 1000
Streptococcus mutans	250	62.5	125	125

Previous studies showed that *Origanum* essential oils have an antibacterial effect. *O. vulgare* essential oil showed strong antibacterial activity against the *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus cereus,* and *Candida albicans* strains. Costa et al. (2009) have reported the antibacterial activity of oregano oil against *E. coli, Enterococcus faecalis,* and Meticillin-resistant *Staphylococcus aureus* (MRSA) [34]. In several studies the major constituents such as thymol, carvacrol, eugenol, linalool and α-terpineol possess antimicrobial properties. Furthermore, linalool as itself showed antibacterial activity against different strains such as *P. aeruginosa* [10]. In controversy, some studies have shown *L. stoechas* has no activity against various pathogens such as *S. aereus, S. epidemidis, E. coli,* and *P. aeruginosa,* however, found active against *Salmonella Typhimurium, and Klebsiella pneumoniae*[33]. According to our results*, L. angustifolia, L. x heterophylla, O. majorona,* and *O. minutiflorum* showed moderate inhibition to MRSA, and *O. vulgare* and *O.onites* showed remarkable inhibition activity. Additionally, all tested *Origanum* essential oils shown inhibitory activity against *S.* mutans.

In this study, we determine the antioxidant activity of commonly used four different *Origanum* and three different *Lavandula* essential oils via bioautography assay to evaluate the major antioxidant capacity of the volatile compounds. In addition to the bioautography assay, we investigated the antibacterial activity against two different pathogens which variously affect a person's health. Between the tested essential oils, *O. minutiflorum* showed the highest activity compared to other oregano EOs, and *L. stoechas* showed the highest activity compared to tested lavender species, against *S. mutans* and most of the tested essential oils had better activity compared to Amoxicillin. Moreover, *O. onites* and *O. vulgare* showed the highest activity against MRSA and all the tested essential oils showed better inhibitory activity against MRSA, compared to the tested antibiotic. Other tested *Lavandula* and *Origanum* EOs also

showed high antibacterial activity against MRSA compared to Amoxicillin. The results of the bioautography assay highlighted that the antioxidant activity was majorly caused by the linalool and carvacrol. However, other undefined spots also have significant antioxidant activity. These antibacterial and antioxidant activities may be related to the complexity of volatile constituents, and bioautography assay also indicated this assumption. Previous studies showed the major components of EOs and their biological activity. Antimicrobial, antioxidant, and insecticide activities proved for linalool and carvacrol. Essential oils and their major constituents, effectively enhance the safety and quality of food products, due to their antimicrobial and antioxidant activity capacities[23]. With the bioautography screening of antioxidant compounds and the antimicrobial assay led to the identification of carvacrol and linalool as the major antioxidant constituent of the tested essential oils. The results obtained indicate that oregano and lavender essential oils are a good source of natural antioxidants with potential application in food and pharmaceutical industries, and a good antibacterial agent, they can be a safer alternative to synthetic agents.

As further studies, we aim to investigate the undefined antioxidant constituents and will try to match them with the GC-MS analyses.

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Parosmia and COVID-19 from the lens of google trends: infodemiology study

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ABSTRACT

Parosmia is a subcategory of olfactory hallucinations and refers to a distorted ability to detect the right smell in the presence of a stimulus. The study aims to investigate the relationship between COVID-19 and parosmia by calculating the interest search volume of parosmia using google trends. Google trends was used to investigate trends in searches regarding parosmia and to track these search engine terms against the coronavirus outbreak in France, Sweden, the United States [USA], and Türkiye. The terms utilized in the search were "Parosmia" and "anosmia" and the data were collected between March 20, 2020, to July 25, 2021. Parosmia searches increase with time in all the countries and the correlation significance values were obtained for France, Sweden, USA, and Türkiye to be Rs 0.660, P-value 0.0038 "Moderate correlation"; Rs 0.566, P-value 0.017 "Moderate correlation"; Rs 0.842, P-value 0.0001 "Strong correlation"; Rs 0.800, P-value 0.0001" Strong correlation" respectively. Relative search volume of parosmia and anosmia changed significantly with time may point out that there are some late COVID-19 complications that haven't been detected yet, and with the pandemic still ongoing, more complications could be discovered by analyzing the trends.
Keywords: Olfactory disorders, quality of life, disease severity, COVID-19

INTRODUCTION

The occurrence of olfactory dysfunction following viral infections is a common phenomenon that can affect an individual's sensory perceptions and cognitive abilities from mood swings to suppressing the ability to detect danger, impairing the gustatory system and by extension food enjoyment, to influencing overall health and quality of life [1,2].

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Among the most common olfactory dysfunctions that affect patients' post-viral infections are parosmia and phantosmia; both conditions affect 56% of patients following viral infections. The SARS-CoV-2 virus trigger an outbreak of the coronavirus disease, which the World Health Organization (WHO) declared a pandemic on March 11, 2020 [3]. Olfactory disorders before the covid-19 pandemic were largely unrecognized, and often underestimated by researchers. During the COVID-19 pandemic, about 52.73% of cases result in loss of smell "anosmia" [4]. Although a large proportion of them recovered within weeks, some of the patients reported persistent problems including parosmia and phantosmia which aren't always associated with additional nasal disorders such as nasal obstruction and rhinorrhea [5]. While sufficient evidence remains scarce regarding the relationship between COVID-19 and olfactory dysfunction; the plethora of report studies and self-reports exchanged on social media platforms show that there's a direct bond between the two.

All olfactory dysfunctions fall into two categories, quantitative olfactory disorders which environ anosmia and hyposmia and qualitative olfactory disorders which environ parosmia and phantosmia. Anosmia is an olfactory dysfunction that refers to a complete loss of the smelling sensation, whereas hyposmia refers to a decrease in the ability to detect scents. On the other hand, there's parosmia, which is a subcategory of olfactory hallucinations and refers to a distorted ability to detect the right smell in the presence of a stimulus. Parosmia can present itself as either pleasant scents referred to as troposmia or unpleasant scents referred to as euosmia. Similarly, phantosmia is another type of olfactory hallucination that triggers the detection of scents in the absence of a stimulus. Both parosmia and phantosmia typically exist in relation to quantitative olfactory dysfunctions, however, they can also exist individually, although rarely [6].

At the start of the COVID-19 pandemic, many studies from all over the globe noted the frequent occurrence of olfactory dysfunction in patients affected by the disease [7]. For instance, some case reports indicate that some perfectly healthy individuals experienced olfactory hallucinations post recovering from COVID-19 where they express their perception of all smells as dirty, rotten, sewage, or smoky [8]. These complications imply the presence of a condition referred to as the Rare Late Complication of COVID-19, which is still unpopular.

The mechanism by which viruses impair or distort olfactory sensations is through damaging the neurons responsible for olfactory sensations and the upper respiratory tract. As for the olfactory dysfunction following a COVID-19 infection, there are a few possible hypotheses that attempt to explain the formation after treatment from the disease. The first hypothesis attributes the

damage to mechanical obstruction suggesting that the formation of inflammation surrounding the olfactory cleft prevents odors from binding to the receptors [9]. The second hypothesis proposes that the direct infection of the ACE-2 cells by the SARS-CoV-2 is the reason [10] and the 3rd hypothesis refers the reason to the direct invasion of olfactory neurons by COVID-19 virus, which impedes the mechanism of conducting olfactory sensations [11].

While the estimated number of cases that reported parosmia post recovering from COVID-19 has been limited by a lack of either reliability or availability of testing; there are several anecdotal reports that conclude the presence of sudden olfactory changes after recovering from COVID-19. For instance, many patients reported having a distorted sense of smell, some of them displayed a persistent smell of a burnt rubber scent, while others displayed the persistent presence of an onion odor [12].

Under times of pressure in a rapidly spreading viral outbreak around the world, the need for equally rapid and fast-paced research technologies and real-time data collection becomes clear. Google Trends is an online tracking system of the biggest search engine that proved to be a powerful tool for epidemiologic surveillance in previous studies specifically in the rhinology field [13]. The study aims to investigate the relationship between COVID-19 and parosmia by calculating the interest search volume of parosmia using google trends.

METHODOLOGY

Google Trends is the master of the materials employed in this research which is a system that tracks internet activity relevant to any topic on Internet hit-search volumes. The selection of searches is predetermined by the portal to be inclusive of user-specified terms searched on Google. Relative search volume (RSV) is a tool from Google trends that suggest the query share of a specific term at a certain location during a certain time period; executed on a scale of 0 to 100 that is later normalized in opposition to the highest query share of the given term over a specific duration. Each individual point has divided by the highest point, which is conventionally 100 to generate the final graph values [14].

Google trends have been used to investigate trends in searches regarding parosmia and to track these search engine terms against the coronavirus outbreak in France, Sweden, the United States (USA), and Türkiye. To make the data comparison process three different countries besides Türkiye were selected randomly using a computer program as it's difficult to collect and cover all the countries around the world.

The terms utilized in the search were "Parosmia OR change of smell" and "anosmia OR loss of smell" for (USA and Sweden), "Parosmi OR kokuları farklı

algılama" and "Anozmi OR ansomi OR koku kaybı" for Türkiye. Lastly, "Parosmie" and "anosmie" for France. All the used data were collected between March 20, 2020 to July 25, 2021 in the countries mentioned previously.

The results were displayed as a monthly search volume score and to investigate the correspondence between parosmia and anosmia, a null hypothesis was constructed, and the p-values was established. A Spearman's correlation was then employed to evaluate the potential significance of the score trends between the relative search volume (RSV) of parosmia and time progression. The data entered was assessed using IBM SPSS (Statistical Package for the Social Sciences) version 20.

RESULTS AND DISCUSSION

Using the search terms "Parosmia" "anosmia", trajectories for the frequency of the search items were examined from March 20, 2020 to July 25, 2021. Figure 1 - 4 shows the total monthly RSVs during the duration mentioned above in the selected countries.

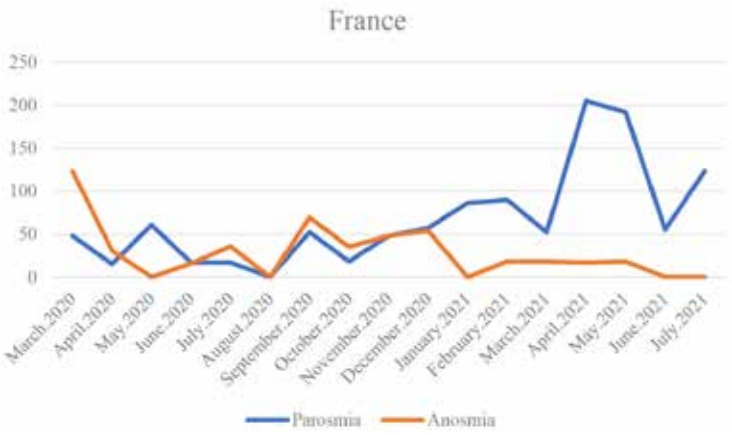

Figure 1. The total monthly RSVs between March 20, 2020 to July 25, 2021 in France

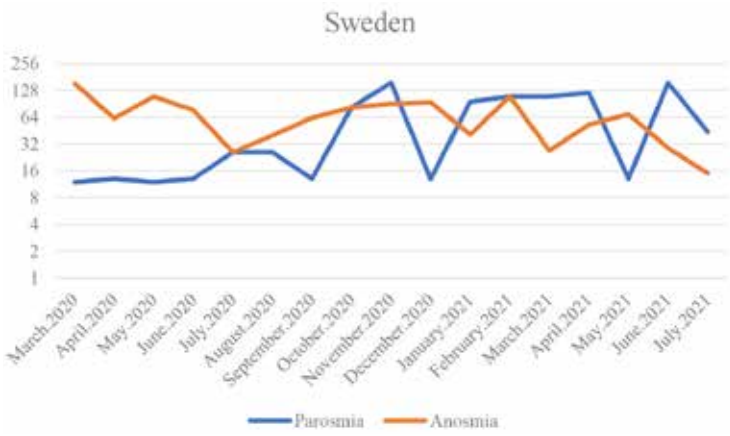

Figure 2. The total monthly RSVs between March 20, 2020 to July 25, 2021 in Sweden

Figure 3. The total monthly RSVs between March 20, 2020 to July 25, 2021 in Unites States

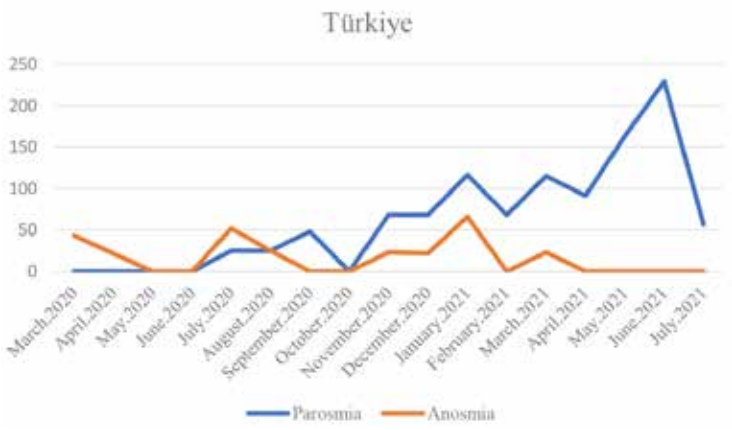

Figure 4. The total monthly RSVs between March 20, 2020 to July 25, 2021 in Türkiye

In regards to hypothesis testing on a population proportion, the null hypothesis HO was rejected indicating the presence of a statistically significant impact between parosmia and anosmia in France, USA, and Türkiye. However, in Sweden the null hypothesis HO has been accepted for the data implying the absence of a statistical significance between parosmia and anosmia.

All the data procured from varying countries indicate the presence of a statistically significant difference ($p<0.05$) between anosmia and parosmia during the pandemic with the exception of Sweden's data. Table1 shows the *P*-values between the selected terms in all the selected countries.

Table 1. Determining the significant difference between the parosmia and anosmia during Covid-19 between March 20, 2020 to July 25, 2021

	France		Sweden		USA		Türkiye	
	Mean ± SD	P-value	Mean ± SD	P-value	Mean ± SD	P-value	Mean ± SD	P-value
Parosmia	66.82 ± 58.49	0.023*	59.58 ± 54.13	0.63	43.76 ± 46.85	0.03*	63.11 ± 64.67	0.007*
Anosmia	28.41 ± 32.00		67.11 ± 36.79		76.00 ± 36.67		16.23± 21.03	

*Significant at the 0.01 level (2-tailed); SD = standard deviation; mean= average search volume per month

=In order to investigate the correlation between the escalating RSV of parosmia and the progression of time, the Bivariate Spearman correlation coefficient was calculated. The correlation significance values obtained for France, Sweden, USA and Türkiye were Rs 0.660, P-value 0.0038 "Moderate correlation"; Rs 0.566, P-value 0.017 "Moderate correlation"; Rs 0.842, p-value 0.0001 "Strong correlation"; Rs 0.800, P-value 0.0001 "Strong correlation" respectively. All countries in the study displayed a significant correlation. Table 2 shows the correlation between the selected variables.

Table 2. Correlations between the time progression and parosmia RSV

	France		Sweden		USA		Türkiye	
	Rs	P-value	Rs	P-value	Rs	P-value	Rs	P-value
Parosmia	0.660	0.0038*	0.568	0.017*	0.842	<0.001*	0.801	<0.001*
Anosmia	-0.474	0.055	-0.509	0. 037	-0.680	0.003	-0.314	0.219

*Correlation is significant at the 0.01 level (2-tailed); Rs = Spearman's correlation;
Corelation done between total monthly RSV and the time progression which ranges between March 20, 2020 to July 25, 2021

By collecting and analyzing data using Google Trends, this study was able to uncover the link between COVID-19 and parosmia as a late COVID-19 consequence. The consequences are far-reaching. There is already good evidence supported by a statistically significant effect from Italy, South Korea and China that significant numbers of patients with proven COVID-19 infection have developed anosmia/hyposmia. In Germany, it is reported that anosmia affects more than two out of every three verified cases [7]. While most people with COVID-19 olfactory dysfunction recover rapidly "within four weeks for 79 % of people" [15,16], some with long COVID-19 smell disorders are detected unpleasant scents after recovery [17].

Our findings show that the RSV of parosmia and anosmia changed significantly with time. This suggests that, around the time the coronavirus first appeared in the world, there was an upsurge in the number of people looking for information about the loss of their sense of smell. Although the RSV of anosmia was decreasing with time, the RSV of parosmia was increasing. This could be supported by a study done in 2021 concluded that, the onset of parosmia started within 3 – 5 months after the smell disorder[12], whereas reporting of anosmia onset began 4.4 days after infection initiation. According to Klopfenstein et al, the average duration of anosmia was 8.9 days, and 98 percent of patients recovered by 28 days[18]. The increase of the parosmia RSV and decrease in the anosmia search volume could be explained clearly via the results of the mentioned studies, as the onset of anosmia begins and ends entirely in 28 days,

whereas the onset of parosmia begins after 3 months of recovery. That's why people have started to search for parosmia term lately while the anosmia term RSV has increased since the initial days of the Covid-19 outbreak.

According to our findings, there is a statistically significant difference $p < 0.05$ present between anosmia and parosmia across all the selected countries except Sweden. A study has been done on 268 patients reported that all patients suffered from parosmia, they were suffering from hyposmia or anosmia prior to developing it. This supports our hypothesis that there is a statistically significant and unambiguous relationship between anosmia and the development of parosmia later on[16].

Our data illustrates a clear strong to moderate correlation in all the countries between the increase of the google search volume about parosmia regarding the time progression. That's could be explained as there is a directly proportional relation between time progression and parosmia given the strength and consistency of the Rasheed et al. study outcomes that patients with COVID-19 started to suffer from parosmia after 3.434 ± 0.4886 months after the disappearance of anosmia or getting better from COVID-19 [16]. Another study of COVID-19 patients found that after 8 weeks of follow-up, 30.9 percent of the study group had developed parosmia[19].

This finding could point to the fact that there are some late Covid-19 complications that haven't been detected yet, and with the SARS-CoV-2 pandemic still ongoing, more complications could be discovered by analyzing the trends or waiting for future case reports.

Despite all our efforts, our findings must be interpreted with caution as the data was collected regarding the RSV. Any data analysis based on electronic search volume must admit the inherent bias of a population sample drawn specifically from those who are educated, have enough money to access the internet, and use Google as their search engine of choice.

Our study suggests that members of the public have noticed a change in their sense of smell and have been obliged to look for answers on google and it shows that the interpretation of Google Trends data could have been useful to investigate the medical issues that the population might be suffering from around the world. Olfactory dysfunction is a remarkable problem that happened in the patient during and after Covid-19. Healthcare providers should play a role in the pandemic by following up with patients to report post-Covid-19 complications and assist patients in overcoming olfactory dysfunction in order to improve their quality of life.

STATEMENT OF ETHICS

No need.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

All authors contributed to the concept and design of the study. Material preparation, data collection, analysis and the draft of the manuscript were performed by [N.A] and [N.O]. The final manuscript has been reviewed and approved by all writers.

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Characterization, antimicrobial and catalytic activities of silver nanoparticles biosynthesized using aqueous extract of *Euphorbia graminea*

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ABSTRACT

Phytosynthesis of silver nanoparticles (AgNPs) is not only affordable and eco-friendly but provides a means of synthesizing phytochemical capped AgNPs with predefined characteristics. The objective of this study was the green synthesis of AgNPs that possess antimicrobial and catalytic activities using aqueous extract of *Euphorbia graminea*. Reactions parameters critical to the yield, size and morphology of the biosynthesized AgNPs were optimized using UV spectroscopy. The UV-visible spectra analysis of the biosynthesized AgNPs showed surface plasmon resonance occurred at 462 nm. Scanning Electron Microscopy with Energy dispersive X-ray analysis revealed the characteristic absorption band of AgNPs at 3 KeV and confirmed 73.66% composition of particles as metallic silver. The AgNPs appeared as well-separated, quasi-spherical particles with narrow size distribution of 6.77±0.89 nm when examined with Transmission electron microscopy. X-ray diffraction confirmed the crystallinity of the AgNPs with mean crystallite size of 7.65 nm. The biosynthesized AgNPs showed broad-spectrum antimicrobial activity against bacteria and fungi. The rate constant of the degradation of methylene blue in the presence of as-synthesized AgNPs was increased several folds to sec^{-1} from sec^{-1} in its absence. The prepared AgNPs could find applications as therapeutic coats in medical devices and in effluent treatment of chemical industries.

Keywords: Silver nanoparticles, methylene blue, catalytic activity, antimicrobial activity, *Euphorbia graminea*

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INTRODUCTION

Nanotechnology involves the manufacture, control of characteristics and applications of structures with nanoscale dimensions, typically between 1-100nm [1]. Nanoparticles are broadly of two types, which are the carbon-incorporated organic structures, and the more varied inorganic nanoparticles that include semi-conductor, metallic and magnetic nanoparticles. Metal nanoparticles because of their increased surface-to-volume ratio exhibit significantly altered physical, chemical and biological characteristics. Silver nanoparticles (AgNPs) for example, have substantial surface zone that show improved chemical stability, electrical conductivity, biochemical and catalytic activities when compared with larger-than-nanoscale structures of similar composition [1-2]. Silver nanoparticles have therefore found potential applications as functional units of electronic sensors and optical devices, antimicrobial agents, biological sensors and industrial catalysts [1,3,4]. The catalytic activity of AgNPs have been employed in the degradation of organic dyes used in industries such as the pharmaceutical, food, textile, paper and print [5-6]. Organic dyes when discharged into wastewater without proper or adequate treatment persist in the ecosystem and can constitute, in addition to severe ecological disruption, health hazards to human. Majority of conventional wastewater treatment methods that have been employed such as flocculation, coagulation, electrochemical degradation, precipitation, adsorption and reverse osmosis remain expensive and often lead to secondary pollution because of excessive use of reagents required to destroy the aromatic stability of the dye [6]. Nanocatalysts, which lower the activation energy required for the breakdown of the organic dyes, will be useful in the safe, efficient and affordable disposal of these environmental chemical hazards. In this regard, several studies have demonstrated the potential application of AgNPs in the degradation of synthetic dyes including methylene blue and methyl orange [5,7,8]. Silver nanoparticles are unique in nanoscale system due to the ease in its synthesis and chemical modifications. Chemical reduction of silver ions remains the most viable means of synthesizing AgNPs with the use of a variety of organic and inorganic reducing agents reported in literature [9]. However, the green synthesis of AgNPs using microorganisms and plants is preferred because the approach is eco-friendly, energy saving and easily scalable. In addition, the presence of plants metabolites as capping agents allow the synthesis of nanoparticles with predefined characteristics [4]. Silver nanoparticles have therefore been successfully biosynthesized using microorganisms including *Ganoderma enigma*ticum [10], *Oscillatoria limnetica* [11] as well as parts from plants such as *Phyllanthus emblica* [12], *Cucumis prophetarum* [4], *Salvia spinosa* [13].

Euphorbia graminea Jacq (Euphorbiaceae) is an annual plant in Nigeria measuring 15-30 cm from the base with leaves that are alternate and may be ovate or oblong, elliptic to linear [14]. The plant which has been previously described as a potentially invasive herbaceous plant in Nigeria has been documented to possess antioxidant activity as well as antiproliferative potential against human breast (MCF-7) cancer cell lines [15,16]. Antimicrobial screening of chromatographic fractions of the plant showed mild inhibitory activity against *Staphylococcus aureus*, *E. coli* and *Candida albicans* [14]. The phytochemicals obtained from the plant include alkaloids, anthraquinones, flavonoids, tannins and terpenes. Biomolecules containing hydroxyl and carbonyl functional groups can serve as reducing and stabilizing agents in the synthesis of AgNPs from ions.

The objective of this study was therefore to employ the aqueous extract of the whole plant of *Euphorbia graminea* in the green synthesis of AgNPs that possess antimicrobial and catalytic activities.

METHODOLOGY

Materials

Euphorbia graminea was collected from the botanical garden of the University of Ibadan, Ibadan. A trained botanist authenticated the collected plant sample, and a voucher specimen (UIH-23130) was deposited for future reference. Silver nitrate was sourced from Sigma Aldrich while methylene blue and sodium borohydride were products of BDH UK.

Preparation of *E. graminea* extract

The stem and leaves of *E. graminea* were thoroughly washed with distilled water and then cut into small pieces with the aid of a steel knife. A quantity of the plant parts equal to 40 g was heated with 400 mL of distilled water in a beaker at 60 for 30 minutes [2]. On cooling, the mixture was filtered using Whatman filter paper No.1 and the filtrate stored in amber-colored bottles at 4 until required.

Phytochemical screening of extract *E. graminea*

Preliminary phytochemical screening was carried out on freshly prepared plant extract to determine presence of phenols, tannins, flavonoids, triterpenoids, and alkaloids using standard procedures [2,4].

Synthesis of Ag-NP using aqueous *E. graminea*

The optimized procedure involved the addition of 10 mL of the aqueous extract to 90 mL of 1 mM $AgNO_3$ in a conical flask covered with aluminum foil.

The reaction mixture was then continuously stirred at about 1000 rpm using a magnetic stirrer for 24 hours. The reduction of silver ions to AgNPs was monitored by acquiring UV-visible data of aliquots taken from the reaction mixtures at regular intervals. Preliminary visual check of color change from light green to brownish-red in the reaction mixture was also used to monitor reduction [1].

After completion of the reaction, nanoparticles were purified by first centrifuging the mixture at 15,000 rpm for 5 minutes to obtain pellets. The pellet was re-dispersed in distilled water using a vortex mixer and then centrifuged. Washing with water was done thrice and then with ethanol.

Optimization of $AgNO_3$ concentration

While keeping the ratio of volumes of the extract to silver nitrate solution as 1:9, the effect of the concentration of silver nitrate solution on formation of nanoparticles was investigated by varying its concentration (0.25, 0.5 and 1mM).

Optimization of ratio of volumes of $AgNO_3$ and extract

Extract-to-silver nitrate volume ratios of 1:9; 3:7 and 5:5 were differently employed for nanosynthesis in order to determine the ratio of volumes optimal for nanoparticle formation.

Characterization of synthesized AgNPs

UV-visible spectroscopic data of the reaction mixture were acquired at regular intervals using a Perkin Elmer Lambda 250 Model in the range of 200-800 nm and 1 nm resolution. The functional groups present in the phytoconstituents of the plant that were responsible for the reduction and capping of nanoparticles were analyzed by FT-IR measurements using a Nicolet iS10 FT-IR spectrophotometer. The size and morphology of the AgNPs were determined by Transmission Electron Microscope (NanoMill 1040 model) while the elemental composition was analyzed using scanning electron microscope integrated with energy dispersive X-ray analysis. Dynamic light scattering (DLS) data were acquired at 25°C using Malvern Zetasizer NanoS90 (UK) with a detector set at right angles [2].

The crystallinity of the biosynthesized AgNPs was confirmed by XRD analysis using Cu K alpha radiation set at 40kV and 20mA.

Antimicrobial Assay of phytosynthesized AgNPs

The antimicrobial screening of the phytosynthesized AgNPs was carried out by determining the minimum inhibitory and bactericidal/fungicidal concentrations against seven test microorganisms including: one Gram-positive (*Staphylococcus aureus – ATCC 29213*), five Gram-negative bacteria (*Klebsiella*

pneumoniae – ATCC 7006303, *Clostridium faecalis* – ATCC 8090, *Escherichia coli* - 2348, *Salmonella typhi* – ATCC 14028 and *Pseudomonas aeruginosa* – ATCC 27853) and one fungi (*Candida albicans*). The minimum inhibitory concentration of the as-synthesized AgNPs against the test microorganisms was carried out as described by Mogana et al., 2020[17] with some modifications using broth micro-dilution method in 96-well microtiter plates. Bacteria and fungi stock suspensions were diluted using Mueller Hinton broth and tryptic Soy broth respectively to give 0.5 McFarland standard. To each of the well containing graded concentrations of AgNPs corresponding to 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95 and 0.98 µg/mL, 20 of the standardized microbial suspension were added and incubated at 37°C for 24 hours for bacteria and at 25°C for up to 48 – 72 hours for fungi. A check for microbial growth in each well was done. The minimum bactericidal/fungicidal concentration (MBC/MFC) for the test samples was determined by swabbing broth from the 96-well plates that did not show any visible growth on the surface of freshly prepared Mueller Hinton (for bacteria) and Sabouraud dextrose (for fungi) agar plates by surface spreading method using sterile cotton swabs. The plates were thereafter incubated at 37°C for up to 72 hours. The lowest concentration of the test sample from the MIC assay that that did not show any microbial growth on the agar plates were taken as the MBC/MFC for that organism[10].

Catalytic activity of as-synthesized AgNPs

The biocatalytic efficiency of synthesized AgNPs in the borohydride-reduction of methylene blue was investigated by monitoring the degradation of the dye with time [3,6]. Reaction variables including the concentrations of sodium borohydride, methylene blue and AgNPs were optimized. In the final optimized procedure, 5mL of 10mM methylene blue was added to 2mL of 0.2M sodium borohydride and the volume made up to 50 mL with distilled water after which 10 mg AgNPs was added. The absorption spectra of aliquots periodically drawn from the reaction mixture was then acquired. A blank set up without the nanocatalyst was similarly prepared and analyzed.

RESULTS AND DISCUSSION

Phytochemical Analysis

The phytochemical analysis of the plant extract revealed the presence of several phytoconstituents as shown in Table 1. Similar phytochemical composition of the plant has been previously reported [14]. These secondary metabolites mediate the reduction of silver ions and capping of resultant AgNPs.

Table 1. Phytochemical constituents of aqueous extract of *E. graminea*

Secondary metabolite	Quantitative presence
Tannins	+
Flavonoids	+
Cardiac glycosides	++
Saponins	++
Terpenoids	++
Phenol	+
Alkaloids	++

Synthesis and optimization of reaction variables

The bioreduction of silver ions to AgNPs was associated with a color change from greenish yellow of the plant extract to deep red as the AgNPs were formed. The color of colloidal solutions of AgNPs have been reported to vary depending on their particle size and morphology [18]. This in turn often depends on the conditions of synthesis. UV spectroscopy was therefore utilized to monitor biosynthesis as AgNPs can interact with light of specific wavelengths thereby causing the conduction electrons on the surface of the metal to collectively oscillate in a phenomenon known as surface plasmon resonance. As depicted in Figure 1, the surface plasmon resonance of AgNPs biosynthesized using aqueous extract of *E. graminea* occurred at 462 nm, suggestive of a particle size of about 70 nm [18]. However, the actual particle size will be determined using TEM and XRD analyses. The use of 1mM silver nitrate solution was critical to formation of small-sized AgNPs in high yields as the use of lower concentrations resulted either in lower yields or bathochromic shifts of the surface plasmon resonance that is indicative of the formation of larger-sized nanoparticles.

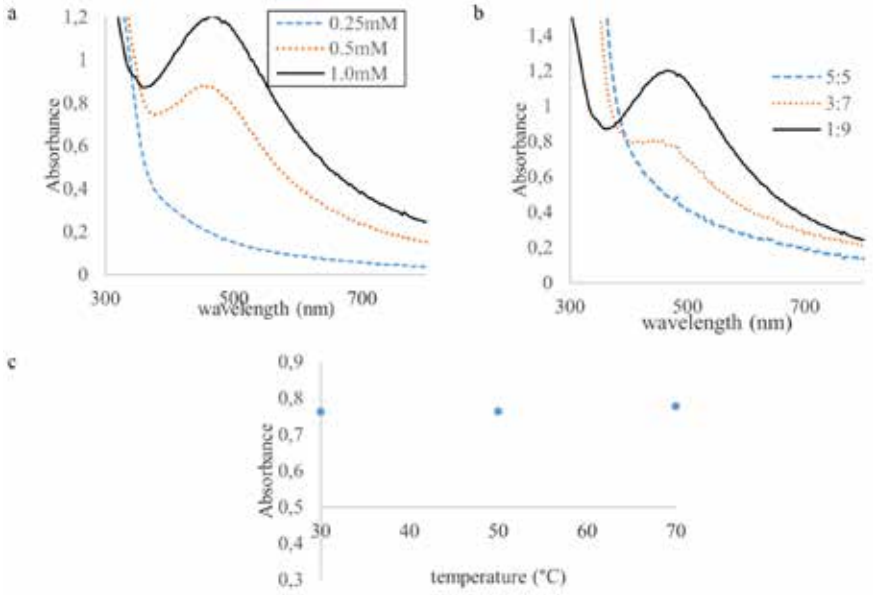

Figure 1. Optimization of critical reaction parameters including (a) concentration of $AgNO_3$ (b) extract-to-$AgNO_3$ volume ratios and (c) temperature

Similarly, an extract-to-silver nitrate volume ratio of 1:9 was found optimal in the biosynthesis of smaller-sized AgNPs in high yields as shown in Figure 1b. As depicted in Figure 1c, no advantage, as regard the particle size or yields, were observed when the reaction was conducted at higher temperatures and as such 30 was adopted for the synthesis.

Characterization of biosynthesized AgNPs

FT-IR Measurement

FTIR analysis was carried out to identify the likely phytochemicals involved in the bioreduction of silver ions to Ag and capping of the AgNPs biosynthesized from the aqueous extract of *E. graminea*. The FTIR spectra of the AgNPs is depicted in Figure 2 below.

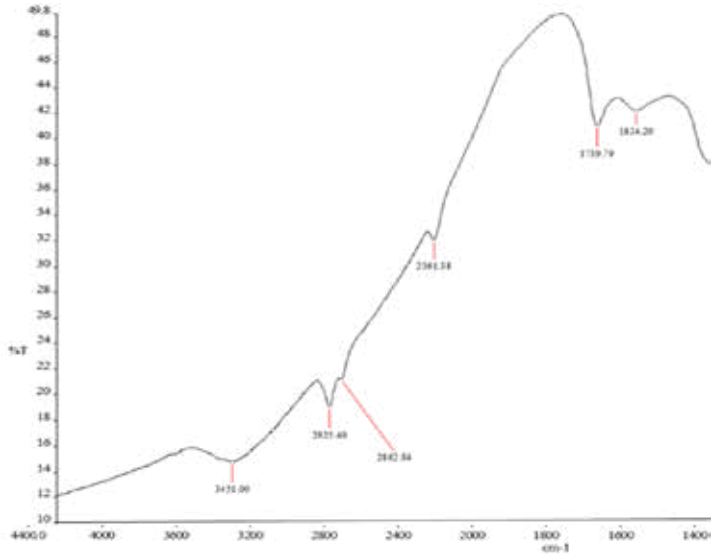

Figure 2. FTIR spectrum of biosynthesized AgNPs using *E. graminea* aqueous extract

Broad and strong peaks at 3451cm^{-1} can be attributed to the O-H stretch of phenolic compounds. This could also be attributed to hydrogen-bonded O-H stretch of alcohols or N-H stretch of amine salts. The bands at 2925.40 and 1634.20 cm^{-1} have been assigned to C-H stretching and bending vibrations respectively of conjugated alkenes. The latter frequency could also designate N-H bending vibrations of amines or their salts which characteristically are found between 1650 and 1580 cm^{-1} depending on their chemical environment[5]. The medium bands at 2862.84 cm^{-1} corresponds to C-H stretch frequencies in sp^3 hybridized hydrocarbons. Thus, the biosynthesized AgNPs are surrounded by metabolites such as terpenoids and flavonoids with functional groups of alcohols, alkanes, alkenes, and amines. These functional residues showed the capability to stabilize and prevent further agglomeration of biosynthesized AgNPs [19].

SEM and EDX analyses

When the green-synthesized nanoparticles were examined using SEM, they appeared as highly aggregated polymorphs with shapes mostly irregularly granulated as shown in Figure 3a. Similar results were obtained with the biosynthesis of AgNPs using *Cucumis prophetarum* [4].

The characterization of the biosynthesized AgNPs by EDX analysis revealed the presence of the characteristic peak of elemental silver as depicted in Figure 3b. The as-synthesized AgNPs displayed an absorption band at 3KeV which is typical of metallic silver nanoparticles as a result of their surface plasmon resonance[20].

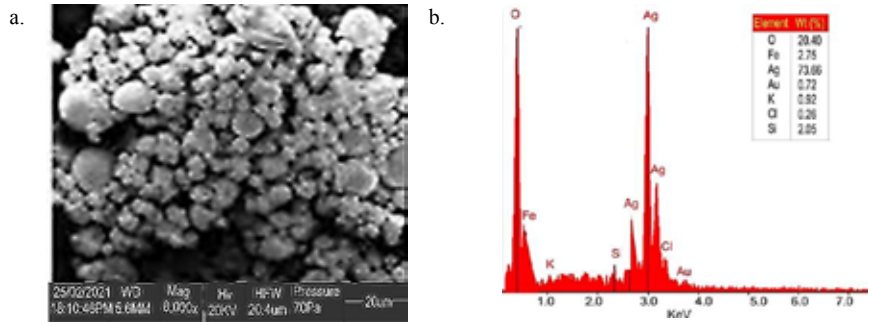

Figure 3. SEM (a) and EDX (b) Analyses of AgNPs biosynthesized using *E. graminea*

The elemental analysis of the biosynthesized AgNPs revealed the predominance (73.66%) of elemental silver particles confirming the high efficiency of *E. graminea* as a reductant of silver ions. In addition to silver, other elements including oxygen, iron, silicon etc. were also present.

Determination of AgNPs size and morphology by TEM

The TEM micrograph as depicted in Figure 4 revealed well-separated, quasi-spherical shaped AgNPs. The average particle size of the AgNPs as determined by TEM was 6.77±0.89 nm. When compared with previously reported AgNPs size ranges of 12-36, 11-83 and 10-180 nm that were obtained using *Ficus panda*, *Terminalia mentaly* and *Clinacanthus nutans* respectively, our new method successfully produced small sized nanoparticles with narrower size distribution [2,19,20].

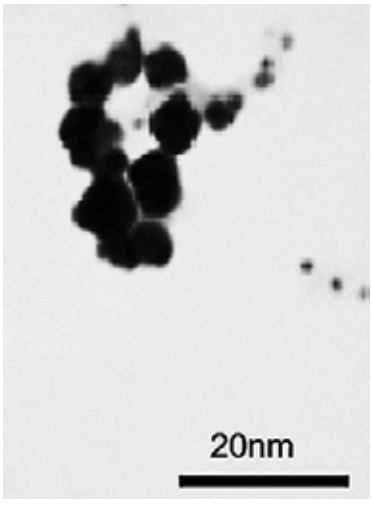

Figure 4. Transmission electron microscopy of biosynthesized AgNPs

Dynamic light scattering analysis

Dynamic light scattering (DLS) measures the correlation coefficient of intensity functions which can be mathematically converted to intensity, volume, or number size distribution [21-22]. The technique is very useful for estimating particle size via the analysis of the modulation of the intensity of laser light passing through a colloidal solution. The particle size distribution of the biosynthesized nanoparticles was therefore determined using DLS with the size distribution by intensity depicted in Figure 5. The DLS data showed that the Z-average size of the particles was 43.14 nm with a polydispersity index of 0.467. The polydispersity index (values 0 to 1) describes the width of particle size distribution with scale ranging from monodisperse to polydisperse particles. Monodispersity arises from zeta potential values that are more negative than -30 mV or more positive than 30 mV and is thus indicative of nanoparticles colloid stability [4,21]. The particle size estimated by DLS was expectedly larger than the nominal size by TEM[23]. This is because DLS measure the hydrodynamic size which is dependent on particle morphology. In particular, intensity-sized distribution can be strongly influenced by the presence of a few large particles, aggregates or dust[24]. In addition, the hydration layer around the nanoparticles and the phytochemicals of the extract may contribute to the hydrodynamic size [4].

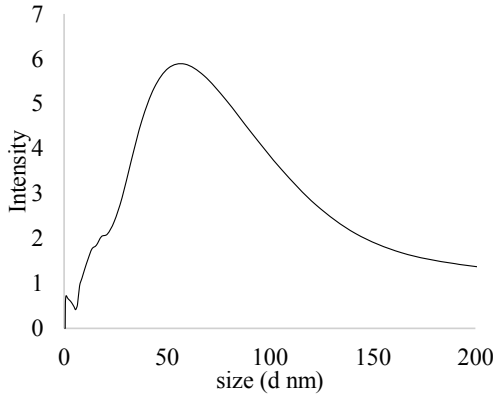

Figure 5. Size distribution of biosynthesized AgNPs by intensity

Crystallinity of AgNPs using XRD

As depicted in Figure 6, the XRD pattern of the biosynthesized AgNPs revealed four intense peaks at 2= 38.21, 44.43, 64.79 and 77.86 which corresponds to (111), (200), (220) and (311) lattice planes. The observed lattice planes were indexed based on the face centered cubic structure of silver as found in standard

data file JCPDS No. 04-0783. The XRD pattern thus clearly confirmed the crystallinity of the AgNPs synthesized in this study. The maximum intensity was observed with the (111) plane indicating the AgNPs were predominantly distributed in the (111) plane.

The average crystallite size of the AgNPs was calculated using the Debye-Scherrer formula below

$$d = \frac{0.9\lambda}{\beta cos\theta} \qquad (1)$$

where, d is the mean crystallite diameter, is the X-ray wavelength,
The mean crystallite size of the silver nanoparticles was determined to be 7.65 nm.

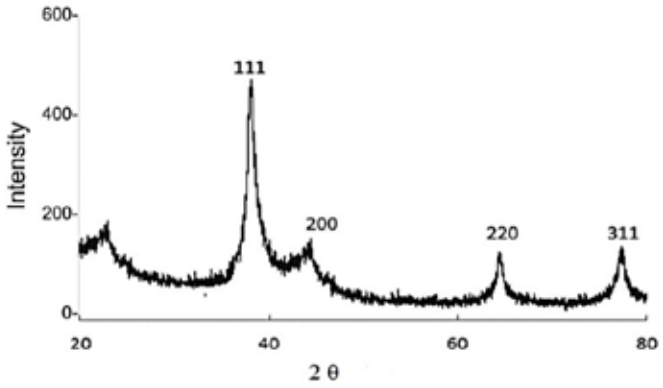

Figure 6. XRD pattern of biosynthesized AgNPs

Antimicrobial assay

The antimicrobial activities of the green-synthesized AgNPs against the various test organisms are shown in Table 2. The AgNPs exhibited broad-spectrum activity against both Gram positive and negative bacteria as well as fungi.

Table 2. Minimum Inhibitory and bactericidal/fungicidal concentrations of green-synthesized AgNPs against test organisms

Test organisms	Minimum Inhibitory Concentration (µg/mL)	Minimum Bactericidal/ Fungicidal Concentration (µg/mL)
Klebsiella pneumonia – ATCC 7006303	125	250
Pseudomonas aeruginosa – ATCC 27853	31.25	500
Staphylococcus aureus – ATCC 29213	125	125
Escherichia coli - 2348	31.25	125
Salmonella typhi – ATCC 14028	62.5	125
Clostridium faecalis – ATCC 8090	31.25	250
Candida albicans	62.5	>500

The antimicrobial activity of the biogenic AgNPs were more pronounced against Gram-negative bacteria including *P. aeruginosa*, *E. coli* and *C. faecalis* (MIC 31.25 /mL) than Gram-positive *S. aureus* with MIC of 125 µg/mL. This pattern can be attributed to the mechanism of antibacterial activity of AgNPs that involves the electrostatic attraction and transfer of silver ions to the negatively charged bacteria cell wall leading to a change in its composition and permeability [25]. The combined effect of the subsequent loss in the ability of the organism's DNA to replicate and the inactivation of enzymes required for ATP production will eventually lead to bacterial death. Gram-negative bacteria with a single layer of peptidoglycan in their cell wall membrane are therefore more susceptible to the inactivating action of silver ions than Gram-positive bacteria with multiple layered peptidoglycan cell membrane [4]. Many previous works have only reported inhibitory activity of biosynthesized AgNPs against only two to four microorganisms. In contrast, the as-synthesized AgNPs reported in this study exhibited a broader range of activity against microorganisms including fungi. A survey of the MIC values reported for AgNPs synthesized using different plant extracts against the more commonly tested *S. aureus* and *P aeruginosa* is depicted in Table 3. The results showed the considerable inhibitory effect of AgNPs synthesized using *E. graminea* and the potential of the nanoparticles for future antibacterial applications.

Table 3. A comparison of the antibacterial activity of different plant-mediated AgNPs

Plant used	Mean particle size	MIC		Reference
		S. aureus	*P. aeruginosa*	
Camellia sinensis	3.9±1.6	250	30	26
Crocus sativus	12-20	No inhibition	250	27
Crocus Haussknechtii Bois	10-25	26.9	20.2	28
Terminalia mentaly	11-83	12.5	Not reported	20
Euphorbia graminea	6.77±0.89	125	31.25	This study

AgNP-catalysed degradation of methylene blue

The biocatalytic activity of as-synthesized AgNPs in the redox reaction between methylene blue and borohydride ions was also investigated. The progressive degradation of methylene blue characterized by loss of its intense blue color was monitored by acquisition of the UV –visible spectra data of the reaction mixture at regular time intervals. The changes in the UV-visible spectra of methylene blue in the presence and absence of the nanocatalyst are depicted in Figure 7a and 7b respectively. Both reactions fitted a pseudo first order kinetics model as shown in Figure 7c.

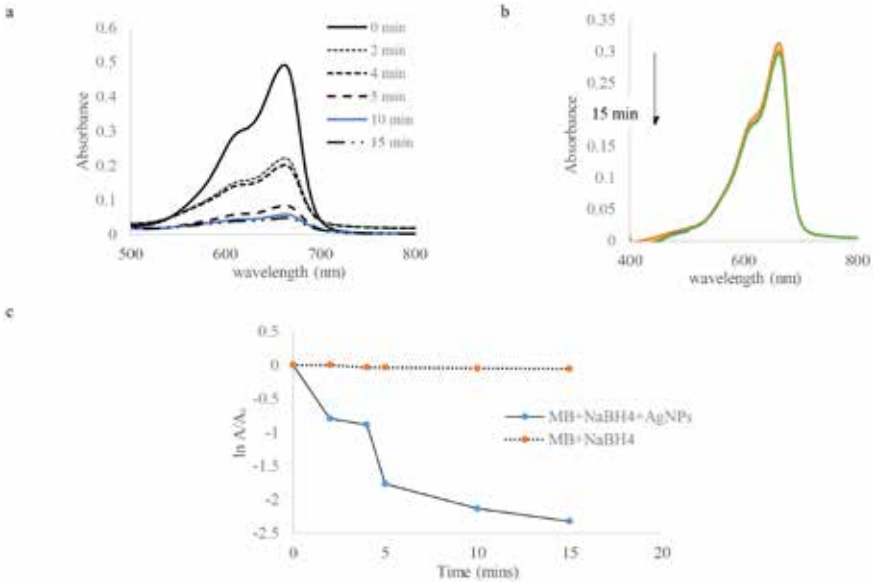

Figure 7. Successive acquisition of UV-visible spectra at specified time intervals showing reduction of methylene blue by NABH4 at ambient temperature: (a) with nanocatalyst, (b) without nanocatalyst and (c) plots of against time

Rate constants were estimated from the regression of relative absorbances of the dye with time. The rate constant of the degradation of methylene blue in the presence of nanocatalyst was sec^{-1} compared to sec^{-1} in the absence of the nanocatalyst. The rate of degradation of the dye was therefore several folds faster in the presence of nanocatalyst with more than 90% degradation achieved within 15 minutes. In contrast, less than 6% degradation was achieved in that time without the inclusion of AgNPs as catalyst.

Degradation of methylene blue requires an electron transfer from borohydride ions to the dye. The nanocatalyst serve as an intermediate in the electron transfer process between the acceptor and donor molecules [19]. The mechanism of catalysis by AgNPs therefore involve an improvement in the efficiency of electron transfer as well as the lowering of the bond dissociation energy during the chemical reaction between the dye and borohydride ions that is required for breaking/forming of bonds. As depicted in Table 4, when compared with other studies, AgNPs biosynthesized using *E. graminea* exhibited excellent catalytic efficiency. The enhanced catalytic efficiency can be attributed to the nanoparticles' quasi-spherical shape and small size that have been reported to improve catalytic efficiency. Similarly, the presence of hetero atoms such as oxygen, which is the next most abundant element (20.40% in EDX analysis) in the phytochemical-capped nanoparticles, have been reported to improve the access of the dye molecules to the catalytic sites leading to accelerated degradation [6].

Table 4. A comparison of catalytic efficiency of biosynthesized AgNPs

Plant used	Mean particle size (nm)	Rate constant	Time of degradation (at least 90%)	Reference
Alstonia scholaris		50 0.0007 sec^{-1} Not specified		29
Caulerpa racemosa	25	0.0011 sec^{-1}	30 min	30
Imperata cylindrica	31	0.137 min^{-1}	14 min	31
Euphorbia graminea	6.8	0.0025 sec^{-1}	15 min	This study

In conclusion, a facile, eco-friendly process involving the use of aqueous extract of *E. graminea* for the green synthesis of stable silver nanoparticles has been developed. The nanoparticles were crystalline, predominantly quasi-spherical shaped and with an average particle size of 6.77±0.89 nm. The prepared AgNPs showed good catalytic activity in the sodium borohydride-degradation of meth-

ylene blue as well broad-spectrum antimicrobial activity against bacteria and fungi. The nanoparticles can therefore find useful applications in the effluent treatment of chemical industries and as therapeutic coats in medical devices.

STATEMENT OF ETHICS

Not applicable as no human or animal subjects were involved in the study.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest associated with this study.

AUTHOR CONTRIBUTIONS

OET was involved in study design, data collection, analysis, and preparation of manuscript draft. OSA and PEO were involved in data collection and analysis. All authors contributed to the revision of the draft, read, and approved the final manuscript.

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Syzygium aromaticum essential oil ameliorates levofloxacin-induced hepatic injury in rats: antioxidant, biomarker and histopathological analysis

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ABSTRACT

Prolonged levofloxacin treatment can trigger hepatotoxicity. This study aimed to examine *Syzygium aromaticum* L. essential oil effect on levofloxacin-induced hepatic injury in rats**.** The chemical constituents and radical scavenging activity were also examined. Rats (n=30) were grouped to receive per oral administration of either placebo, levofloxacin (93 mg/kg), *S. aromaticum* L. oil (10, 25, or 50 mg/kg), or curcuma (6 mg/kg) with levofloxacin for 28 days. Serum liver biomarkers, liver malondialdehyde (MDA) levels, and histopathological changes were analyzed. *S. aromaticum* L. oil contained eugenol (63.63%) and the IC_{50} was 35.1 µg/mL. Levofloxacin induced significant increases in serum biomarker and tissue MDA levels ($p < 0.05$), with significant damage in hepatocytes. The oil treatment (10 mg/kg) reduced the levels of alanine aminotransferase, total bilirubin, lactate dehydrogenase, tissue MDA ($p < 0.05$), and the hepatic injury. In conclusion, *S. aromaticum* L. oil alleviated levofloxacin-induced liver injury, possibly related to its eugenol content and radical scavenging activity.

Keywords: Clove essential oil, hepatotoxicity, levofloxacin, *Syzygium aromaticum* L.

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INTRODUCTION

Levofloxacin is a broad-spectrum antibiotic that clinicians widely prescribe to treat various infections. Moreover, it is commonly used in the second-line anti-tuberculosis (AT) regimen in patients who are intolerant to first-line AT agents, have latent tuberculosis infection (LTBI), or multidrug-resistant tuberculosis (MDR TB), for up to 24 months [1, 2]. Levofloxacin is considered well-tolerated; however, there is evidence that levofloxacin treatment can trigger hepatotoxicity [3]. Although unusual, fatality can result [4]. The prolonged use of levofloxacin in TB patients has raised concerns about the increased risk of levofloxacin-induced hepatotoxicity [5]. Studies on the use of levofloxacin regimen have shown hepatotoxicity in 9.7% to 14.3% of MDR TB patients [6, 7].

Even though the exact mechanism of levofloxacin-induced liver injury remains elusive, many believe it relates to the increased release of intracellular reactive oxygen species (ROS) that leads to mitochondrial damage [8]. The alteration of mitochondrial electron transport chain activity may also result in ROS accumulation [9]. Since oxidative stress is involved in the pathogenesis of levofloxacin-induced liver injury, using antioxidants may relieve the damage.

Antioxidants obtained from natural sources might serve as alternatives to standard antioxidants. These antioxidant compounds are abundant in essential oils from spices or herbs [10]. *Syzygium aromaticum* L. (clove), a member of the Myrtaceae family native to eastern Indonesia [11], is one of the most valuable kitchen spices and has been used in folk medicines for centuries. Its essential oil has demonstrated very potent antioxidant activity comparable to, and even superior to, butylated hydroxytoluene (BHT), a synthetic antioxidant [12, 13]. The main active compound in *S. aromaticum* L. oil, eugenol, has demonstrated a protective effect in induced hepatotoxicity in many animal models [14, 15]. The purpose of this study is to examine the potential use of *S. aromaticum* L. essential oil as an antioxidant to reduce levofloxacin-induced hepatic injury in rats.

METHODOLOGY

Drugs and chemicals

Diethyl ether, formaldehyde 10%, sodium carboxymethyl cellulose (Na CMC), methanol, hematoxylin, and eosin were purchased from a registered chemical store in Makassar, Indonesia. Levofloxacin tablets (Hexpharm Jaya®) and Curcuma tablets (Soho®) were purchased from a licensed pharmaceutical store in Makassar, Indonesia. 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 1,1,3,3-tetramethoxypropane (TMP) were purchased from Sigma Aldrich. Diagnostic kits for aspartate aminotransferase (AST), alanine aminotransferase (ALT),

total bilirubin, lactate dehydrogenase (LDH) were purchased from Human Diagnostics Worldwide (Magdeburg, Germany).

The essential oil was purchased from Happy Green®, Indonesia, batch No. 20171526-08-23). This oil was obtained from flower buds of *Syzygium aromaticum* L. plants from Indonesia using steam distillation. The oil is a dark, clear liquid and *S. aromaticum* L. bud odor, with a certificate of analysis from the manufacturer showing a specific gravity (20°C) of 1.0639, a refractive index (20°C) of 1.5217, and a density (20°C) of 1.0620.

Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS analysis was performed to obtain the profiles of chemical constituents in the *S. aromaticum* L. essential oil by using the Trace 1310 GC coupled with TSQ 8000 Evo MS (Thermo Scientific, USA). The column used was TG-5MS with a size of 20 m x 0.18 mm. The oven had an initial temperature of 50°C that increased to 150°C at a rate of 25°C/min, then to 230°C at a rate of 15°C/min, and finally to 330°C at the rate of 10°C/min. The holding time was 5 min. The sample volume was 1 µL, and helium was used as the carrier gas.

Free radical scavenging assay

The free radical scavenging activity was examined using a 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method as described in a previous study[16]. DPPH reagent (5 mg) was dissolved in 100 mL of methanol to obtain a DPPH concentration of 50 µg/mL. The *S. aromaticum* L. oil solution was prepared in methanol with a series of concentrations: 10, 20, 30, 40, and 50 µg/mL. Each concentration of *S. aromaticum* L. oil was pipetted (1 mL) and mixed with DPPH solution to a total volume of 5 mL in the measuring flask. The prepared mixtures were incubated at room temperature for 30 minutes (mins), and the absorbances were measured with a UV spectrophotometer (Shimadzu, Japan) at a wavelength of 517 nm. The same treatment was carried out on the control solution (4 mL of DPPH solution and 1 mL of methanol). All sample measurements were carried out in triplicate. The percentage of inhibition was calculated using the equation:

$$\% \text{ Inhibition} = \frac{A_1 - A_2}{A_2} \times 100\%$$

A_1: the absorbance of the control
A_2: the absorbance of samples

The value of IC_{50} (inhibitory concentration at 50%) was obtained using a linear regression equation between sample concentration (x) and % inhibition (y).

Animal preparation

Albino Wistar rats (200–250 g) were procured from a rodent breeding house in Yogyakarta, Indonesia. Prior to the experiment, the rats were acclimatized for 14 days to adapt to the laboratory environmental conditions (25°C with a 12-hour light/dark cycle). Rats were provided with regular food pellets and water *ad libitum*.

Evaluation of hepatoprotective effects

Thirty male Wistar rats were assigned to one of the six groups. The control group only received per oral (p.o) administration of a placebo (1% Na CMC suspension), while the LFX group received only p.o administration of levofloxacin. The *S. aromaticum* L. (clove oil, CO) groups (CO 10 + LFX, CO 25 + LFX, and CO 50 + LFX) received p.o administration of *S. aromaticum* L. oil at a dose of 10, 25, or 50 mg/kg, respectively, followed by levofloxacin administration with a 2-hour interval. Another group received curcuma (Curcuma + LFX) as a comparison to *S. aromaticum* L. oil. Curcuma was applied in this study as the comparison treatment since it has been used in a clinical setting to treat patients with hepatotoxicity[17].

Levofloxacin and curcuma tablets were prepared in 1% Na CMC suspension, while the *S. aromaticum* L. oil was diluted in corn oil to facilitate oral administration. Levofloxacin and curcuma doses were chosen based on human daily doses (93 mg/kg for levofloxacin and 6 mg/kg for curcuma) as explained in Nair and Jacob[18]. The dose of *S. aromaticum* L. oil was chosen based on the pilot study, where three daily doses were compared (10 mg/kg, 50 mg/kg and 100 mg/kg). From the pilot study, it was found the 10 mg/kg dose had the most efficacy in preventing hepatotoxicity (unpublished data). Therefore, in this present study, 10 mg/kg, 25 mg/kg and 50 mg/kg were used. The treatments were administered for 28 days. The rats were sacrificed 24 hours after the last treatment using diethyl ether and cervical dislocation. A laparotomy was performed, and the liver was harvested.

Analysis of serum biomarkers

Before and after 28 days of treatments, the blood samples (3 mL) were collected from the lateral saphenous veins of the rat using vacuum tubes containing an anticoagulant. This method allows rapid blood sampling and the site of puncture can be repetitively used to withdraw blood if needed[19]. All animals were anesthetized with diethyl ether prior to blood withdrawal. Blood samples were centrifuged at 3000 rpm for 15 mins to separate serum and blood cells. The serum was analyzed spectrophotometrically (Humalyzer 3500) using reagent kits for AST, ALT, total bilirubin, and LDH (Human Diagnostics Worldwide, Germany). All protocols were performed according to the manufacturer's instructions.

Analysis of liver malondialdehyde

Malondialdehyde (MDA) was analyzed using a thiobarbituric acid reactive substance (TBARS) method as explained in a previous study[20]. 1,1,3,3-tetramethoxypropane (TMP) was used to obtain the standard curve. The liver tissues were weighed (400 mg), homogenized with 2 mL of phosphate-buffered saline (PBS), and then centrifuged at 3000 rpm for 10 mins. The supernatant (0.5 mL) was mixed with 1 mL of 1% thiobarbituric acid and 1 mL of 10% trichloroacetic acid. The mixture was heated at 90°C in a water bath for 10 mins before centrifugation to remove precipitation. The absorbance of the supernatant was measured using a spectrophotometer (Agilent, USA) at 531 nm. The MDA concentration of the samples was obtained by plotting the absorbances against the standard curve, multiplied by the dilution factor. The final concentration of liver MDA was expressed as ng/mL.

Examination of liver histopathology

Once harvested, the liver tissues were rinsed in normal saline and immersed in 10% formaldehyde for 48 hours. The liver tissues were then processed as described in previous studies[21, 22], and stained with hematoxylin-eosin. The histopathological analysis was conducted by a forensic pathologist blinded to the treatments using a light microscope coupled to a camera (Olympus).

Statistical analysis

The data (presented as mean ± SEM) were analyzed for normal distribution using the one-sample Kolmogorov-Smirnov test. If the data were normally distributed ($p > 0.05$), a one-way ANOVA analysis was performed, followed by Tukey's post hoc test to determine any significant differences among groups. A significant difference was indicated by a p-value < 0.05.

RESULTS AND DISCUSSION

We used *S. aromaticum* L. oil as a protective agent to alleviate levofloxacin hepatotoxicity. Previously, hepatotoxicity-induced levofloxacin has been demonstrated in rats [20], which shown intense degeneration of hepatocytes accompanied by elevation of serum ALT, AST and GGT levels.

Chemical composition and free radical scavenging activity of *S. aromaticum* L. oil

The acceptable daily human intake of *S. aromaticum* L. or clove oil, used in food additives and medicines for centuries, has been established by the World Health Organization (WHO) at 2.5 mg/kg of body weight [13].

The quality of *S. aromaticum* L. oil is determined by the content of its phenolic compounds, especially eugenol as the main constituent. Based on the GC-MS analysis (Table 1), the *S. aromaticum* L. oil had 23 constituents with 4 major compounds: eugenol (63.63%), 3-allyl-6-methoxyphenyl acetate (20.53%), caryophyllene oxide (4.75%) and phenol,2-methoxy-3-(2-propenyl)-acetate or eugenol acetate (2.44%).

Table 1. The chemical compounds of the *S. aromaticum* L. oil based on GC-MS analysis

No	Retention Time	Molecular Formula	Compound Name**	%Area
1	4.56	$C_8H_{14}O$	5-Hepten-2-one, 6-methyl-	0.10
2	4.93	$C_{10}H_{16}$	D-Limonene	0.11
3	5.92	$C_9H_{10}O_2$	Benzoic acid, ethyl ester	0.10
4	6.51	$C_9H_{10}O$	Phenol,4-(2-propenyl)-	1.41
5	6.12	$C_8H_8O_3$	Methyl salicylate	1.74
6	7.08	$C_7H_{12}O_5$	Glycerol 1,2-diacetate	0.80
7	7.62	$C_{10}H_{12}O_2$	Eugenol	63.63
8	8.54	$C_{12}H_{14}O_3$	3-Allyl-6-methoxyphenyl acetate	20.53
9	9.06	$C_{15}H_{24}O$	Caryophyllene oxide	4.75
10	9.99	$C_{10}H_{12}O_3$	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	0.10
11	10.39	$C_{15}H_{24}O$	Caryophyllene oxide	0.12
12	10.67	$C_{15}H_{24}O$	Isoaromadendrene epoxide	0.28
13	11.10	$C_{15}H_{26}O_2$	4,4,8-Trimethyltricyclo[6.3.1.0(1,5)]dodecane-2,9-diol	0.39
14	11.24	$C_{11}H_{18}O_6$	1,3-Dioxolane-2,2-diacetic acid, diethyl ester	0.27
15	12.63	$C_{21}H_{36}O_4$	9,12,15-Octadecatrienole acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	0.11
16	15.55	$C_{11}H_{16}O$	3-Methyl-2-pent-2-enyl-cyelopent-2-enone	1.16
17	15.41	$C_{19}H_{24}$	Benzene, 1,2,4,5-tetramethyl-3-(3-phenylpropyl)-	0.16
18	15.88	$C_{10}H_{12}O_2$	Phenol,2-methoxy-6-(1-propenyl)-	0.10
19	16.17	$C_{15}H_{20}O_3$	Butanoic acid,2-methyl-2-methoxy-4-(2-propenyl) phenyl ester	0.19
20	17.20	$C_{12}H_{14}O_3$	Phenol,2-methoxy-4-(2-propenyl)-, acetate	0.91
21	17.74	$C_{12}H_{14}O_3$	Phenol,2-methoxy-3-(2-propenyl)-acetate	2.44
22	18.91	$C_{27}H_{54}O_4Si_2$	1-Monolinoleoyglycerol trimethylsilyl ether	0.05
23	19.20	$C_{27}H_{54}O_4Si_2$	1-Monolinoleoyglycerol trimethylsilyl ether	0.09
		Total		**100**

Radical scavenging activity was determined to evaluate the ability of *S. aromaticum* L. oil to halt free radical reactions in biological systems. In the DPPH assay, *S. aromaticum* L. oil reduced the radical DPPH to diphenyl-picrylhydrazine, as demonstrated by the yellow color in the solution. At a concentration of 10 to 50 µg/mL, *S. aromaticum* L. oil demonstrated a powerful reducing capacity with an IC_{50} of 35.1 µg/mL (R^2 = 0.9963) (Figure 1). This value suggests a potent radical scavenging capacity, which can protect liver cells against the damaging effects of free radicals and oxidative reactions.

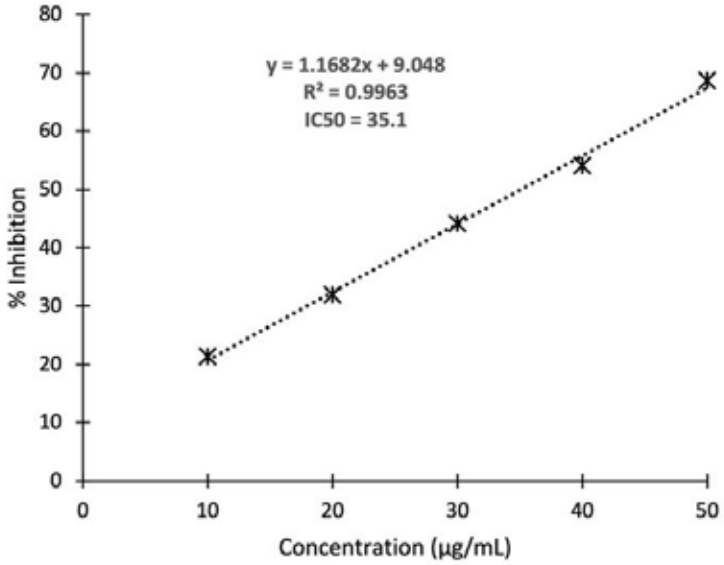

Figure 1. The dose-response curve of free radical scavenging activity of the *S. aromaticum* L. oil using 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) assay

The antioxidant activity of *S. aromaticum* L. essential oil is mainly derived from its eugenol content, but other polyphenols may also contribute [23]. Eugenol is a very potent antioxidant and can reduce ROS production and significantly improve oxidative stress [24]. It is believed that the antioxidant activity of eugenol strongly contributes to its pharmacological effects, such as anti-inflammatory, analgesic, anticancer, and anti-dyslipidemia [25, 26]. Apart from eugenol, three other major components were found in *S. aromaticum* L. oil: 3-allyl-6-methoxyphenyl acetate, caryophyllene oxide, and eugenol acetate. These compounds are also known to have pharmacological activities. A study by Kamadatu and Santoso shows that 3-allyl-6-methoxyphenyl acetate possesses a potent cytotoxicity against human breast cancer cell line [27]. Caryophyllene oxide has also been found to elicit anticancer and analgesic activities [28]. Whereas, in sepa-

rate studies, eugenol acetate and caryophyllene have been reported to possess nematocidal and antifungal properties [29, 30].

Hepatoprotective effect of *S. aromaticum* L. oil

The results of the liver function test and liver MDA analysis are shown in Figure 2. Significant increases in the levels of ALT (77.9 ± 8.4 U/L), AST (245.6 ± 21.4 U/L), total bilirubin (1.32 ± 0.15 mg/dl), and LDH (841.8 ± 97.4 mg/dl) were observed in the group treated with levofloxacin (LFX) for 28 days compared to the controls ($p < 0.05$). The *S. aromaticum* L. oil pre-treatment, especially at the dose of 10 mg/kg, significantly reduced the elevation of (53.2 ± 2.2 U/L), total bilirubin (0.35 ± 0.11 mg/dl), and LDH (440.7 ± 69.2 mg/dl) levels ($p < 0.05$), although the AST level (214.2 ± 10.0 U/L) was not significantly lowered. However, the reduction of ALT, bilirubin, and LDH were not observed with *S. aromaticum* L. oil treatment at the higher doses (25 mg/kg and 50 mg/kg). Nevertheless, the liver MDA levels in all *S. aromaticum* L. oil groups improved compared to the LFX group ($p < 0.05$). As a comparison, curcuma pre-treatment was also found to significantly reduce the total bilirubin, LDH, and liver MDA levels in LFX-treated rats ($p < 0.05$).

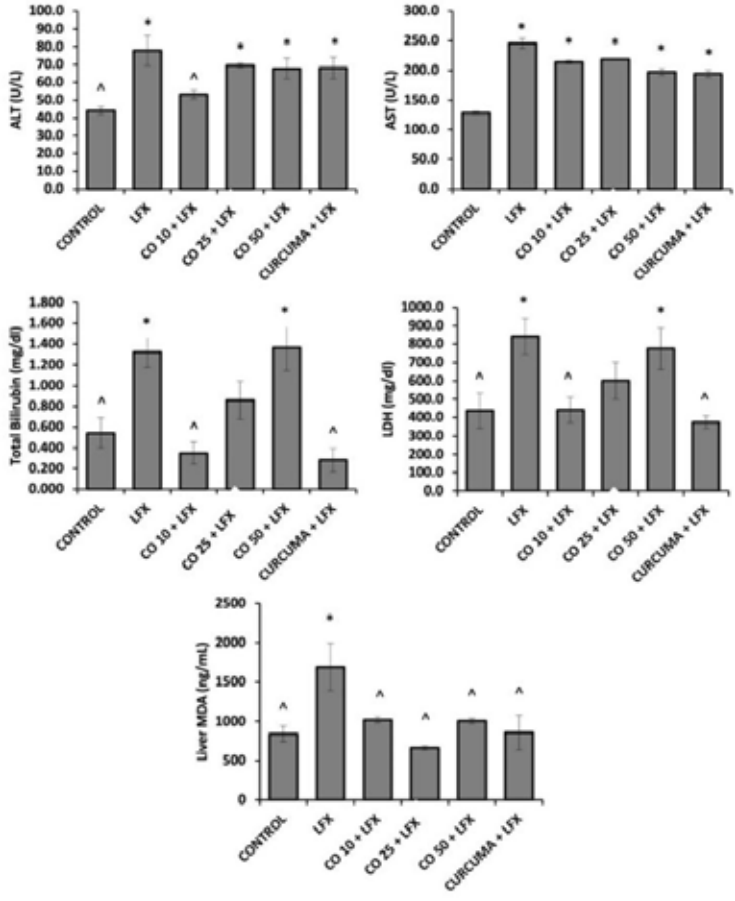

Figure 2. The levels of liver injury biomarkers following 28 days of different treatment administration in rats. A. Alanine aminotransferase (ALT), B. Aspartate aminotransferase (AST), C. Total bilirubin, D. Lactate dehydrogenase (LDH), and E. Liver malondialdehyde. (MDA). LFX: levofloxacin; CO: clove oil. *p<0.05 compared to the control group; ^p<0.05 compared to the LFX group

Photomicrographs of rat liver histology are shown in Figure 3. The liver histology of the control tissues demonstrates a normal architecture of hepatocyte lobules with portal triads at the vertices. The sinusoidal space was clear and sometimes contained Kupffer cells and blood cells. The hepatocytes had eosinophilic cytoplasm and clear nuclei containing nucleoli (Figure 3a). In contrast, the LFX-treated rats had significant histopathological changes in their liver tissues. Congestion of hepatic veins and hyperplasia of the bile ducts were evident (Figure 3b), along with infiltration of inflammatory cells (Figure 3c). Some areas of hepatocyte lobules experienced both hydropic and lipid degen-

eration (Figure 3d). Curcuma treatment led to improved liver histology (Figure 3e) similar to that of tissues treated with *S. aromaticum* L. oil at 10 mg/kg dose (Figure 3f). Pre-treatment with *S. aromaticum* L. oil at higher doses also led to improved hepatocyte structures, although some liver sections still presented hepatic degeneration and an increased number of Kupffer and inflammatory cells in the sinusoid (Figure 3g, h).

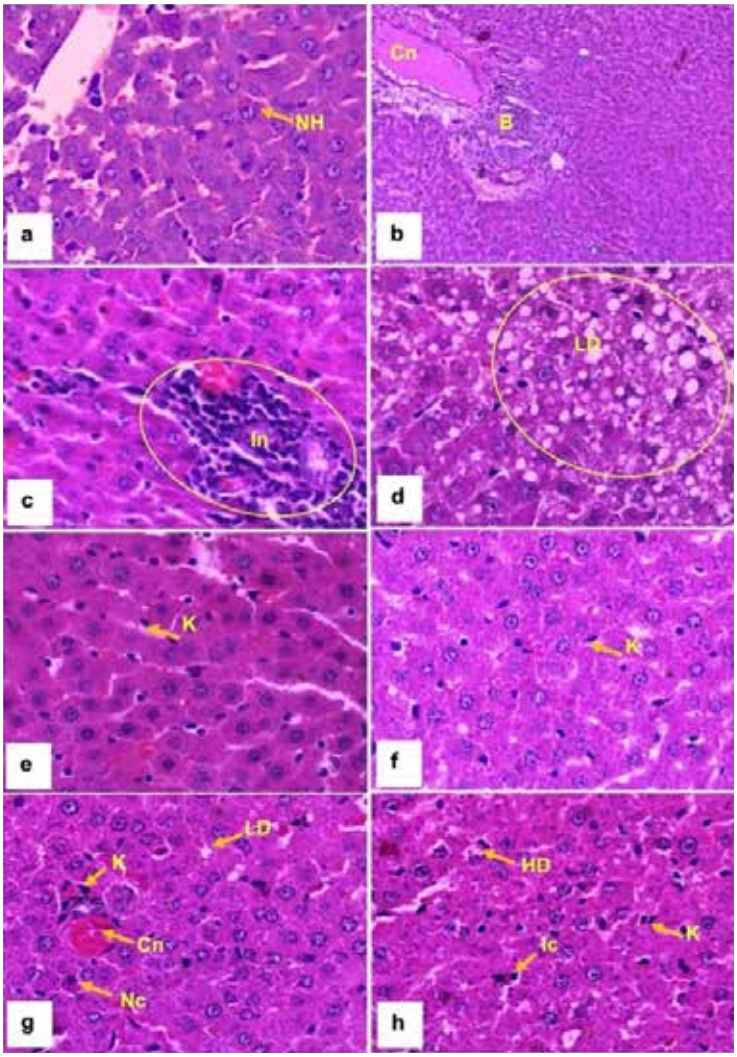

Figure 3. Representative photomicrograph of rat liver tissue in the control (a), LFX (b,c,d), Curcuma + LFX (e), CO 10 + LFX (f), CO 25 + LFX (g), CO 50 + LFX (h) groups. LFX: levofloxacin; CO: Clove oil; NH: normal hepatocyte; Cn: congestion; B: proliferation of biliary ducts; In: Inflammation; LD: lipid degeneration; HD: hydropic degeneration; K: Kupffer cell; Nc: necrotic cells

Hepatotoxicity was evident in levofloxacin-treated animals after 28 days of administration, as demonstrated by marked elevation of liver enzyme, total bilirubin, and oxidative product (MDA) levels. The histological examination supported the serum biomarker and liver oxidant levels. The signs of liver tissue injury were prominent, characterized by marked hepatocyte degeneration and infiltration of inflammatory cells in many areas of the hepatocyte lobules. The main finding of this study is that the liver damage induced by levofloxacin was alleviated by a pre-treatment with *S. aromaticum* L. oil, especially at a preventative dose of 10 mg/kg. Administration of *S. aromaticum* L. oil treatment not only allowed for recovery of serum biomarker levels and MDA but also improved liver histological features. Notably, the present study found that the low dose of *S. aromaticum* L. oil (10 mg/kg) provided better protection than the higher doses. The 10 mg/kg *S. aromaticum* L. oil returned ALT, bilirubin, LDH, and liver MDA levels to near normal, comparable to those of the curcuma treatment. Curcuma is a hepatoprotective agent that has been clinically used to treat hepatitis or drug-induced liver injury [17], hence, it was used in this study as a comparison.

The hepatoprotective effect of *S. aromaticum* L. oil had been evaluated previously against carbon tetrachloride-induced hepatotoxicity in rats [31], where the essential oil was administered 3 times a week (for 4 weeks) using higher doses (100 mg/kg and 200 mg/kg). However, carbon tetrachloride-induced hepatotoxicity may have different pathogenesis from that of levofloxacin. Since every drug has its own particular mechanism, it is important to mimic the mechanism of drug-induced liver injury, levofloxacin in this case, to examine if *S. aromaticum* L. oil can still elicit liver protection. In our study, daily administration of 10 mg/kg dose for 28 days (4 weeks) was sufficient to provide liver protection against levofloxacin-induced toxicity.

It is important to acknowledge that although it is considered safe for ingestion, some evidence has pointed out possible inducement of liver dysfunction by *S. aromaticum* L. oil in high doses. Shalaby et al.[32] showed that the sub-chronic administration of *S. aromaticum* L. oil at > 300 mg/kg dose per day might induce the elevation of ALT and AST levels and congestion and inflammation in the liver tissue. Meanwhile, a subacute toxicity study suggested that the no-observed-adverse-effect level (NOAEL) of eugenol was 50 mg/kg in rats, whereas higher doses might trigger unwanted effects, including liver injury [33].

The present study showed that 10 mg/kg *S. aromaticum* L. oil was the only dose effective at ameliorating levofloxacin-induced hepatotoxicity. Higher doses were not necessarily superior to the lower dose. Consistent with this

finding, an earlier study showed that 10 mg/kg of *S. aromaticum* L. oil treatment was more effective at reducing renal damage than greater doses (25 mg/kg and 50 mg/kg) [34]. Unfortunately, the toxic effects of *S. aromaticum* L. essential oil in rats, using daily administration for a longer period, were not evaluated. Further investigation of the effects of *S. aromaticum* L. oil treatment is needed to conclusively resolve the safe use parameters of *S. aromaticum* L. oil as a hepatoprotective agent.

STATEMENTS OF ETHICS

All animal protocols were performed according to the guide for the care and use of laboratory animals, and the study was granted an institutional ethical clearance (# UH20050197) in the Faculty of Medicine, Hasanuddin University, Indonesia.

CONFLICT OF INTEREST

Authors declare to have no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept: YYD; Design: YYD, AF, SL, AS; Data Collection or Processing: AF, FZ, NF; Analysis or Interpretation: YYD, AF, SL, AS; Literature Search: YYD, AF, FZ, NF; Writing: YYD, AF, FZ, NF; Revision and Proofreading: YYD, AF, SL, AS, FZ, NF

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Evaluation of venous thromboembolism prophylaxis in cancer patients: a retrospective study

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ABSTRACT

This study aims to evaluate the prophylaxis of venous thromboembolism (VTE) in cancer patients. This retrospective study included 100 adult patients of different ages and genders who applied to the University Hospital between January and November 2021, who were diagnosed with cancer, admitted to the intensive care unit (ICU), and received chemotherapy. The patients were evaluated by clinical pharmacists during their ICU hospital stay in accordance with recommendations from the National Comprehensive Cancer Network, the American Society of Clinical Oncology, and the International Society for Thrombosis and Homeostasis Recommendations. Anticoagulant prophylaxis was indicated in all of our patients (100 patients) admitted to the ICU, and 38 (38%) of our patients received anticoagulant prophylaxis during their hospital stay.

Enoxaparin sodium was the preferred anticoagulant drug for 38 patients. During outpatient cancer treatment of patients, 27 of our 100 patients had a Khorana risk score greater than 2 and these patients were recommended to receive prophylactic anticoagulant therapy during outpatient treatment.

Results of this study showed that, oncology team members should be educated about factors that significantly increase VTE risk, and there is an urgent need to improve VTE awareness and practice of thromboprophylaxis in clinical practice.

Keywords: Venous thromboembolism, cancer, thromboprophylaxis, anticoagulants

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INTRODUCTION

Venous thromboembolism (VTE), which includes deep vein thrombosis and pulmonary embolism, is a disease associated with high morbidity and mortality in cancer patients. It occurs in about 10% of cancer patients and is the second leading cause of death after cancer [1]. The risk of developing VTE, recurrent VTE and bleeding complications during VTE treatment is 4 to 7 times higher in cancer patients than in people without cancer, depending on age and gender [2].

Multiple mechanisms are believed to underlie the pathogenesis of the hypercoagulable state. The etiology is diverse and attributable to patient, cancer, and therapeutic factors. Cancer cells can directly or indirectly activate the coagulation pathway [3]. The direct mechanism involves the appearance of procoagulant factors such as tissue factor. Indirect mechanisms include the production of cytokines such as Interloukine-2 (IL2), Tumor necrosis factor-alpha (TNF-α), and vascular endothelial growth factor (VEGF) that activate monocytes, platelets, and endothelial cells that induce expression of the procoagulant phenotype. In addition, cancer cells have surface adhesion molecules that can bind to monocytes, platelets, and endothelial cells that activate and stimulate fibrin production [4].

Cancer treatments such as conventional chemotherapy, hormone treatments, and biologics can increase the risk of VTE by up to 15% per year, depending on the type and combination of drugs chosen or the addition of radiation therapy to the treatment. Chemotherapy can contribute to the development of VTE by causing acute or delayed damage to the vessel walls. In addition, certain predisposing factors such as hospitalization, systemic inflammatory state, and tumor compression stasis may increase prothrombotic risk. Furthermore, the significant improvement in cancer patient survival in recent years prolongs the time that cancer patients may be exposed to VTE [4,2].

Adequate knowledge of VTE risk assessment and VTE sensitization in cancer patients is essential. Cancer patients should be assessed for VTE risk using an approved risk assessment tool, particularly at the start of systemic cancer treatment, during hospitalization and periodically thereafter [5].

National Comprehensive Cancer Network (NCCN), American Society of Clinical Oncology (ASCO), and International Society for Thrombosis and Homeostasis guidelines for cancer-associated VTE in hospitalized cancer patients with underlying medical conditions and/or restricted mobility in the absence of other contraindications recommend thromboprophylaxis. Careful selection of treatment modalities with optimal efficacy in terms of safety is crucial to achieve the best outcomes [3,6,7].

The 2021 update made significant changes to the NCCN guidelines by adding detailed information on prophylactic dosing of anticoagulants based on inpatient/outpatient status for surgical and cancer patients. Dose adjustments for conditions such as obesity, renal failure and thrombocytopenia are the main changes to consider [3].

Although thromboprophylaxis is effective in reducing VTE risk, current guidelines do not advocate its routine use in cancer outpatients, possibly due to the large number of patients requiring treatment, the risk of bleeding, and the difficulty of daily administration of low molecular weight heparin (LMWH) by injection [8].

Risk stratification tools guide the selection of cancer patients at high risk of VTE. An ideal tool should help identify negligible and very high-risk patients in need of intervention. It should also ensure that patients who could benefit most from VTE prophylaxis are identified [9]. To date, only the Khorana score has been successfully used in prospective randomized trials on thromboprophylaxis for risk assessment. The Khorana score is the most well-known risk stratification tool that has been included in the latest updated ASCO and NCCN guidelines for assessing VTE risk in community cancer patients. Five clinical and biological parameters are evaluated prior to chemotherapy [10,11]. According to the updated 2019 ASCO guideline, patients with a low VTE risk are those with a Khorana score less than 2 who do not require routine VTE prophylaxis. Patients at intermediate or high risk of VTE are those with a Khorana score of 2 or greater and VTE prophylaxis for up to 6 months or longer should be considered. In the previous version of the Khorana score, VTE prophylaxis was recommended for patients with a score of 3 or higher [6].

The 2021 NCCN guideline recommends additional dose adjustments for outpatients. Prophylactic anticoagulant therapy should be excluded in cancer patients with platelet counts below 50,000/μl, and a reduced dose of enoxaparin can be used in patients with platelet counts between 50,000 and 75,000/μl^3. Although the relationship between cancer and VTE is well established, it is interesting to note that patient and physician knowledge of VTE risk is extremely low[3]. This study reports the status of prophylaxis demand and adherence to guidelines in cancer patients with VTE prophylaxis at ICU admission. The aim is to assess the status of the considered deficiency by assessing the VTE risk during the outpatient treatment period.

METHODOLOGY

Our group of patients undergoing chemotherapy was admitted to the ICU for various diseases; consists of patients for whom VTE prophylaxis is indicated by National Comprehensive Cancer Network (NCCN), American Society of Clinical Oncology (ASCO) and International Society for Thrombosis and Homeostasis guidelines.

This study included 100 adult patients of different age groups and genders who have applied for admission to the University Hospital with ethical approval of the Hospital Ethics Committee (İstanbul Medipol University Non-Interventional Clinical Studies, E-10840098 -772.02-4020, 08/ 24/2021) who had cancer between January 2021 and November 2021, were admitted to the intensive care unit and received chemotherapy. As the study was retrospective no informed consent was obtained.

Hospital data collection parameters were patient data, medical history (especially VTE history and type of cancer), laboratory values (serum creatinine to calculate Khorana score [*The Khorana score is a guideline-recommended point-based risk score used to estimate the risk of incident VTE in ambulatory cancer patients*], platelet count, hemoglobin and white blood cell count before chemotherapy), body mass index, glomerular filtration rate (GFR) value, medical history, metastatic status, surgical planning status, anticoagulant used in VTE prophylactic treatment, and dose and duration of administration. Among these data, our primary priority is to understand and evaluate the efficacy of anticoagulant use in the prophylactic treatment of VTA, and only the results of the statistical evaluation of this are highlighted here.

Patients in the study were evaluated by clinical pharmacists for VTE prophylaxis during their hospitalization in the ICU according to National Comprehensive Cancer Network, American Society of Clinical Oncology and International Society of Clinical Oncology recommendations and homeostasis (Table 1 and Table 2) [3,4].

Table 1. VTE prophylaxis options for hospitalized medical oncology patients[3]

Agent	Standart dosing[a,b]	Renal dose	Obesity dosing (BMI ≥40 kg/m^2)[c]
Dalteparin	5,000 units SC daily	Avoid if CrCl<30 mL/min	Consider 7,500 units SC daily or 5,000 units SC every 12 hours or 40-75 units/kg SC daily
Enoxaparin	40 mg SC daily	Recommended 30 mg SC daily if CrCl<30 mL/min	Consider 40 mg SC every 12 hours or 0.5 mg/kg SC daily
Fondaparinux	2.5 mg SC daily Avoid in patient weighing<50 kg	Caution if CrCl 30-49 mL/min Avoid if CrCl<30 mL/min	Consider 5 mg SC daily
Unfractionated Heparin (UFH)	5,000 units SC every 8-12 hours	Same as standard dose	Consider 7,500 units SC every 8 hours

CrCl: Estimated creatinine clearance
SC: Subcutaneous
BMI: Body mass index

[a]Recommendation derived from patients hospitalized with medical illness (Hospitalized>6 days, immobile/bed rest >3 days, age≥ 40 years, plus additional risk factors for VTE)

[b]Thromboprophylaxis for duration of hospital stay or 6-14 days or until patient is fully ambulatory

[c]Limited data available to support recommendations. Recommended doses are derived from non-oncology populations[3].

Table 2. Treatment of established VTE[4]

Agent		Dosing
Initial	UFH	80 U/kg IV bolus, then 18 U/kg/h IV and adjust dose based on aPTT[k]
	Dalteparin[j,l,m]	100 U/kg every 12 hours 200 U/kg once daily
	Enoxaparin[j,l,m,n]	1 mg/kg every 12 hours 1,5 mg/kg once daily
	Tinzaparin[j,l,m,o]	175 U/kg once daily
	Fondaparinux[j,l,p]	< 50 kg: 5.0 mg once daily 50-100 kg: 7.5 mg once dailiy >100 kg: 10 mg once daily
	Rivaroxaban	15 mg orally every 12 hours for 21 days
Long Term[p,q,r]	Dalteparin[l,m,s]	200 U/kg once daily for 1 month, then 150 U/kg once daily
	Enoxaparin[l,m,n]	1.5 mg/kg once daily 1 mg/kg every 12 hours
	Tinzaparin[m,o]	175 U/kg once daily
	Warfarin	Adjust dose to maintain INR 2-3
	Rivaroxaban[m,t]	15 mg orally every 12 hours for 21 days, followed by 20 mg once daily there after (both doses with food)
	Edoxaban[m,t]	Needs at least 5 days of parenteral anticoagulation prior to its started, then switch to 60 mg orally once daily or 30 mg orally daily in those weighing≤ 60 kg, who have creatinine clearance between 30 and 50 mL/min, or who need concomitant use of a P-glycoprotein inhibitor

aPTT: Activated partial thromboplastin time
INR: International normalized ratio
IV: Intravenous
UFH: Unfractionated heparin
VTE: Venous thromboembolism

[j]Parenteral anticoagulants should overlap with warfarin for 5-7 days minimum and should be continued until the INR is in the therapeutic range for 2 consecutive days.

[k]UFH infusion rate should be adjusted to maintain the aPTT within the therapeutic range in accordance with local protocols to correspond with a heparin level of 0.3-0.7 U/mL using a chromogenic antifactor Xa assay.

[l]Dependent on significant renal clearance, avoid in patient with creatinine clearance≤ 30 mL/ minor adjust dose based on antifactor Xa levels.

[m]Optimal dose unclear in patient> 120 kg

[n]Twice-daily dosing may be more efficacious than once-daily dosing for enoxaparin based on post hoc. data.

[o]This medicine is not available in the United States.

[p]Fondaparinux had a higher rate of recurrent thrombosis and no difference in hemorrhage compared with enoxaparin in patients with cancer in a post hoc. Subgroup analysis. It is not

a standard option but may be used for long term anticoagulation if standard LMWH or direct oral anticoagulants (DOACs) are not a feasible option for the patient. Dosing for long term treatment with fondaparinux is the same as for initial treatment.

[q]Total duration of therapy depends on clinical circumstances.

[r]Apixaban and dabigatran do not have fully published results from cancer-specific clinical trials. Prospective randomized trial data in patients with cancer with active diseases of cancer therapy are needed prior to their use. Therefore, they are currently not recommended for routine use in patients with cancer with active diseases.

[s]This is the only LMWH with US food and Drug Administration approval for extended therapy to prevent recurrent thrombosis in patients with cancer.

[t]Edoxaban has the highest level of evidence for patients with cancer among all the DOACs, followed by rivaroxaban. Limited data from, small, unpublished patient series suggest that the effectiveness of DOACs in patient with a weight> 120 kg might be reasonable based on anti-Xa levels. The data are very limited, however, and LMWH is still likely to be preferred in this setting. Please refer to the package inserts for detailed information regarding potential dosing adjustment needs, especially as regards renal impairment, liver failure, weight extremes, or drug-drug interaction [4].

During outpatient cancer treatment, a VTE risk assessment was performed at least one month prior to ICU admission, taking into account the Khorana score and National Comprehensive Cancer Network recommendations for further dose adjustments prior to receiving systemic chemotherapy (Table 3 and Table 4) [3].

Table 3. Dosing regimens for prophylaxis VTE in cancer outpatients[b3]

Agent	Dosing
Dalteparin[d,g]	5,000 U once daily
Enoxaparin[d,g]	40 mg once daily
Fondaparinux[d,h]	2.5 mg once daily
Apixaban[d]	2.5 mg orally twice daily
Rivaroxaban[d]	10 mg orally once daily

[b]Duration for medical patients is for the length of hospital stay or until fully ambulatory. For surgical patients, prophylaxis should be continued for at least 7-10 days. Extended prophylaxis for up to 4 weeks should be considered for high-risk patient. Duration for outpatient prophylaxis is somewhat uncertain, as most studies did not assess beyond 6 months. [d]This drug is not approved by the US Food and Drug Administration for this indication.[g] Higher prophylactic doses were used for patients with pancreatic cancer: dalteparin 200 IU/kg once daily for 4 weeks followed by a step down to 150 IU/kg for a further 8 weeks in FRAGEM andenoxaparin 1 mg/kg once daily in CONCO-OO4.[h] Fondaparinux has not been studied in outpatient prophylaxis setting. It should only be considered if the patient has contraindications for other LMWH and DOAC use is considered an inferior option[3].

Table 4. Other dose modifications for ambulatory patients with cancer[3]

Prophylactic anticoagulation therapy should be ruled out in medical oncology patients whose platelet count is less than 50,000/ml
A reduced dose of enoxaparin can be used in patients with platelet count between 50,000 and 75,000/ml

Our study includes the assessment of cancer patients in relation to renal impairment and obesity doses for thromboprophylactic treatment, updated in the 2021 NCCN guidelines [3]. This study may be one of a leading study in the literature as it was conducted during the ICU hospitalization and the risk of VTE during the period when they received outpatient treatment, taking into account the Khorana score and other dose modification updates according to the 2021 NCCN guideline.

Descriptive statistics were analyzed using SPSS Statistics software (version 21.0.). Results are presented as arithmetic means ± standard errors of means where required. The study population was considered as a group and no subgroup analysis was performed.

RESULTS AND DISCUSSION

Inpatients

Of our 100 patients, 50 (50%) were male and 50 (50%) were female. The mean age of our patients was calculated as 59.44 years. All of our patients received chemotherapy. 26% of our patients had lung cancer, 18% had breast cancer, 13% had bladder cancer; 9% had colon cancer, 9% had gynecologic cancer, 5% had pancreatic cancer, 2% had brain cancer, 1% had kidney cancer, 1% had bladder cancer, and 16% had other cancers. In addition, 11 (%11) of our patients had a history of VTE. Anticoagulant prophylaxis was indicated in all of our patients admitted to the ICU and 38 (38%) of our patients received anticoagulant prophylaxis during their hospitalization. All 38 patients preferred enoxaparin sodium as the anticoagulant drug. In 62 patients (62%) no anticoagulant prophylaxis was administered, although it was indicated [3].

The enoxaparin dose had to be increased in 5 of 38 patients due to a history of VTE and the dose had to be reduced in 1 patient due to renal dysfunction. None of our patients required an obesity test. No bleeding, bruising or side effects associated with the administration of antithrombotic prophylaxis have been reported.

All of our patients who received coagulation prophylaxis received prophylactic

treatment during their stay in the intensive care unit. The mean number of days on anticoagulant prophylaxis was 22.55 (minimum: 1 day; maximum: 48 days).

Outpatients

During outpatient cancer treatment, 27 of our 100 patients had a Khorana score greater than 2, and it is recommended that these patients receive prophylactic anticoagulant therapy during outpatient treatment. Prophylactic treatment with anticoagulants according to the Khorana scale was recommended for 2 of the patients; It was also suggested to exclude prophylactic anticoagulant therapy according to the other dose adjustment recommendations in the NCCN 2021 guidelines [3].

In a study with 199 patients (75 men [37.7%] and 124 women [62.3%]), the medical records of the patients with an average age of 50.6 years were reviewed[12]. In another study conducted with 100 inpatients with cancer, 48 (48%) of the patients were women and 52 (52%) were men, with a mean age of 59 years[11].On the other hand, 50% of the patients included in our study were women and 50% men, with a mean age of 59.44 years.

In a study of 100 inpatients with cancer, the majority of patients (n=34, 34%) had gastrointestinal cancers (8 gastric, 8 pancreatic, 8 colon, 4 hepatocellular, 4 esophageal, and 2 cholangiocellular) and Lung tumors diagnosed in 17 (17%) cancer patients. Eleven of the patients (11%) had a history of VTE [11].

In our study, 26% of our patients had lung cancer, 18% breast cancer, 13% bladder cancer; 9% had colon cancer, 9% had gynecologic cancer, 5% had pancreatic cancer, 2% had brain cancer, 1% had kidney cancer, 1% had bladder cancer, and 16% had other cancers. In addition, 11 of our patients had a history of VTE. The two studies are similar in that the most common types of cancer detected are lung cancer and cancer of the gastrointestinal tract.

The results of the DissolVE 2 study, conducted in China, which enrolled 1,535 cancer patients and aimed to assess the VTE risk profile and VTE prophylaxis in inpatient cancer patients, are as follows: 666 patients (93.9%) with high VTE risk had no VTE received VTE prophylaxis and only 11 (1.6%) patients received adequate VTE prophylaxis. The results reflect the poor management of VTE risk in hospitalized cancer patients in China [13]. In a retrospective study conducted at a university hospital in Lebanon, a 2-month retrospective review of hospitalized cancer patients for deep vein thrombosis (DVT) prophylaxis was performed and found that 21 (221%) of 95 patients with indications for DVT prophylaxis received DVT prophylaxis, while only 47.6% of patients receiving anticoagulant therapy received prophylaxis with the right drug and dose. The

results suggest that there is a need to improve awareness and practice of VTE prophylaxis [14].

The findings of our study show that 38 (38%) of 100 patients received anticoagulant prophylaxis and appropriate thromboprophylaxis was applied to 32 of these patients (84.21%). Although it was indicated in 62 patients, no anticoagulant treatment was administered (%62).

An international survey study shows that physicians do not carry out prophylactic treatment in about 30% of hospitalized cancer patients for whom VTE prophylaxis is indicated because of the perceived high risk of bleeding [15]. In another study of 100 inpatient cancer patients, 36 (36%) of the patients did not receive any anticoagulant therapy during the hospital stay [11]. Our study showed more negative results regarding VTE prophylaxis than these two studies, in which 62% of the patients for whom VTE prophylaxis was indicated did not use anticoagulant prophylaxis.

Recent meta-analyses have confirmed previous findings that LMWHs are more effective than vitamin K recurrence in cancer antagonists in reducing VTE patients [6]. In a study of 199 patients, only 2 patients received unfractionated heparin prophylaxis and the remaining 197 patients preferred enoxaparin as thromboprophylaxis. In 3 patients (6.4%) an insufficient dose of the drug was administered. No dose adjustment was made in 2 patients due to renal failure, although it was necessary. In addition, no dose adjustment was made in 1 obese patient. The mean duration of thromboprophylactic treatment was calculated to be 4.1 days (minimum: 1 day; maximum: 36 days). No bleeding, bruising, or serious side effects associated with heparin or enoxaparin administration have been reported. In the study, which included 100 inpatient cancer patients, enoxaparin (96.9%) and apixaban (3%) were the preferred treatment options during hospitalization [12].

In a study from Lebanon, enoxaparin was the most commonly prescribed anticoagulant at 76.2%[14]. In the study, which included 100 cancer inpatients, enoxaparin (96.9%) and apixaban (3%) were the preferred treatment options during the hospital stay. The length of hospital stay was determined to be between 10 and 11 days (minimum: 2 days; maximum: 70 days) [11].

According to the data obtained in our study, enoxaparin sodium was the preferred anticoagulant in the 38 patients receiving prophylactic anticoagulant treatment, consistent with the preferred anticoagulant option in other studies. As a practical clinical decision by the physicians, 5 patients had their enoxaparin dose increased due to a history of VTE, and 1 patient had their dose reduced

due to renal dysfunction. There were no obese patients in our study group and for this reason there was no need for dose adjustment for obese patients according to the guideline[3]. In addition, no bleeding, hematoma or serious adverse events related to the use of thromboprophylaxis were not report. The hospital stay of our patients was calculated to be 22.55 (minimum: 1 day; maximum: 48 days) days.

A single-center retrospective cohort study of patients with pancreatic and gastric cancer aimed to examine the prescription rates of prophylactic anticoagulants in patients at high risk of VTE using the Khorana risk score. Of 437 patients, 181 (41%) had a Khorana score greater than 3 and none had an alternative. No anticoagulant has been used prophylactically without an indication for treatment [3]. In another study that assessed VTE risk in 200 cancer outpatients using the Khorana risk score, 11 (5.5%) patients were at high risk and 117 (58.5%) had an intermediate risk. The consultant was informed of the patient's risk scores; however, anticoagulant therapy was not started because the doctors decided to mobilize the patients[11].In our study, 27 (27%) of 100 patients had a Khorana score greater than 2 (high risk) during their outpatient cancer treatment. It is recommended that these patients receive prophylactic anticoagulant therapy during outpatient treatment. In 2 of 27 patients recommended to receive prophylactic anticoagulation therapy according to Khorana risk score, however, prophylactic anticoagulation was not recommended according to the 2021 NCCN guidelines [3]. Based on a comprehensive literature review, our study is one of the leading to evaluate VTE prophylaxis in cancer patients in the light of recent guideline updates. Although the association between cancer and VTE is well characterized, the awareness of both patients and clinicians about the risk of VTE remains low. Members of the oncology team should be educated about factors that significantly increase the risk of VTE, especially major surgery, hospitalization, and systemic antineoplastic therapy. VTE awareness and prophylaxis in clinical practice urgently need to be improved.

Consistent with the obtain findings of the previous studies, consensus guideline by clinical pharmacists could significantly improve the implementation of appropriate VTE prophylaxis, reduce the development of VTE and have a positive impact on the safety of patients.

The weakness of our study is that it was conducted retrospectively. There is a need for larger studies aiming to improve VTE prophylaxis practices, to identify deficiencies and problems in practice, and to evaluate the effectiveness of therapeutic interventions to address them.

STATEMENT OF ETHICS

We confirm that we have read and understood the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

CONFLICT OF INTEREST

None

AUTHOR CONTRIBUTIONS

Idea/Concept: Neda Taner, Betül Şirin, Design: Neda Taner, Betül Şirin, Data Collection/Processing: Neda Taner, Analysis/Interpretation: Neda Taner, Betül Şirin, Literature Review: Betül Şirin, Drafting/Writing: Neda Taner, Betül Şirin, Critical Review: Neda Taner, Betül Şirin

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