ACTA PHARMACEUTICA SCIENCIA

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Formerly: Eczacılık Bülteni / Acta Pharmaceutica Turcica
Founded in 1953 by Kasım Cemal GÜVEN

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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 Istanbul University, Turkey.

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. Şeref DEMİRAYAK

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.

*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 on-line 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 and Reviews.

1.2.1 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.

1.2.2 Reviews integrate, correlate, and evaluate results from published literature on a particular subject. They expected to report new and up to date experimental findings. They have to have a well-defined theme, are usually critical, and may present novel theoretical interpretations. Up to date experimental procedures may be included. Reviews are usually submitted at the invitation of the Editors. However, experts are welcome to contact the Editors to ensure that a topic is suitable. Approval is recommended prior to submission.

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 authors. 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 state-

ment 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". 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 summarized. 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.

Key words: 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 experi-

mental 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, ºC, 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. Referencing style is given in our website in detail. Citations should be made as superscript arabic numbers. 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. 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. Vancouver style is used in the reference list. However, in-text citations should be given superscript numbers (e.g. 1) according to order in the manuscript. 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.

Journal article examples

Article with two authors example:

Tinworth, C. P., & Young, R. J. (2020). Facts, Patterns and Principles in Drug Discovery: appraising the Rule of 5 with measured physicochemical data. *Journal of Medicinal Chemistry*. doi:10.1021/acs.jmedchem.9b01596

Sahin, Z., Ertas, M., Berk, B., Biltekin, S. N., Yurttas, L., & Demirayak, S. (2018). Studies on non-steroidal inhibitors of aromatase enzyme; 4-(aryl/heteroaryl)-2-(pyrimidin-2-yl)thiazole derivatives. *Bioorganic & Medicinal Chemistry*, 26(8), 1986–1995. doi:10.1016/j.bmc.2018.02.048.

Articles up to 6 authors should be given examples as above, and articles with more than 6 authors should be given as et al. after sixth author.

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 information such as the article's number which is assigned by system, title and formation date. You may delete the articles by using **Delete** button on the right column, if the article is not considered to send into the system.

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.

The required revisions can be seen in "**Notes**" part by clicking the articles title.

In order to send any revision, **Submit Revision** button on the last column should be clicked. This connection will take the author to the first level of **Adding Article** and the author can complete the revision transaction by carrying out the steps one by one. All changes must be made in the registered file and this changed file must be resent. Author's most efficacious replies relating to the changes must be typed in "Cover Letter" part.

If the is transaction is discontinued, the system move the revised article to **Submitted Manuscripts** part and the transaction can be continued from here.

After the transaction was completed, the system moves the revised article to "Submitted Manuscripts" part.

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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Authors should consider that this time may take time because of the reviewer assignments and acceptance for review may take time for some cases.

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ORIGINAL ARTICLES

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In vitro ACE2 Enzyme Inhibitory Activity Evaluation of Different *Salvia* Essential Oils

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ABSTRACT

In this present study, commertially available *Salvia triloba* L., *S.officinalis* L., and *S. sclarea* L. essential oils were evaluated for their *in vitro* angiotensin converting enzyme 2 (ACE2) inhibitory activity. The *Salvia* essential oils compositions were confirmed both by GC-FID and GC/MS. Main components of the *S. triloba* essential oil was characterized as 1,8-cineole (22.8%), camphor (17.2%), α-thujone (15.2 %), β-caryophyllene (11.4 %), and α-humulene (3%). Major constiutents were identified as α-thujone (28.5 %), camphor (20.6 %), 1,8-cineole (10.9%), α-humulene (5%), and camphene (4.9 %) in *S. officinalis* essential oil. Whereas, linalylacetate (56.8 %), linalool (21.1%), α-terpineol (6.1%), geraniol (5%), and β-caryophyllene (3.4%) were the major components of *S. sclarea* essential oil. The essential oils were evaluated using a fluorometric multiplate based enzyme inhibition kit, where the ACE2 inhibitions of *S. triloba, S. officinalis,* and *S. sclarea* essential oils were 50.1%, 60.5%, and 72.1% at a concentration of 20 μg/mL, respectively. As a result, further tests of *Salvia* essential oils supported by *in vivo* studies may have antiviral potential applications against coronaviruses due to ACE2 enzyme inhibitions.

Keywords: *Salvia*, coronavirus, ACE2, essential oil

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INTRODUCTION

Salvia L., the largest genus of the Lamiaceae family, contains more than 900 species spread around the world, where some species are economically important, and as used as a spice and flavoring agent in food, condiments and bewerages, also in cosmetic and pharma industries. *Salvia* essential oils are generally rich in 1,8-cineole and borneol content[1-4].

Ethnobotanical utilization of *Salvia* species are also common worldwide, *Salvia officinalis* L. is widely used in the treatment of cough, bronchitis and colds[5]. In addition, *Salvia sclarea* L. is also used to relieve the upper respiratory tract, especially in the treatment of upper respiratory tract infections[6]. Anatolian *Salvia triloba* L. (Synonym *Salvia fruticosa* Mill.) is commonly used against coughs and colds[7]. In addition, of *Salvia* essential oils are also used due to their antimicrobial and antiviral effects in aromatherapy[8-11].

The aim of the present study was to evaluate the *in vitro* ACE2 enzyme inhibitory potential of *S. triloba*, *S. officinalis*, and *S. sclarea* essential oils associated to their possible antiviral effect against coronavirus. The essential oil composition was identified by GC-FID and GC/MS, respectively.

METHODOLOGY

Chemicals and Plant Material

Commercial *S. triloba, S. officinalis,* and *S. sclarea* essential oils were kindly provided by Doallin Ltd., İstanbul, voucher samples are deposited at IMEF Herbarium (Herbarium No: IMEF 1146-1147-1148)

GC-MS/GC-FID analysis

An Agilent 5975 GC-MSD system was used for GC/MS analyses. Whereas the Agilent 6890N GC system was used for the GC-FID. FID detector's temperature was set to 300°C. Concurrent auto-injection was applied in two identical columns with the same conditions in the GC/MS system. Relative percentages (%) were calculated using FID chromatograms (Figure 1-3). Relative retention times were used to characterize the essential oils chemical composition. This process was held either by authentic samples or analyzing relative retention index (RRI) of n-alkanes, along with GC/MS Library, MassFinder 3 Library, in-house "Başer Library of Essential Oil Constituents" [12].

ACE2 Enzyme Inhibition Activity

The essential oils were dissolved initially using DMSO [< 1% (v/v)]. The *in vitro*

enzyme inhibition was performed according the manufacturer's instructions for the "Angiotensin II Converting Enzyme (ACE2) Inhibitor Screening Kit (Bio-Vision, K310)" and the enzyme inhibition of the essential oils were measured with Ex/ Em = 320/420 nm wavelength using a multimode microplate reader (SpectraMax i3). The enzyme inhibition of the essential oils were calculated by comparing with standards included in the kit and the percentage inhibition (%I) values were calculated as mean values resulting from triplicate data for all samples as previously reported [12].

Statistical analysis

The statistical analysis was carried out using the GraphPad Prism, Version 7.02 (La Jolla, California, USA). *In vitro* data was expressed as mean ± standard deviation (Mean± SD). The statistical significance was analyzed by One-way ANOVA (followed by Dunnett's post hoc test) and Paired Samples T-Test. The $p<0.05$ was accepted as statistically significant.

RESULTS and DISCUSSION

GC-MS/GC-FID analysis

The phytochemical constituents of the *Salvia* essential oils were confirmed by using GC-FID and GC-MS. *S. triloba* essential oil contained the main constituents 1,8-cineole (22.89%), camphor (17.15%), α-thujone (15.18%), and β-caryophyllene (11.43%), respectively. Major components of *S. officinalis* were identified as α-thujone (28.46%), camphor (20.58%), 1,8-cineole (10.45%), and α-humulene (5%); whereas *S. sclarea* essential oil contained linalylacetate (56.8%), linalool (21.06%), α-terpineol (6.05%), and geraniol (15.18%) among others (Figure 1-3).

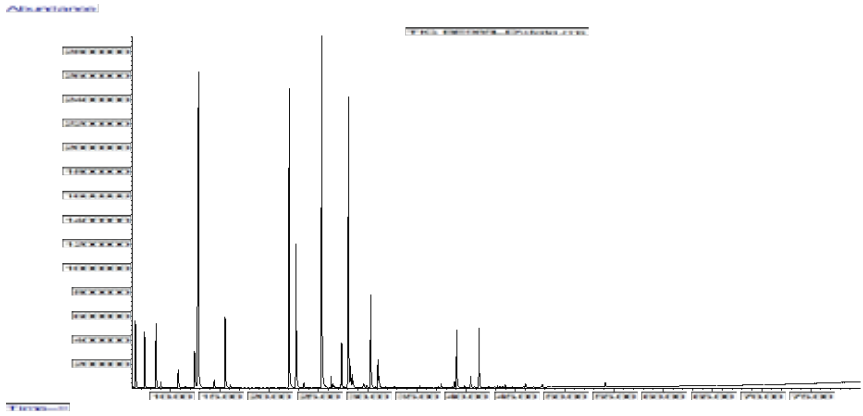

Figure 1. GC Chromatogram of *Salvia officinalis* essential oil

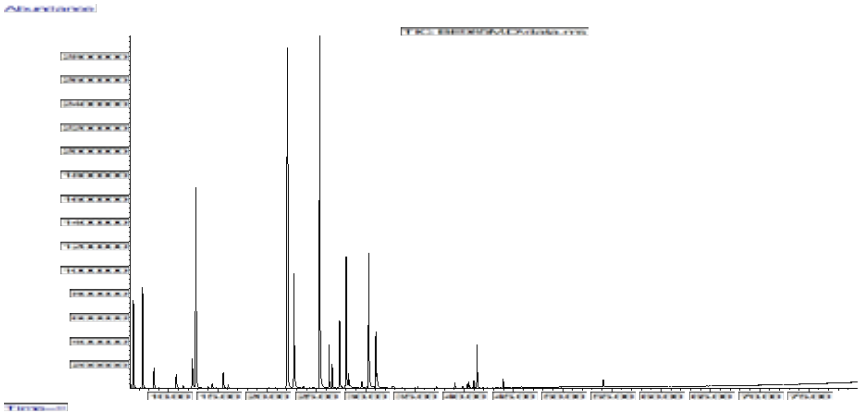

Figure 2. GC Chromatogram of *Salvia triloba* essential oil

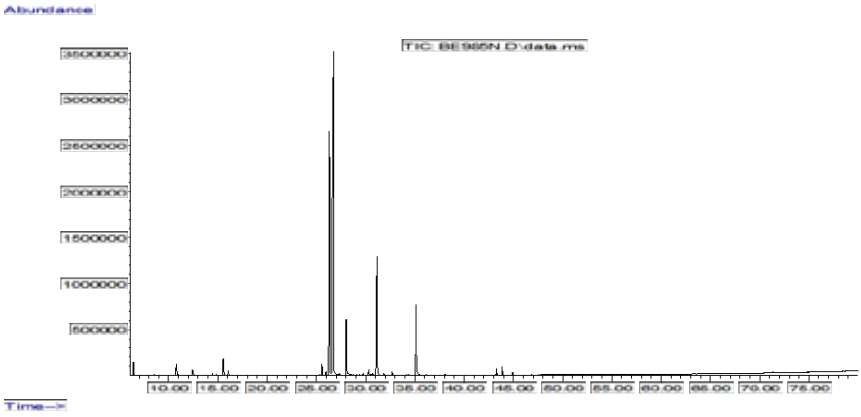

Figure 3. GC Chromatogram of *Salvia sclarea* essential oil

Recent literature on *S. triloba, S. officinalis,* and *S. sclarea* essential oils from Turkey show that major components of the species have similarity [13]. Compared to *Salvia officinalis*, *Salvia triloba* essential oil has a very low thujone content. *S. triloba* essential oil, known as Turkish sage, is a non-toxic alternative to *S. officinalis* as it does not contain thujone. In addition, *S. triloba* is richer in 1,8-cineol than *S. officinalis*. *S. sclarea*, on the other hand, has a rich content of linalool and linalyl acetate.

ACE2 Enzyme Inhibition Activity

The enzyme inhibition assay was performed at a concentration of 20 µg/mL for all tested *Salvia* essential oils using a a fluorometric multiplate based enzyme inhibition kit, where the *in vitro* ACE2 inhibition rates of *S. triloba*, *S.*

officinalis, and *S. sclarea* essential oils were 50.07 ± 2.99%, 60.45 ± 1.82%, and 72.12 ± 0.90%, respectively as also illustrated in Figure 4.

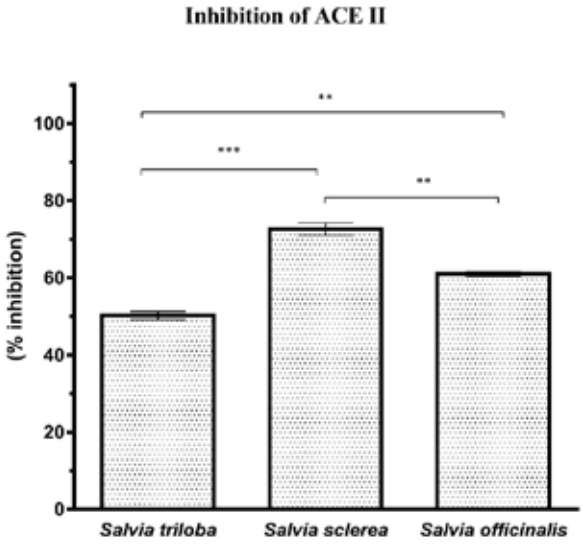

Figure 4. ACE2 Enzyme Inhibition of *Salvia Essential* Oils (at 20 µg/mL concentration)

ACE2 was proved to be the receptor for the SARS-CoV, the human respiratory coronavirus NL63, and the novel coronavirus 2019 nCoV/SARS-CoV-2[14].It is also known that previous studies identified that ACE2 is the essential and important receptor for coronavirus types to enter into the cell [15-16]. The highest ACE2 enzyme inhibition was observed in *S. sclarea* essential oil. *S. sclarea* was found to be effective against many viruses and human pathogenic microorganisms in previous studies [17-18]. In another detailed study on *S. sclarea* essential oil, it was revealed that the essential oil has a better antimicrobial effect compared to food preservatives. In addition, in this study, it was shown that the essential oil mechanism of action of was by damaging the cell membrane and impairing the cell membrane permeability. It is also thought to cause the release of macromolecular substances, even substances such as ATP and DNA, inside the cell. In general, the antimicrobial-antiviral effect of the essential oil of *Salvia sclarea* is not only attributable to a single pathway, but is thought to involve a series of events both on the cell surface and in the cytoplasm [19]. Due to the relevance of the prevention of coronavirus and the recovery process of the disease with ACE2, the results of the study can also be associated with the COVID-19 pandemic [20]. Based on all this information and the ACE2 enzyme inhibition of *S. sclarea* essential oil, it can be said that this essential oil may also

be effective against coronavirus.

Its effects on DNA and deformation of the cell membrane make its effectiveness against viruses possible. To the best of our knowledge, this is the first report on the ACE2 enzyme inhibition evaluation of *Salvia* essential oils. Further studies will be continued to evaluate the antiviral potentials of these essential oils, especially *S. sclarea*.

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Cytotoxic activity of the fruit extracts of *Heptaptera anatolica* (Boiss.) Tutin

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ABSTRACT

Cytotoxic activities of the fruit extracts of *Heptaptera anatolica* (Boiss.) Tutin were investigated on the colon cancer COLO205 and KM12 cell lines. The dichloromethane extract of the fruits of *H. anatolica* showed the best cytotoxic activities with IC_{50} values of 17.9 and 15.1 ug/mL on the COLO205 and KM12 cell lines, respectively. Whereas, the ethyl acetate extract of the fruits showed moderate cytotoxic activity with IC_{50} values of 23.4 ug/mL against the KM12 cell lines.

Keywords: Cytotoxic activity, *Heptaptera anatolica*, Apiaceae

INTRODUCTION

Cancer is a major public health problem worldwide and is the second leading cause of death[1]. Natural sources have a great potential for the discovery of new anticancer drugs[2]. As part of our continuing studies on the genus *Heptaptera* (Apiaceae), we report here the cytotoxic activity of *Heptaptera anatolica* fruits.

The genus *Heptaptera* Marg. & Reuter (Apiaceae) is represented by 10 species worldwide, four of them; *H. cilicica* (Boiss. & Bal.) Tutin, *H. anisoptera* (DC.) Tutin, *H. anatolica* (Boiss.) Tutin and *H. triquetra* (Vent.) Tutin are growing in Turkey[3,4]. *Heptaptera* species are known to contain sesquiterpene coumarin derivatives[5-9], these compounds have various biological activities such as; cytotoxicity, P-glycoprotein inhibitory, cancer chemopreventive, anti-inflammatory, antibacterial, antileishmanial, antiviral, antidiabetic, etc.[8-14].

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METHODOLOGY

Plant Material

The fruits of *Heptaptera anatolica* were collected in the vicinity of Izmir in June 2013 and identified by Prof. A. Duran. A voucher specimen (A. Duran 9703) was deposited in the Herbarium of Selçuk University, Faculty of Sciences, Department of Biology (KONYA).

Extraction

Coarsely powdered fruits (50 g) of the plant were sequentially extracted at room temperature with dichloromethane (CH_2Cl_2) and methanol. The extracts were individually concentrated in a rotary evaporator under reduced pressure to dryness. Dichloromethane and methanol extracts of the fruits were 8.31 g, 16.62% and 8.67 g, 17.34 %, respectively. The methanol extract was redissolved in a mixture of methanol/water (10:90) and then partitioned with ethyl acetate (EtOAc), the resulting extracts were separately concentrated in vacuo to dryness. Ethyl acetate and aqueous-methanol extracts of the fruits were 1.33 g, 2.66% and 7.14 g, 14.28%, respectively.

Cytotoxicity Assay on Colon Cancer Cells

The assay used for this study was a two-day, two cell line XTT bioassay[15], an in vitro antitumor colorimetric assay developed by the MTL Assay Development and Screening Section. Colon cancer cell lines used were COLO205 and KM12. Cells were maintained and passed weekly in RPMI-1640 medium with phenol red (Gibco, Carlsbad, CA, USA) and supplemented with 2 mM L-glutamine (Quality Biologicals, Inc., Gaithersburg, MD, USA) and 10% fetal bovine serum (Hyclone, Logan, UT, USA). Cells were placed in a humidified incubator with an atmosphere of 5% CO2 and 95% air and a temperature of 37° C. Cells were placed in a humidified incubator with an atmosphere of 5% CO2 and 95% air and a temperature of 37 °C. Cells used in the assay were harvested with RPMI-1640 medium, without phenol red (Gibco, Carlsbad, CA, USA) and supplemented with 2 mM L-glutamine (Quality Biologicals, Inc., Gaithersburg, MD, USA) and 10% fetal bovine serum without antibiotics. Harvested cells were counted using a Cellometer Auto T4 cell counter (Nexcelom Bioscience LLC, Lawrence, MA, USA) and plated in 384-well flat-bottom polystyrene microtiter plates (Nunc, Nunc A/S, Denmark), at a density of 5000 cells/well for COLO205 and 5000 cells/well for KM12. The cells were incubated in a 5% CO2/95% air and 37 °C incubator for 24 h. After incubation, test samples were added to plates using a Biomek FX robotic liquid handling workstation (Beckman/Coulter, Fullerton, CA, USA). The robot performed eight 2-fold serial dilutions of the sample and

then transferred the sample from the source plate to the assay plate. The plates used were Costar 384-well round-bottom plates (Corning Inc., Corning, NY, USA). Cells were further incubated with samples for 48 h, at which time the XTT reagent was added. Viable cells reduced the XTT to a colored formazan product, and after an additional 4 h incubation period the amount of formazan produced was quantified by absorption at 450 nm, using a 650 nm reference. Sanguinarine was used on each plate as a positive control.

RESULTS and DISCUSSION

This is the first report on the cytotoxic activity of the fruits of *H. anatolica*. The dichloromethane extracts of the fruits exhibited strong inhibitory activity on the colon cancer COLO205 and KM12 cell lines. The ethyl acetate extract of the fruits exhibited moderate inhibitory activity on the KM12 cell lines. The cytotoxic activities observed with these extracts are shown in Table 1.

Table 1. Cytotoxic activities of the extracts

	Cytotoxic activity (IC50 values in ug/mL)	
Extracts		
	COLO205	KM12
1	17.9	15.1
2	> 50	23.4
3	> 50	> 50

1: CH_2Cl_2 extract of the fruits; 2: EtOAc extract of the fruits; 3: aqueous-methanol extract of the fruits

The dichloromethane extract of the fruits of *H. anatolica* showed the best cytotoxic activities with IC_{50} values of 17.9 and 15.1 ug/mL on the COLO205 and KM12 cell lines, respectively. The ethyl acetate extract of the fruits showed moderate cytotoxic activity with IC_{50} values 23.4 ug/mL on the KM12 cell lines and a weak cytotoxic activity against the COLO205 cell line with IC_{50} value greater than 50 ug/mL. Previously, Appendino *et al.* reported samarcandin, samarcandone, conferone, feselol and more polar compounds 9,10,11-trihydroxyumbelliprenin and 9,10,11-5'-tetrahydroxyumbelliprenin from the chloroform extract of the fruits of *H. anatolica* collected from Mardin in June 1991[6]. Cytotoxic activity of certain sesquiterpene coumarins were described earlier[8-10], thus, the cytotoxic compound(s) of the fruits of *H. anatolica* may be this type of compound(s). Bioactivity guided fractionation of the dichloromethane extracts of the fruits of *H. anatolica* is planned to isolate and identify their cytotoxic principles.

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Artemisia campestris and *Artemisia herba-alba*: LC-HRESI-MS Profile Alongside Their Antioxidant and Antimicrobial Evaluation

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ABSTRACT

Artemisia species have been traditionally used to treat various ailments and health problems like colds, digestive troubles, gastric ulcer, menstrual pain, and diarrhea. In our study, total phenolic and flavonoid contents of 80% aqueous methanol extracts of *Artemisia campestris* and *Artemisia herba-alba* plants were investigated. Furthermore, their *in vitro* antioxidant and antimicrobial activities were evaluated. Also, their phytochemical profiling was performed via using LC-HRESI-MS analysis. Both plant extracts showed strong antioxidant activity using DPPH, ABTS, and phosphomolybdenum assays. The results revealed *A. herba-alba* extract showed moderate antimicrobial activity against bacteria including *Staphylococcus epidermidis* and *Staphylococcus aureus*. While *A. campestris* extract exhibited antimicrobial activity against different microbial populations such as *Pseudomonas aeruginosa* and *Candida albicans*. Also, the results revealed that *A. herba-alba* extract contains high amounts of 3-*O*-methylquercetin, eupatilin and acacetin (ranging from 22.04 to 31.88 mg/g), while *A. campestris* extract contains significant amounts of 3-*O*-methylquercetin, rutin and chlorogenic acid (82.98, 79.44 and 29.54 mg/ g,

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respectively). In conclusion, the aqueous methanol extracts of both studied *Artemisia* species could be promising candidates for treating microbial infections and oxidative stress.

Keywords: *Artemisia campestris*, *Artemisia herba-alba*, polyphenolics, antioxidant, antimicrobial

INTRODUCTION

The high infection rates with infectious diseases as well as the extreme resistance of the pathogenic microbial organisms against the current antibiotics encouraged scientists to search for alternative drugs from safe natural sources such as medicinal plants, marine organisms and fungal extracts [1-4]. On the other hand, the over-production and the accumulation of free radicals inside the body leads to a phenomenon known as oxidative stress that causes many serious diseases such as cancer, cardiovascular diseases, and inflammations. Moreover, the harmful effects of this phenomenon can be diminished via using naturally occurring antioxidant compounds as free radical scavengers [5-8]. Additionally, the polyphenolic compounds especially flavonoids and phenolic acids have characteristic and optimum structural criteria to exert strong free radical scavenging activity among them heavy hydroxylation pattern, and extended conjugation [9].

The genus *Artemisia* (Asteraceae) comprises about 400 species, broadly disseminated in different regions around the world like Northern Africa, Western Asia, and Mediterranean area [10, 11]. Traditionally, different *Artemisia* species have been used to treat numerous ailments and health disorders like colds, coughing, intestinal disturbances, febrifuge, vermifuge, bronchitis, digestive troubles, gastric ulcer, menstrual pain, and diarrhea [12-18]. *A. herba-alba* is a perennial herb, known as "Chih", in Arabic and as "Armoise blanche" in France [11, 19]. In the same context, *A. campestris* L. is also a perennial herb, commonly known as field wormwood [11]. Numerous biological activities have been reported on the plant among them are antiproliferative [11], anti-tyrosinase [11], anti-cholinesterase [11], antioxidant [18], and antimicrobial activities [20]. Regarding cytotoxicity and toxic effects, Lahna et al (2020) reported that *A. herba-alba* aqueous extract showed LD_{50} value >2 g/kg bw, and the extract was toxic at the dose up to 2 g/ kg bw [21]. Moreover, the essential oils from *A. herba-alba* and *A. campestris* possess anti-leishmanial activity against *Leishmania infantum* promastigotes with IC_{50} values of 68 µg/mL and 44 µg/mL, respectively [22]. Furthermore, the essential oil of *A. herba-alba* possessed antiproliferative activity against the acute lymphoblastic leukaemia tumor cell line with IC_{50} value of 3 µg/mL [23]. From the phytochemical point of view, different classes of secondary metabolites have

been reported in the plant such phenolics [20], flavonoids [24], coumarins [25], and essential oil [11]. Therefore, the current study aims to investigate the *in vitro* antimicrobial and antioxidant potentials of the 80% aqueous methanol extracts of *A. campestris* and *A. herba-alba* as well as their chemical profiling via using LC-HRESI-MS analysis.

METHODOLOGY

Plant material

Flowering aerial parts of two plants from genus *Artemisia*, namely *A. campestris* and *A. herba-alba* were collected from Laghouat region, Southern Algeria (Latitude: 33° 47′59", Longitude: 2°51′54", Altitude: 764 m) during the spring season 2018. Collected plants have been kindly verified and authenticated by Dr. Mohamed Kouidri, Botanist, Department of Agronomy, Faculty of Sciences, University of Laghouat, Algeria, with the numbers LGP Ac/04/18 and LGP Aha/05/18, respectively.

Preparation of the aqueous methanol extracts

Dried and powdered 90 grams of the two plants were separately macerated in 500 mL of 80% methanol for 24 hours. The maceration solutions were concentrated by a rotary evaporator and the obtained aqueous methanol extracts were subjected to defatting process via using *n*-hexane to get rid of unwanted fatty compounds. Then, the defatted aqueous methanol extracts were dried under liquid nitrogen and stored for chemical and biological investigations.

Determination of total phenolic content

Approximately, 100 µL sample of each plant was added to 1.9 mL of diluted Folin-Ciocalteu's reagent (1:10, v/v) and was incubated for 5 minutes at room temperature [26]. The mixture was then added to 1.5 mL of 75 g/L Na_2CO_3. The absorbance of the mixture was measured at 765 nm using a spectrophotometer (OPTIZEN 2120UV Single beam UV/Vis spectrophotometer, Korea) after 30 minutes of incubation. The content of total phenolics was expressed as mg of gallic acid equivalent (GAE) per amount of sample in gram.

Determination of total flavonoid content

Aluminium chloride colorimetric method was used with some modifications to determine total flavonoid content [26]. One milliliter of the aqueous methanol extract was mixed with 3 mL of methanol, 0.2 mL of 10% aluminium chloride, 0.2 mL of 1M potassium acetate and 5.6 mL of distilled water and remains at room temperature for 30 minutes. Sample blank was prepared in a similar way by replacing aluminium chloride with distilled water. The absorbance was measured

at 420 nm. Rutin was used as standard (1 mg/mL). Flavonoid contents were determined from the standard curve and were expressed as rutin equivalent (RE) as mg/g sample.

In vitro antioxidant activities

DPPH assay

Aqueous methanol extracts were analyzed for their capacity to scavenge the stable DPPH radical according to Boulanouar et al (2013). The inhibition (IC_{50}) of free radical DPPH was calculated in percentage: $IC_{50} = [(A_{blank}-A_{sample})/A_{blank}] \times 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test extract), and A_{sample} is the absorbance of the test extract. The concentration of the test extract providing 50% inhibition (IC_{50}, expressed in μg/mL) was calculated from the graph plotted with inhibition percentage against the extract concentrations [26].

ABTS assay

The free-radical scavenging capacity was measured using the ABTS discoloration method according to the reported procedures [26]. The radical scavenging activity was calculated as a percentage of ABTS discoloration using the equation: % radical scavenging activity = $[(A_{ABTS}-A_S)/ A_{ABTS}] \times 100$, where A_{ABTS} is the absorbance of the ABTS solution and A_S is the absorbance of the solution containing the extract. The result was expressed as IC_{50} value in μg/mL calculated from the graph of ABTS scavenging percentage activity against extract concentrations.

Phosphomolybdenum assay

Phosphomolybdenum assay was carried following the reported procedures, [27] *i.e.*, formation of green phosphate/Mo (V) complex at acidic pH (reduction of Mo (VI) to Mo (V) by the sample). An aliquot of 0.1 mL of sample solution, containing 10 to 300 μg /mL of the tested aqueous methanol extract in ethanol, was combined in a tube with 1 mL of reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The mixture was incubated in at 95°C for 90 min. After that allowing the samples for cooling and read the absorbance at 695 nm using a blank contains 1 mL of reagent solution and the suitable volume of ethanol then was incubated under the similar conditions. The concentration of the test extract providing 50% inhibition (IC_{50}, expressed in μg/mL) was calculated from the graph plotted with inhibition percentage against the extract concentrations.

In vitro antimicrobial activity

The tested aqueous methanol extracts were evaluated for their *in vitro* antimicrobial activities against some pathogenic microbial strains including *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 14153, *Enterococcus faecalis* ATCC 29212, *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019, and *Candida tropicalis* ATCC 750 via using the broth micro dilution technique according to the Clinical Laboratory Standards Institute recommendations [28-29]. Mueller-Hinton broth for bacteria and RPMI-1640 medium for the yeast strain were used as the test media. Serial twofold dilutions ranging from 2500 µg/mL to 1.2 mg/L were prepared in the media. The inoculum was prepared using a 4-6 h broth culture of each bacteria and 24 h culture of yeast strains adjusted to a turbidity equivalent of a 0.5 McFarland standard, diluted in broth media to give a final concentration of 5×10^5 cfu/mL for bacteria and 0.5×10^3 to 2.5×10^3 cfu/mL for yeast in the test tray. The trays were covered and placed in plastic bags to prevent evaporation. The trays containing Mueller-Hinton broth were incubated at 35°*C* for 18-20 h while the trays containing RPMI-1640 medium were incubated at 35°*C* for 46-50 h. The minimum inhibition concentration was defined as the lowest concentration of compound giving complete inhibition of visible growth. As a control, antimicrobial effects of the solvents were investigated against test microorganisms. The results were evaluated according to the values of the controls. Ceftazidime, cefuroxime-Na and amikacin for bacteria; clotrimazole and amfotericin B for yeast were used as reference antimicrobials. The minimum inhibition concentration values of the reference antimicrobials were within the accuracy range in Clinical Laboratory Standards Institute throughout the study.

LC-HRESI-MS conditions and preparation of tested extract solutions

A. campestris aqueous methanol extract was prepared as 0.049 g/5 mL, while *A. herba-alba* aqueous methanol extract was prepared as 0.051 g/5 mL in MeOH. The final concentration of the internal standard solution (100 mg/L) is added to the extracts to be 3 ppm. The sample was filtered through 0.45 µ filter and 2 µL from the sample was injected into the instrument [30]. Liquid chromatography high resolution mass spectrometry (LC-HRMS) measurements were made with a Thermo Orbitrap Q-Exactive instrument at ESI source and provided with a Troyasil C18 column (3.0 × 150 mm i.d., 3.0 µm). Mobile phases consisting of 1% formic acid (A) and methanol with 1% formic acid (B) were used in the following

gradient elution method: 0-1 min, 50% B; 3-6 min, 100%; and 7-10 min, %50 B. The flow rate was 0.35 mL/min, the injection volume was 2 µL and the total run time was 10 min. In determinations, the temperature was 22.0 (± 5.0) °C and relative humidity was 50 (± 15) % RH. Ions between *m/z* 85-1500 are scanned in high resolution mode of the device. The identification of the compounds was made by comparing the retention time of the standard compounds (purity in the range of 95%-99%) with the HRMS data of Bezmialem Foundation, University, Drug Application and Research Center Library (İLMER).

Statistical analysis

All measurements were performed in triplicates, with the results were expressed as mean ± SD. Microsoft Excel program was used for statistical data analysis.

RESULTS and DISCUSSION

Total phenolic content, total flavonoid content and *in vitro* antioxidant activities

The total phenolic contents of the 80% aqueous methanol extracts of *A. herba-alba* and *A. campestris* are 304.88 and 212.87 gallic acid equivalent (GAE)/ g dry extract, respectively. While, their total flavonoids contents are 37.74 and 75.96 rutin equivalent (RE)/ g dry extract, respectively. On the other side, the two extracts showed remarkable *in vitro* antioxidant activities using three antioxidant models. In the DPPH assay, their IC_{50} values are 36.5 and 20.2 µg/mL, while in the ABTS assay are 24.10 and 9.50 µg/mL, however in the phosphomolybdenum assay are 43.25 and 30.5 µg/mL for *A. herba-alba* and *A. campestris*, respectively (Table 1).

Table 1. Total phenolic content (TPC), total flavonoid content (TFC), and *in vitro* antioxidant activities (DPPH, ABTS, and PPM) of the 80% aqueous methanol extracts of A. *campestris* and *A. herba-alba*

Sample	TPC (GAE/g dry extract)	TFC (RE/g dry extract)	DPPH assay (IC_{50} µg/mL)	ABTS assay (IC_{50} µg/mL)	PPM assay (IC_{50} µg/mL)
A. herba-alba	304.88 ± 27.05	37.74 ± 5.03	36.5 ± 1.1	24.10 ± 3.5	43.25 ± 2.15
A. campestris	212.87 ± 11.74	75.96 ± 10.40	20.2 ± 0.8	9.50 ± 0.9	30.5 ± 1.25
Ascorbic acid	-	-	5.40 ± 0.2	2.80 ± 0.1	8.58 ± 0.50

Our previous study revealed that the hydroalcoholic extract of *A. campestris* grown in Algeria showed antioxidant oxygen radical absorbance capacity value of 120.5±10.4 µmol Trolox equivalent, with a total phenolic content value of 102.09±1.65 mg/g gallic acid equivalent [18]. Additionally, the methanolic ex-

tracts of Algerian *A. herba-alba* and *A. campestris* were evaluated for their total phenolic and flavonoids contents as well as their DPPH scavenging activity. TPCs values were 8.64 and 20.53 mg GAE/g dry material, while TFCs values were 5.47 and 11.11 mg QE/g dry material, respectively for *A. herba-alba* and *A. campestris*. On the other hand, the extracts showed DPPH scavenging activities with EC_{50} values of 33.71 and 2.47 µg/mL, respectively for *A. herba-alba* and *A. campestris* [31]. Megdiche-Ksouri et al (2015) reported that the methanolic and ethyl acetate extracts of Tunisian *A. campestris* demonstrated high DPPH antioxidant scavenging activity with IC_{50} values of 6.0 and 10.0 µg/mL, respectively) [32]. Additionally, the methanolic extract of Tunisian *A. herba-alba* exhibited DPPH free radical scavenging activity with IC_{50} value of 100.0 µg/mL [33].

Antimicrobial activities

The minimum inhibition concentration of the 80% aqueous methanol extract of *A. herba-alba* is 1250, 156.25, and 312.5 mg/L against *E. faecalis*, *S. epidermidis*, and *S. aureus*, respectively and there is no any recorded activity against the rest of the microbial strains. While the minimum inhibition concentration of the 80% aqueous methanol extract of *A. campestris* is 1250, 156.25, 312.5, and 625 mg/L against *P. aeruginosa*, *S. epidermidis*, *S. aureus*, and *C. albicans*, respectively and there is no any recorded activity against the rest of the microbial strains (Table 2). Both of *Artemisia* species showed antibacterial activity mainly against the gram-positive bacteria strains, especially *S. epidermidis*, with the minimum inhibition concentration values of 156.2 mg/L. These results indicated that both of studied *Artemisia* species had the potency especially for *S. epidermidis* to be further studied. Moreover, several *in vitro* antimicrobial activity studies of *Artemisia* species have reported similar results with our present study. The methanolic extract of fresh leaves of *A. campestris* grown in southern Libya showed *in vitro* antimicrobial activity against five pathogenic microbial strains with minimum inhibition concentration values of 12.5, 12.5, 250, 500, and 500 µg/ml, respectively for *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, and *S. typhi* [34]. Moreover, the different solvent extracts of *A. campestris* grown in south Tunis (e.g., methanolic, ethyl acetate, and water) were evaluated for their antibacterial activities against fourteen bacterial species. The results revealed that the ethyl acetate and methanolic extracts showed promising bacterial inhibition against *L. mono-cytogenes* (13 and 12.7 mm, respectively), and *B. thuringiensis* (18.3 and 13 mm, respectively). While the water extract showed activity against *V. parahaemolyticus* with inhibition zone value of 9 mm at 300 mg/L [32]. In the same context, the methanolic extract of the aerial part of *A. herba-alba* grown in south Tunis was evaluated for its antibacterial activity against gram positive and gram-negative bacteria. The inhibition

Table 2. Minimum inhibition concentrations (MICs) of the 80% aqueous methanol extracts of *A. campestris* and *A. herba-alba* against ten pathogenic microbial strains (mg/L)

Extract	P. aeruginosa ATCC 27853	E. coli ATCC 25922	K. pneumoniae ATCC 4352	P. mirabilis ATCC 14153	E. faecalis ATCC 29212	S. epidermidis ATCC 12228	S. aureus ATCC 29213	C. albicans ATCC 10231	C. parapsilosis ATCC 22019	C. tropicalis ATCC 750
A. herba-alba (mg/L)	-	-	-	-	1250[a]	156.25	312.5	-	-	-
A. campestris (mg/L)	1250	-	-	-	-	156.25	312.5	625	-	-
Controls (mg/L)	2.4[a] Ceftazidime	4.9 Cefuroxime-Na	4.9 Cefuroxime-Na	2.4 Cefuroxime-Na	128 Amikacin	9.8 Cefuroxime	1.2 Cefuroxime-Na	4.9 Clotrimazole	0.5 Amphotericin B	1 Amphotericin B

zone values were 12.0, 15.5, 11.5, and 22.5 mm, respectively for *S. aureus*, *E. coli*, *P. aeruginosa*, and *B. cereus* [33]. Moreover, Trinh et al (2018) reported that the 70% ethanol extract of *A. apiacea* H. grown in Korea showed antimicrobial effect against *A. niger*, *C. albicans*, *B. subtilis* and *S. aureus* with minimum inhibition concentration values ranged from 0.03125 to 4 mg/mL [35]. Additionally, the ethanol, methanol and hexane extracts from *A. absinthium*, *A. annua* and *A. vulgaris* showed antimicrobial activities against five gram-positive bacteria, two gram-negative bacteria and one fungal strain with inhibition zones ranged from 6 to 20 mm [36]. Methanol extracts from aerial parts of *A. diffusa*, *A. oliveriana*, *A. scoparia* and *A. turanica* were tested for their antimicrobial activities against *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, and *C. albicans* with inhibition zones ranged from 9.4 to 18.4 mm [37]. Moreover, various solvent extracts of *A. parviflora* were tested for their antimicrobial potential against some pathogenic microorganisms including *B. subtilis*, *S. aureus*, *E. coli*, *Y. enterocolitica*, *P. vulgaris*, *P. aeruginosa*, *K. pneumoniae*, *S. flexneri*, *E. faecalis*, and *E. aerogenes* with minimum inhibition concentration values ranged from 32 to 256 µg/mL [38].

[a]The results of the antimicrobial activity of both controls and the aqueous methanol extracts can be seen in the appendix in mg/L. Both of the extracts have activity against gram-positive microorganisms at a moderate level. *A. campestris* extract also has a low level of gram negative (*P. aeruginosa*) and yeast (*C. albicans*).

LC-HRESI-MS chemical profiling and quantification of secondary metabolites

In this work, LC-HRESI-MS analysis of the 80% aqueous methanol extracts of *A. campestris* and *A. herba-alba* led to the identification of 22 secondary metabolites. Based on their retention times and fragmentation patterns these compounds were classified as flavonoids (aglycones, glycosides), phenolic acids as well as their derivatives and triterpenoids (Table 3, Figures 1-3). LC-HRESI-MS analysis revealed that *A. campestris* extract contains high amounts of 3-*O*-methylquercetin, rutin, nepetin and chlorogenic acid (82.98, 79.44, 32.36 and 29.54 mg/g, respectively). While *A. herba-alba* extract was found to contains quite high amounts of 3-*O*-methylquercetin, eupatilin, acacetin and nepetin (31.88, 29.64, 22.04 and 15.47 mg/g, respectively). Moreover, both plants were found to contain cynarin which is caffeoylquinic acid derivative, as clearly seen in LC-HRESI-MS chromatograms (Figures 1 and 2). However, since we could not provide the standards of caffeoyl quinic acids, we could not determine the exact quantification. The two peaks seen in these chromatograms most likely belong to 1,3- and 3,5-dicaffeoylquinic acid derivatives. In the literature,

LC-DAD-MS/MS analysis of the methanolic extract from the aerial part of *A. herba-alba* grown in Tunisia led to the identification of ten polyphenolic compounds namely chlorogenic acid, apigenin-6,8-di-*C*-glu, apigenin-6-*C*-ara-8-*C*-glu, apigenin-6-*C*-glu-8-*C*-ara, apigenin-6-*C*-pent-8-*C*-glu, apigenin-6-*C*-glu-8-*C*-pent, quercetin-rha-glu, 1,4-dicaffeoylquinic acid, 3,4,5-tricaffeoylquinic acid, and 3,4-dicaffeoylquinic acid [33]. Also, LC/MS analysis of different solvent extracts of *A. campestris* grown in Tunisia led to tentative identification of thirty-nine compounds comprising coumarins, flavones, flavonols, phenolic acids, and sesquiterpenes. The main ingredients are luteolin-7-*O*-rutinoside, rhamnetin, isorhamnetin, hydroxycoumarin, kaempferol rutinoside, and di-*O*-caffeoylquinic acid isomers [32]. On the other hand, HPLC-PDA-ESI/MS-MS analysis of 70% ethanol extract from the aerial part of *A. annua* led to the identification of certain classes of secondary metabolites among them are flavonoid glycosides, caffeoyl- and feruloylquinic acid derivatives [39]. Mouton et al (2014) reported the identification of eleven compounds from the aqueous extract of German *A. annua* namely scopolin, *cis*-melilotoside, chlorogenic acid, 5-feruloylquinic acid, *trans*-melilotoside, scopoletin, 3,5-dicaffeoylquinic acid, rutin, caffeoyl feruloylquinic acid, chrysosplenol D, and chrysosplenetin [40]. Moreover, six methoxylated flavones *viz*., jaceosidin, hispidulin, eupalitin, eupatorin, casticin, and acacetin as well as two hydroxycinnamic acids namely caffeic and chlorogenic acids were detected via LC-MS analysis in the aerial parts of *A. annua*, *A. vulgaris,* and *A. absinthium* grown in Romania [41]. Furthermore, umbelliferon, chlorogenic acid, rutin, di-caffeoylquinic acid isomers, scopolin, scopoletin, 4-hydroxycoumarin, 3-hydroxycoumarin, luteolin, isorhamnetin, apigenin, and rhamnazin were detected via LC-MS analysis in the ethanolic and chloroform extracts from *A. gmeilinii* grown in Kazakhstan [42]. In the same context, HPLC-DAD-MS study and quantitative determination of polyphenols in the aerial parts from *A. absinthium*, *A. annua*, and *A. vulgaris* led to identification of gentisic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, hyperoside, isoquercitrin, rutoside, fisetin, quercitrin, quercetin, patuletin, luteolin, kaempferol, and apigenin [41]. HPLC-MS/MS analysis of polyphenols from *A. argyi* H. grown in Korea led to identification of caffeoylquinic acid isomer, 6,8-di-*C*-glucosylapigenin, 6-*C*-arabinosyl-8-*C*-glucosylapigenin, secoisolariciresinol, amentoflavone isomer, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucuronide, dicaffeoylquinic acid, 3,4,5-*O*-tricaffeoylquinic acid, quercetin dimethyl ether, skullcapflavone II, and calcelarioside A [43]. A study carried out by Lee and co-authors regarding the simultaneous determination of the phytoconstituents in *A. apiacea* via HPLC-DAD-UV/Vis revealed that calibration data of the three standard compounds showed good linearity (R^2>0.9994)

in a relatively wide concentration range. Also, limits of detection (LOD) and limits of quantification (LOQ) values of all standard compounds were in the range 0.55-7.07 μg/mL and 1.67-21.44 μg/mL, respectively [44].

Table 3. Compounds determined in *A. campestris* and *A. herba-alba* 80% aqueous methanol extracts and their amounts (mg/g extract)

Compounds	A. campestris	A. herba-alba
(-)-Catechin gallate	0.01	<LOD
(+)-Trans-taxifolin	3.54	<LOD
3-O-methylquercetin	82.98	31.88
Acacetin	2.97	22.04
Apigenin 7-glucoside	0.16	6.48
Apigenin	0.25	0.49
Caffeic acid	2.58	2.24
Chrysin	0.01	0.10
Dihydrokaempferol	3.81	10.75
Eupatilin	7.70	29.64
Fumaric acid	1.02	7.86
Hederagenin	<LOD	0.19
Hyperoside	6.52	1.79
Isosakuranetin	2.50	0.39
Luteolin-7-rutinoside	0.17	2.47
Myricetin	0.11	0.17
Naringenin	5.37	0.61
Nepetin	32.36	15.47
Quercetin	7.56	0.11
Quillaic acid	<LOD	0.92
Rutin	79.44	3.37
Chlorogenic acid	29.54	0.48

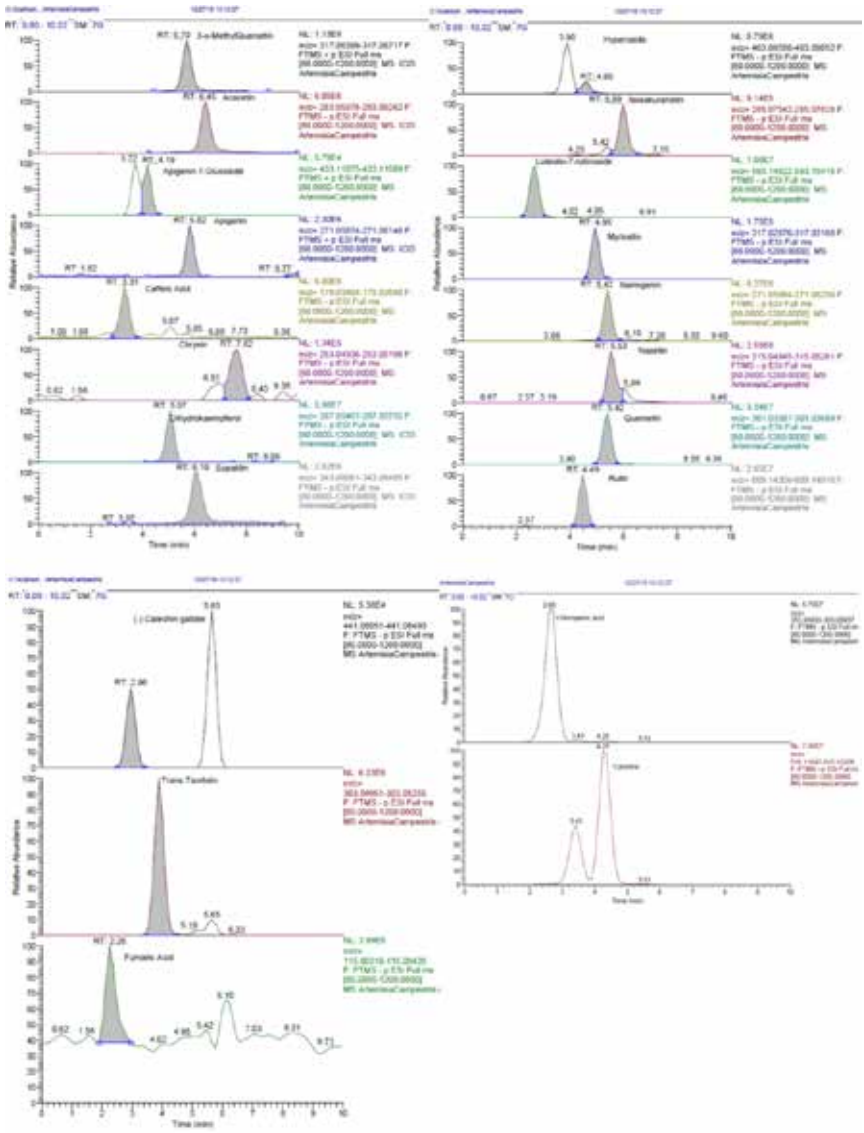


Figure 1. LC-HRESI-MS chromatograms of *A. campestris* 80% aqueous methanol extract

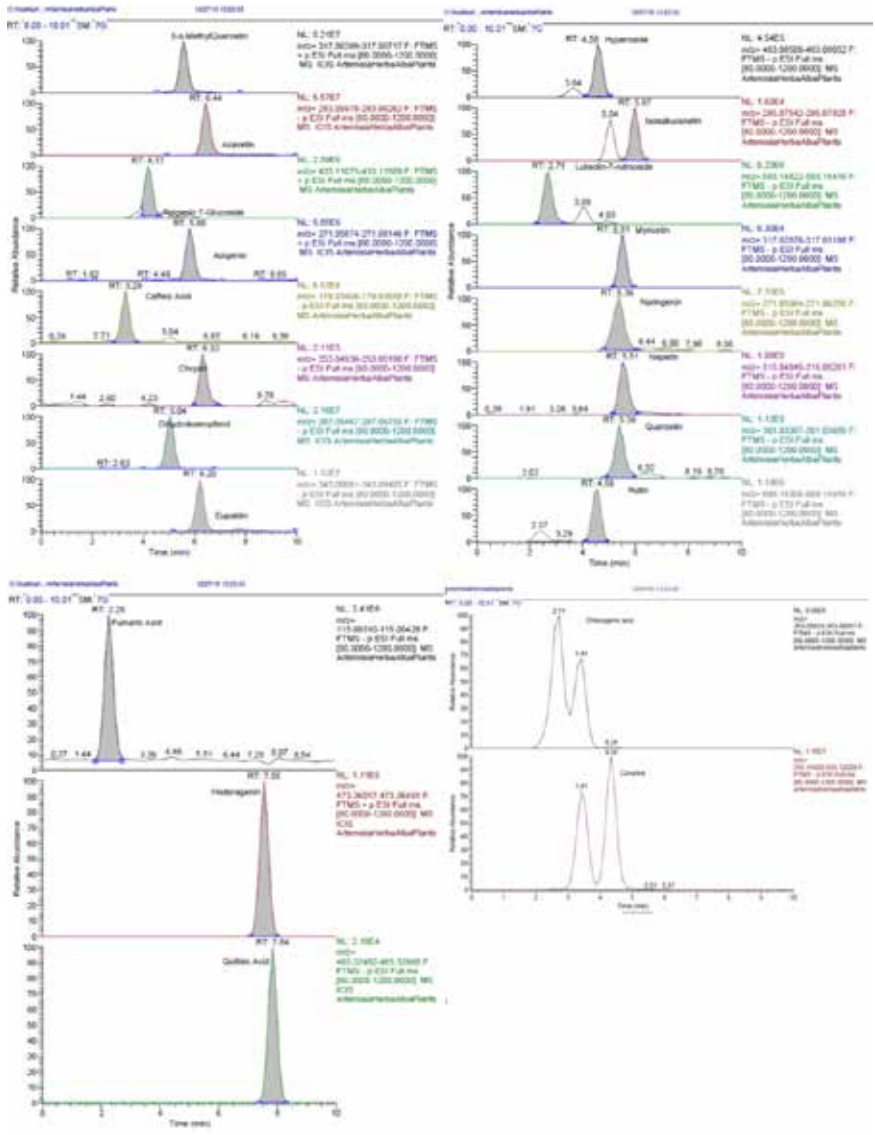
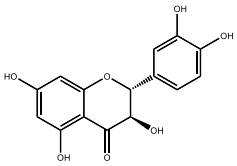
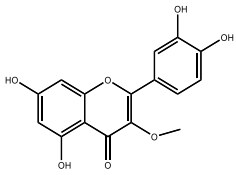


Figure 2. LC-HRESI-MS chromatograms of *A. herba-alba* 80% aqueous methanol extract



Taxifolin



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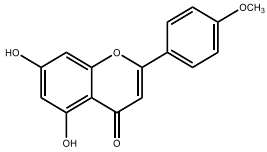

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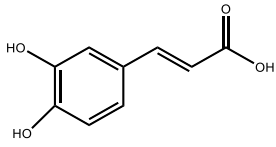

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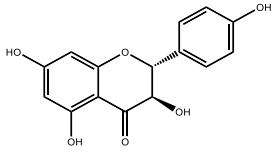

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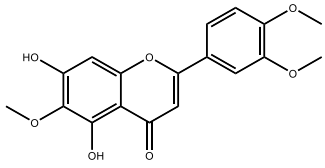

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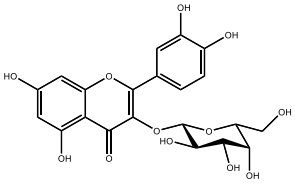

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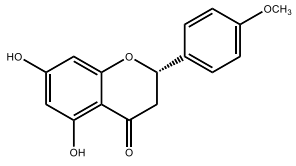

Isosakuranetin

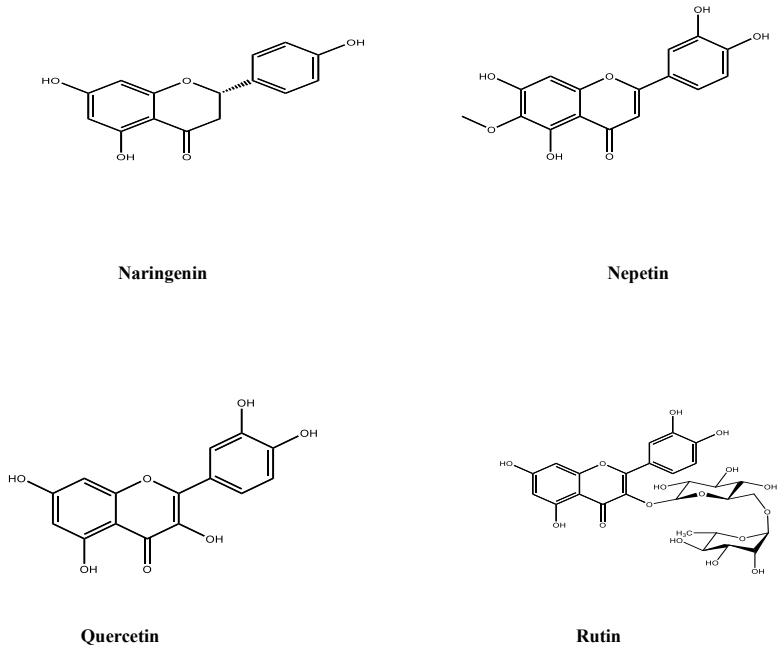

Figure 3. Chemical structures of some major identified compounds in the 80% aqueous methanol extracts of the two species

Method validation for LC-HRESI-MS analysis

The calibration curve was obtained by plotting the detector responses corresponding to the concentrations of the standard compound solutions, separately and relative regression coefficient (R^2) was calculated to authenticate its linearity. The calibration data of the standard compounds showed suitable linearity (R^2> 0.993) in a relatively broad concentration scale. The LOD and LOQ values of all standard compounds are in the range 0.08-2.56 mg/L and 0.28-8.53 mg/L, respectively (Table 4). The recovery was in the range of 95.67% to 106.37%. Measurement uncertainty was determined according to GUM and EA-4/02 documents [45-47].

Table 4. The regression data, *LOD, LOQ* of twenty-two compounds in the 80% aqueous methanol extracts of *A. campestris* and *A. herba-alba*

Compound	Ionization mode	m/z	Linear regression equation	R^2	LOD/ LOQ* (mg/L)	Recovery %
(-)-Catechin gallate	Negative	441.0827	y=1.065e-2X + 6.756e-4[a]	0.999	0.11/0.38	96.8
(+)-Trans-taxifolin	Negative	303.0510	y=1.289e-2X + 2.513e-3	0.999	0.14/0.47	99.3
3-O-methyl quercetin	Positive	317.0656	y=1.129e-2X + 1.507e-3	0.993	0.22/0.75	101.7
Acacetin	Negative	283.0612	y=1.867e-2X - 1.874e-3	0.998	0.13/0.42	99
Apigenin 7-glucoside	Positive	433.1129	y=2.935e-3X + 2.157e-4	0.996	0.18/0.60	102.47
Apigenin	Positive	271.0601	y=6.223e-2X + 1.074e-2	0.998	0.22/0.72	99.6
Caffeic acid	Negative	179.0350	y=1.68e-2X + 5.922e-3	0.999	0.19/0.62	102.3
Chrysin	Negative	253.0506	y=2.735e-2X - 1.414e-3	0.996	0.21/0.69	97.17
Dihydrokaempferol	Negative	287.0561	y=1.34e-2X + 5.461e-3	0.999	0.11/0.36	104.23
Eupatilin	Negative	343.0823	y=3.182e-3X - 5.419e-5	0.999	0.1/0.33	100.3
Fumaric acid	Negative	115.0037	y=1.855e-3X + 5.312e-4	0.997	0.26/0.88	97.27
Hederagenin	Positive	473.3625	y=3.913e-4X + 6.82e-4	1.000	2.56/8.53	99.33
Hyperoside	Negative	463.0882	y=2.326e-3X - 2.487e-4	0.989	0.33/1.09	95.67
Isosakuranetin	Negative	285.0769	y=2.6e-3X + 4.973e-4	0.995	0.23/0.77	106.37
Luteolin-7-rutinoside	Negative	593.1512	y=5.179e-3X + 8.77e-4	0.997	0.22/0.73	102.4
Myricetin	Negative	317.0303	y=1.229e-2X - 1.743e-3	0.998	0.13/0.45	97.53
Naringenin	Negative	271.0612	y=1.08e-2X + 1.351e-3	0.997	0.2/0.67	97.73
Nepetin	Negative	315.0510	y=5.633e-2X + 8.265e-3	0.997	0.12/0.40	100.43
Quercetin	Negative	301.0354	y=3.326e-2X + 5.001e-3	0.998	0.16/0.54	99.03
Quillaic acid	Negative	485.3273	y=5.453e-3X + 9.866e-5	0.999	0.08/0.28	102.9
Rutin	Negative	609.1461	y=2.365e-3X + 7.711e-4	0.993	0.25/0.85	98.4
Chlorogenic acid	Negative	353.0878	y=0.00817x+0.000163	0.999	0.02/0.06	99.8

[a]*y*: Peak area; *X*: Amount (mg/L), Limits of detection (*LOD*), Limits of quantification (*LOQ*)

Limitations of the Study

In this study, the 80% aqueous methanol extracts of *A. campestris* and *A. herba-alba* plants that macerated with aqueous methanol were examined, but not with any other polar solvents. In addition, methods such as hot and supercriti-

cal fluid extraction could be tried in extraction of plants to possibly contribute to more efficient and/or better investigation of chemical component content. Due to the lack of sufficient financial resources, biological tests such as anti-alzheimer and cytotoxicity screening of plant extracts, which were found to be rich in flavone and phenol components, could not be performed. Also, the mechanisms of action of the extracts against microbial strains have not been investigated. Further studies are needed for these issues.

The present study disclosed that the main identified compounds in the aqueous methanol extracts of the two plants are 3-*O*-methylquercetin, eupatilin, acacetin, rutin and chlorogenic acid. Additionally, remarkable antimicrobial potential was detected from the two species. In conclusion, the aqueous methanol extracts of the both studied *Artemisia* species could be considered as important antioxidant and antibacterial sources and also demonstrate the importance of these medicinal plants in the food industries.

ABBREVIATIONS

LC-HRESI-MS: Liquid chromatography–high resolution electrospray mass spectrometry; CEM: Acute lymphoblastic leukaemia tumor cell line; TPC: Total Phenolic Content; UV/Vis: Ultraviolet–visible spectrophotometry; GAE: Gallic acid equivalent; TFC: Total Flavonoid Content; RE: Rutin equivalent; DPPH: 2,2-diphenyl-1-picrylhydrazyl; IC_{50}: The half maximal inhibitory concentration; ABTS: 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid; RSA: Radical scavenging activity; ATCC: American Type Culture Collection; CLSI: Clinical Laboratory Standards Institute; MIC, The minimum inhibition concentration; ESI: Electrospray ionization; HPLC-DAD-ESI-MS: High performance liquid chromatography-Diode array detection-Electrospray ionization-Mass spectrometry; LOD: Limits of detection; LOQ: Limits of quantification.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

B.B. and M.A.G.: Conceived and designed the experiments, searched information, analyzed it, wrote the original paper and revised the final version; A.C.G, Z.A. and E.M.: Performed the LC-MS analysis and revised the manuscript; A.C.G, Z.A. and E.M.: Performed the biologic assays and statistical analysis. All authors have read and agreed to the published version of the manuscript.

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Formulation and Evaluation of Sugar Spheres Containing Antiemetic Drug

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ABSTRACT

The aim of the research study was to formulate and evaluate Metoclopramide loaded sugar spheres for oral drug delivery. Sugar spheres were prepared by extrusion-spheronization method. Drug-excipients characterization studies were done by FTIR and UV analysis. Formulations were subjected to various evaluation parameters such as particle size, SEM (Scanning Electron Microscopy), drug content and *in vitro* drug release studies. Anti-emetic study was carried out by using rat model. Surface morphology of sugar spheres by SEM, showed it was spherical. Drug content was found to be 93.7%. The results of *in vitro* drug release data of the optimized sugar spheres formulation F8 showed drug release up to 98%. From the animal study, it was observed that F8 formulation showed better antiemetic activity. Stability studies were carried out for F8 formulation, revealed that there were no noticeable changes in drug content and *in vitro* drug release study. Hence, it is suitable candidate for the treatment of emesis, special focus on pediatrics.

Keywords: Sugar spheres, metoclopramide, emesis, extrusion-spheronization

INTRODUCTION

Metoclopramide is an antiemetic drug which is substituted benzamide and a derivative of para-amino benzoic acid (PABA), is structurally related to procainamide. The available marketed Metoclopramide dosage form is in liquid form are undergoing hydrolysis and oxidation slowly, which decreases the shelf life of the product. Hence it would be beneficial to improve the patient compliance and enhance the oral solubility of Metoclopramide by sugar spheres or pellets, which

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would in turn lead to probable improvement in the bioavailability of the drug[1-3]. Pellets are the aggregates of granules or fine powder mixture of drug and excipients; they are small spherical or semi spherical free flowing solid units ranging from 0.5 to 3 mm, which are usually used for oral administration. The pellets are usually compressed into tablets or filled into hard gelatin capsules, they are usually formulated as immediate release or sustained release dosage forms, and they can also be coated to target a particular site for its action[4]. Extrusion-Spheronization is a multistep process, the advantage of this method is to fabricate the spheres with high drug loading i.e.; up to 90%. This process involves the dry mixing of the drug and excipients to achieve a homogenous mixture, then followed by wet massing of the dry mixture, then it is granulated, extrusion of the wet mass is carried out where noodle like extrudates are obtained, then this mass is transferred into the spheronizer which produces the spherules, then these spherules are dried in dryer followed by screening is done to obtain a required particle size. This process is called as mass extrusion-spheronization[4, 5]. Our present work is to formulate and evaluate sugar spheres containing antiemetic drug for paediatric administration as conventionally available formulation for pediatrics' are liquid dosage form. These formulations undergo oxidative and hydrolytic degradation. It has less bioavailability, as it undergoes first pass metabolism. Therefore, to overcome these problems mouth dissolving sugar spheres loaded with Metoclopramide was formulated. The main objective of the research work was to formulate and evaluate sugar spheres containing antiemetic drug for treatment of emesis [6-8.]

METHODOLOGY

Metoclopramide were procured from All-well Pharmaceuticals Chandigarh India, β-Cyclodextrin and other excipients from CDH Bengaluru, India. Other solvents and chemicals are analytical grades.

Preformulation study

A preformulation study was useful to identify the Metoclopramide drug and studied the compatibility studies with the excipients. Preliminary solubility studies were carried out for known quantity of drug with different solvents. Melting point of drug was determined by using Thiele tube apparatus. Determination of λ_{max} was analyzed using UV spectrophotometer using phosphate buffer pH 6.8. Calibration curve was constructed using phosphate buffer pH 6.8, after suitable dilutions absorbance was taken using UV visible spectrophotometer at 240 nm[9, 10].

Drug-excipient Compatibility Studies (FTIR)

Metoclopramide was placed in the sample port of FTIR; the spectrum of Metoclopramide was recorded. Compatibility studies were carried out to know the possible interactions between Metoclopramide and β- cyclodextrin, Drug: β-cyclodextrin complex with 1: 4 ratio was considered to know the interactions, based on high drug loading capacity[11].

Preparation of Metoclopramide complex with β-Cyclodextrin (β-CD) by Kneading Method

Metoclopramide and β-cyclodextrin in molar ratios of 1:1, 1:2, 1:3 and 1:4 were taken in mortar and pestle, and then wetted with appropriate quantity of IPA to obtain a paste. All 1:1, 1:2, 1:3 and 1:4 molar ratios were then subjected to kneading by trituration for 30 min. After that it was dried at 50°C, crushed, sieved and stored at temperature of 25±2.0°C and relative humidity between 40-50% RH[12-14].

Preparation of Metoclopramide sugar spheres by Extrusion-Spheronization method

Formulation chart was prepared by using drug-complex with different ratios of disintegrants & other excipients in F1-F9 formulations as shown in the Table 1. Extrusion-Spheronization are a multistage process for obtaining pellets with uniform size from wet mass using non-aqueous solvents. The method involves the following steps. The dry mixing of the drug and excipients, in order to achieve homogenous powder dispersions[15-18]. The powder mixture was wet mixed with IPA as a solvent to form a sufficiently damp mass. In extrusion stage, the wet mass is converted into cylindrical segments with a uniform diameter of 3 mm at 50 rpm. In spheronization stage, the small cylinders are rolled into solid spheres (spheroids) at 500 rpm. The drying of the spheroids at 45°C in order to achieve the desired final moisture content. Screening (optional), to achieve the desired narrow size distribution [19- 23].

Table1: Formulation chart of Sugar spheres containing Metoclopramide

Ingredients	F1	F2	F3	F4	F5	F6	F7	F8	F9
Drug complex (1:4) g	8.02	8.02	8.02	8.02	8.02	8.02	8.02	8.02	8.02
Sucrose (g)	58.98	56.98	54.98	52.98	50.98	55.98	52.98	49.98	46.98
MCC (g)	30	30	30	30	30	30	30	30	30
Sodium starch glycolate (%)	0	2	4	6	8	-	-	-	-
Cross carmellose sodium (%)	-	-	-	-	-	3	6	9	12
Preservatives (%)	1	1	1	1	1	1	1	1	1
Methyl cellulose (%)	2	2	2	2	2	2	2	2	2
IPA	QS	QS	QS	QS	QS	QS	QS	QS	QS
Flavor (Vanillin)	QS	QS	QS	QS	QS	QS	QS	QS	QS
Color	QS	QS	QS	QS	QS	QS	QS	QS	QS
Batch size	100 g								

EVALUATION OF SUGAR SPHERES

Physicochemical characteristics of pellets[24]:

The porosity, compressibility and density of prepared pellets were defined by bulk density (BD), tapped density (TD) and porosity was determined by using digital density apparatus (Innovative XCN 77). In density apparatus about 10 g of pellets were added into a 100 mL calibrated measuring cylinder. The initial volume (bulk volume) is noted down. After 100 tapping further final volume were noted, the following equations were used for calculating the BD and TD. Then, flow properties were calculated by using Hausner's ratio formulas given below.

$$\text{B}ulk\ Density = Weight\ of\ pellets/Bulk\ volume$$

$$Tapped\ density = Weight\ of\ pellets/(Tapped\ volume)$$

$$Porosity = Volume\ of\ voids/Total\ volume$$

$$Hausner's\ ratio = Bulk\ density/Tapped\ density$$

Angle of repose[24]

The angle of repose was calculated using the fixed funnel method. The pellets, were measured exactly weight equivalent to 10 g, had been transferred to the funnel. The funnel height was set in such a way that the funnel tip reached the heap apex of the pellets. The pellet was allowed to flow through the funnel freely on to the surface. Finally, the diameter of pellets cone was measured. The angle of repose was calculated using following formula.

$$Angle\ of\ repose\ (\theta) = tan - 1h/r$$

Where, *'h'* and *'r'* are height and radius of the pellet cone.

Friability

Friability of the pellets was performed by using USP Roche friabilator (FE2, Electro lab, Mumbai India). Pre-weighed about 5 g pellets were placed in the plastic chamber, along with pellets around 10 g of glass pellets were added to increase the stress level pellets. It was then operated for 100 revolutions. Pellets were dropping from a distance of six inches with each revolution, after completion of desired cycles; pellets were removed from friabilator, dusted and % friability was calculated by using formula [24].

$$\%\ Friability = Initial\ weights\ of\ pellets - Final\ weight\ of\ pellets/Initial\ Weight\ of\ pellets \times 100$$

Particle size: Particle size was determined by using sieve analysis using sieve shaker machine.

Drug Content [24]

About 10 g of pellets were crushed and powdered. Weighed accurately the quantity equivalent to 5 mg of drug and taken in 10 mL volumetric flask and volume was made up to the mark with phosphate buffer pH 6.8 and filtered through 0.45 µm Whatman filter paper and 1mL of this solution was taken and volume made up to 10 mL of phosphate buffer pH 6.8. The absorbance was measured at 240 nm using UV Spectrophotometer (Shimadzu 1800, Japan). Percentage drug content is calculated by using formula.

$$Drug\ content = Concentration \times Dilution\ factor$$

$$\%Drug\ content = Drug\ content(mg)/Label\ Claim\ (mg)$$

Disintegration test [25, 26, 27]

Disintegration test was performed using disintegration tester (USP) (Electrolab, ED 2L, India). About 1 g of pellets were weighed and placed in all the 6 tubes and the disc was placed at the top, the beaker was filled with phosphate buffer pH 6.8 solution, the disintegration was carried out and the bath temperature was maintained at 37±2°C. Disintegration time was recorded. The pellets were considered to be completely disintegrated as no residue remains on the screen.

Scanning electron microscopy

Scanning electron microscope, it produces images of samples by scanning it with focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that can be detected and that contain information about the samples surface topography. SEM photographs were taken for the prepared sugar spheres using a scanning electron microscope (Carl Zeiss FESEM model number: ULTRA 55 USA), at room temperature. The photographs were observed for morphological characteristics. Photographs were taken at the magnifications of 22.00 X and 38.00 X[25].

In vitro drug release studies

Dose equivalent to 5 mg of drug containing Metoclopramide pellets using USP 29 type I apparatus (Dissolution Tester EDT 08Lx, Electrolab, Mumbai, India) stirred at speed of 50 rpm in 900 mL of phosphate buffer pH 6.8 at 37±0.5°C. About 1 mL sample was withdrawn at predetermined time intervals and it was replaced with fresh dissolution media to maintain the sink condition. The withdrawn sample were filtered through 0.45 µm membrane filter and the volume was made up to 10 mL using volumetric flask and analyzed the absorbance periodically by using UV spectrophotometer (UV1700, Shimadzu, Japan) at 240 nm[25].

Antiemetic activity in rat model

The experimental protocol for the antiemetic activity was approved by Institutional Animal Ethical Committee IAEC/ABMRCP/2019-2020/5. Antiemetic activity was carried out using 8 female rats as they are more sensitive compared to male rats. Each rat weighing between 200-250 g were selected. Animals were divided into 3 groups. Out of 3 groups, 3 animals in emetic group, 3 animals in antiemetic group and 2 animals in control group. Kaolin pellets and saccharine solution were placed on the stainless-steel grid cover of the cage for 6 days prior to 5-flurouracil to allow the rats to adopt to its presence. All the animals were given oral administration of physiological saline for 3 days (dosage 2 mL/day) prior to administration of the 5-flurouracil. On 4th and 5th day, for Metoclopramide group, Metoclopramide solution (0.45 mg/kg body weight; dosage

0.5 mL/day), given through oral administration, whereas the other 2 groups continued to receive physiological saline. On the 7th day, 5-flurouracil (0.2 mL through oral route) was administered to all rats, except blank group. After 1 hour subsequent to the oral administration, Metoclopramide was administrated to emetic group. The kaolin, saccharin solution and normal feed containers were removed each day (at 10 am). The kaolin and normal feed were collected and weighed, and the weight of the bottle of saccharin was also being determined. The quantity of kaolin, normal feed and saccharin solution consumed during each 24 h period was determined by the weights with the initial weights[28].

The following formula was used to determine the ultimate weight value. Ultimate weight value (day n) = weight (day n) – weight (day n+1)

Stability studies

The optimized formulation was kept in the foil sachet and sealed tightly and kept in stability chamber (Thermo lab, scientific equipment's Ltd) maintained accelerated stability condition at temperature 40±2°C / 75±5% RH for 6 months. At at the intervals of every 3 months, samples were analyzed for drug content, disintegration time and *in vitro* drug release studies[24].

RESULTS and DISCUSSION

The preliminary solubility of Metoclopramide was found that, drug was soluble in ethanol, chloroform, benzene, 1 M HCl, sparingly soluble in methanol, DCM, DMSO, acetone, 0.5 N NaOH, Metaclopramide hydrochloride is freely soluble in Water and phosphate buffer pH 6.8[9,10,29]. The melting point of Metoclopramide was determined by using Thiele tube method and was found to be in the range of 149-155°C, which complied with European pharmacopoeia standards, thus indicating purity of obtained Metoclopramide sample [9, 10].

The position of peak in FTIR spectrum of pure Metoclopramide is compared with those in FTIR spectrum of Metoclopramide with β-cyclodextrin. The pure drug Metoclopramide peak at 686 cm^{-1} due to Cl bending, 3397 cm^{-1} due to N-H stretching, 1835 cm^{-1} due to C=O stretching, 1590 cm^{-1} due to C=C stretching, 2289 cm^{-1} due to CN stretching, 2763 cm^{-1} due to OCH stretching these are the characteristic peaks of Metoclopramide. It was observed that, there was drug characteristic peaks were observed as shown in the Figure 1a & Figure 1b, which proved that drug and β-cyclodextrin were compatible. The drug release profile for drug complex of all formulations 1:1-1:4 was found to be ranging between 52.7±3.95 to 83.1±7.49%. The results were shown in Figure 2. It was found that the 1:4 ratios showed the better drug release compared to other formulations. Hence it was selected for the formulation of sugar spheres[11, 30].

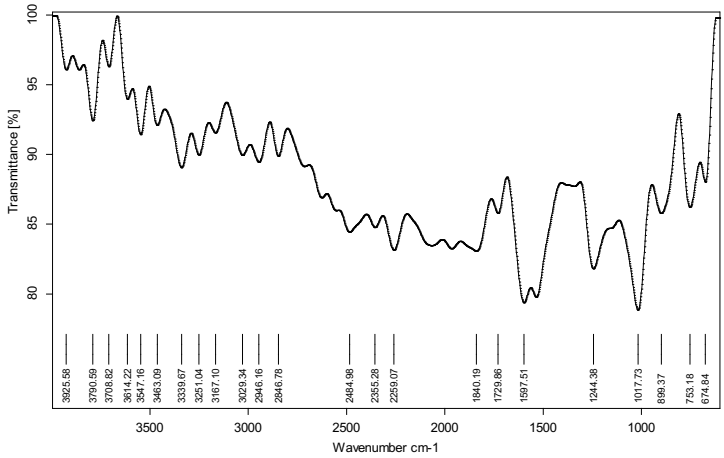

Figure 1a. FTIR Spectrum of Metaclopramide

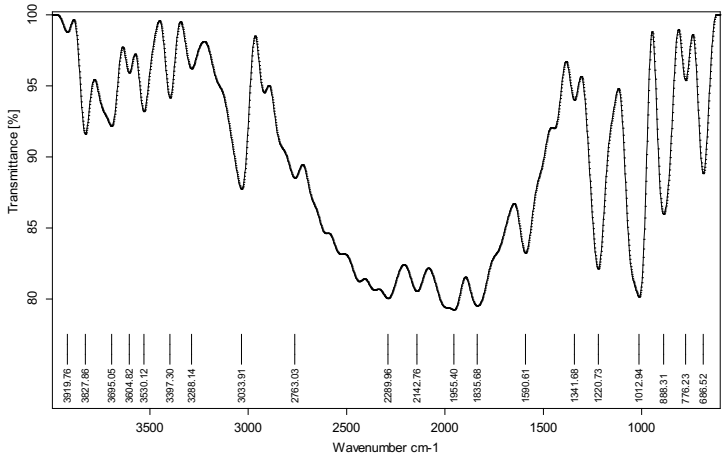

Figure 1b. FTIR spectrum of Metaclopramide with β-Cyclodextrin

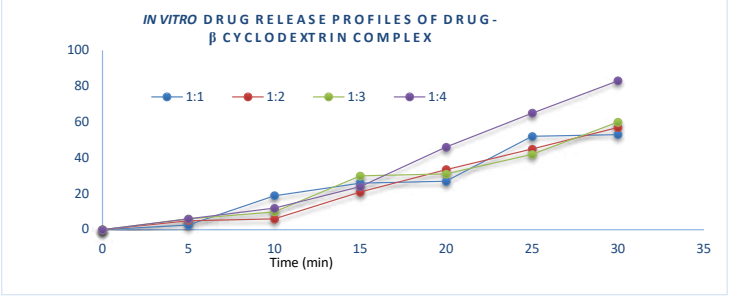

Figure 2. *In vitro* drug release profiles of Metoclopramide drug complex with β- Cyclodextrin (β-CD) by Kneading Method

Evaluation parameters of sugar spheres:

The pellets were tested for bulk density, tapped density, Carr's index, Hauser's ratio, porosity and angle of repose. The angle of repose in F4 to F5 formulations ranges from 26.5±2.05 to 30.11±0.78 i.e. in the normal limits gives excellent flow ability, pellets are free flowing, and it had been compliance with IP standards. Porosity of all batches ranges from 81.2±3.84 to 96.1±2.26%. The bulk density and tapped density ranges from 0.38±0.04 to 0.62±0.03 (g/mL) and 0.4±0.04 to 0.76±0.06 (g/mL) respectively, which is in normal range shows good flow ability. Hausner's ratios of all batches were in normal range i.e. 1.04±0.10-1.23±0.09, shows excellent flowing ability. The resulting values shows good flowability as specified in the Table 2. All formulation batches were evaluated for friability testing in triplicates. Formulations F5-F9 shows the friability 0-0.2%, its well, within the limit, however, the friability values greater in F1 to F4 as shown in the Table 3. The results of friability indicated that the pellets formulations were mechanically stable. Disintegration time of all formulations was performed using USP disintegration test apparatus, temperature at 37±°C using phosphate buffer pH 6.8. Formulated products showing very less disintegrating time, i.e.193-480 seconds. Disintegration is depending abreast on the concentration of disintegrants[31] and when cross carmellose sodium concentration increases the disintegration time was at faster rate, In case of F5 formulation disintegration time was faster, due to sodium starch glycolate concentration was more. Formulations F8 was disintegrated within 193 sec, better disintegration rate when compared to all other formulations. The values of disintegration time were shown in Table 3. Drug loading was found to be 49.2-93.7%, highest percentage was observed in F8, where cross carmellose sodium high concentration was utilized in the formulation, Cross carmellose sodium having high water uptake and swelling characteristics due to the presence of carboxymethyl sodium substituents[32]. The average particle size was found to be 1.0±0.01 to 1.0±0.02 mm. The scanning microscopy (SEM) analysis is vital for determining the surface morphology, size, and shape. SEM images of the formulated F8 pellets at different magnification were as shown in Figure 3, Surface morphology of pellets studied by SEM, indicated that the pellets were spherical shaped with rigid surface and were discrete, isolated images observed from SEM, predicted that pellets are free flowing, and number of micro porous surface structure was observed.

Table 2: Flow properties of pellets

Formulations	Angle of repose (ɵ) Mean±SD *	Bulk density (g/mL) Mean±SD *	Tapped density (g/mL) Mean±SD *	Hausner's ratio (%) Mean±SD *	Porosity (%) Mean±SD *
F1	11.3±1.10	0.55±0.03	0.62±0.10	1.12±0.06	88.8±2.80
F2	5.71±1.73	0.38±0.04	0.4±0.04	1.04±0.10	96.1±2.26
F3	20.8±0.98	0.58±0.10	0.66±0.07	1.13±0.07	88.2±2.26
F4	26.5±2.05	0.55±0.06	0.62±0.10	1.12±0.13	88.8±1.41
F5	25.6±2.22	0.52±0.04	0.55±0.05	1.05±0.04	94.7±2.81
F6	27.9±0.69	0.55±0.05	0.58±0.16	1.05±0.17	94.4±1.80
F7	28.8±0.83	0.52±0.06	0.58±0.07	1.11±0.13	89.4±5.71
F8	30.1±0.78	0.62±0.03	0.76±0.06	1.23±0.09	81.2±3.84
F9	28.4±1.49	0.45±0.09	0.5±0.06	1.10±0.09	90.9±1.49

*n=3 Mean±SD

Table 3: Physicochemical parameters for prepared pellets

Formulation code	Avg Particle size (mm)	Friability (%) Mean±SD*	Disintegration time (sec) Mean±SD*	Drug content (%)
F1	1.0±0.01	2.9 ±0.35	480sec ±0.40	49.2
F2	1.0±0.02	1.7 ±0.28	399sec ±0.57	58.8
F3	1.0±0.01	1.2 ±0.21	326sec ±0.76	78.6
F4	1.0±0.01	10.2 ±0.76	258sec ±0.64	77.2
F5	1.0±0.01	0.1 ±0.04	256 sec ±1.70	76.4
F6	1.0±0.01	0 ±0.04	371 sec ±0.38	83.0
F7	1.0±0.02	0.2 ±0.17	247sec ±0.36	83.1
F8	1.0±0.01	0 ±0.04	193sec ±0.38	93.7
F9	1.0±0.01	0 ±0.04	194sec ±0.48	91.6

*n=3 Mean±SD

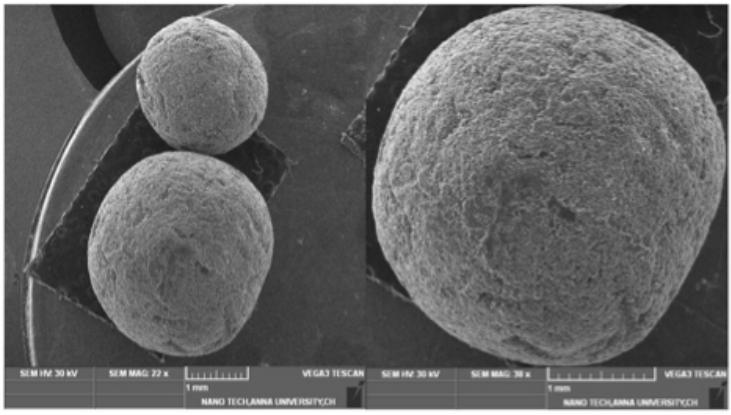

Figure 3: SEM Photograph of F8 magnification at 22.00 X & 38.00 X

In vitro drug release profiles:

In vitro drug release study was carried out for 30 min for formulation F1-F9. The amount of drug released at the end of 30 min is varying from 69.77 to 98.13%. The formulations contain 2-8% of sodium starch glycolate and 3-12% of cross carmellose sodium. The results of *in vitro* drug released were found to be lowest 69.77% in F1 formulation, which contained 2% sodium starch glycolate and the highest 98.13% drug released in F8 formulation, which contained 9% cross carmellose sodium. Sodium starch glycolate and Cross carmellose sodium are superdisintegrants which helps in faster disintegration followed by drug release from the pellets. Overall F8 formulation showed the optimum results. The results were as shown in the Figure 4.

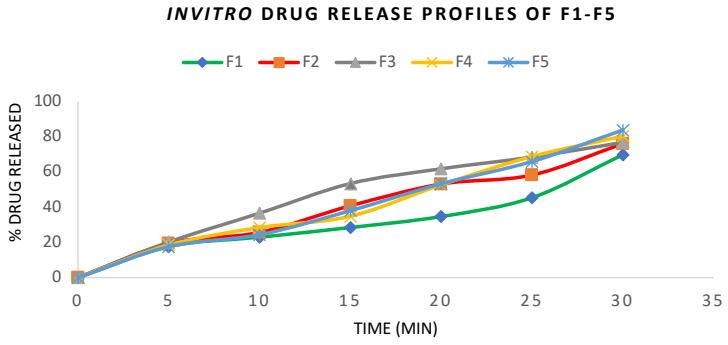

Figure 4a. *In vitro* drug release profiles of F1-F5

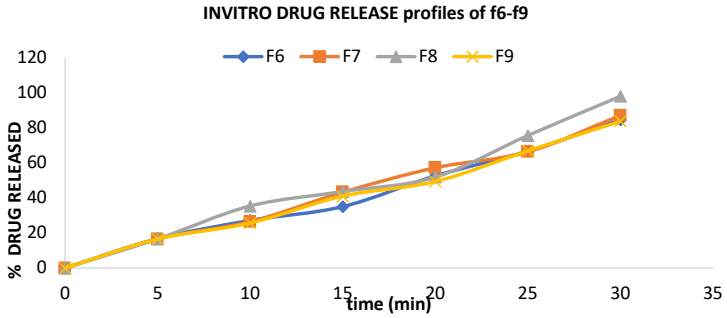

Figure 4b. *In vitro* drug release profiles of F6-F9

Anti-emetic studies using rat model:

Anti-cancer drug 5-fluorouracil unwanted side effect is severe emesis. According to American Society of Clinical oncology guidelines, 5-Fluorouracil causes acute and chronic emesis. Delayed vomiting occurs after 24 h, it persists for a week. In contrast to rodent species possess an emetic reflex, rat's exhibits pica behavior to stimuli that induce emesis. Nonnutritive substance such as Kaolin is a phenomena of emetic reflux[28].

From the animal study it was observed that kaolin consumption was apparently enhanced 1st day which indicated that the rats showed the pica behavior, whereas after Metoclopramide administration, initially till 24 h; 2.08±082 g kaolin consumption from the rat group was observed, followed by after 48 h Kaolin consumption was gradually decreases to 0.82 ±0.62 g, after 72 h still decreases to 0.43±0.72 g as shown in the Table 4.

In case of Sacharin solution, after 24 h, consumption with blank was 35.46±0.34 mL, with Control Saccharin solution consumption was 20.26±10.56 mL, followed by with Metoclopramide 28.66±0.65 mL, it was gradually decreased to 24.63±0.86 mL (*Mean ± SEM n=3/group) after 72 h of study.

On normal feed consumption induced by administration of 5-Flurouracil in rats was studied. With control consumption of feed was 8.19±0.87 g, it was gradually decreased to 6.63±0.96 g after 72 h of study. On Metaclopramide administration rat group feed consumption on 1st day was 9.24±0.45 g was consumed by the rats, followed by on 3rd day, gradually food intake was increased to 10.61±0.57 g. By the above observation it was concluded that F8 formulations the antiemetic activity. The results were tabulated in the Table 4-6.

Table 4. Kaolin consumption induced by administration of 5 fluorouracil in rats

Kaolin consumption (g)				
Group	n	0-24 h	24-48 h	48-72 h
Blank	2	0.58±0.22	0.36±0.14	0.49±0.72
Control	3	3.65 ±0.92	0.84 ±0.52	0.53±0.82
Metoclopramide	3	2.08±0.82	0.82 ±0.62	0.43±0.72

*Mean ± SEM (Standard Error Mean)

Table 6. Normal feed consumption induced by administration of 5 fluorouracil in rats

Normal feed consumption (g)				
Group	n	0-24 h	24-48 h	48-72 h
Blank	2	20.61±0.65	22.48±0.98	20.33±0.78
Control	3	8.19±0.87	5.76±0.74	6.63±0.96
Metoclopramide	3	9.24±0.45	10.07±0.86	10.61±0.57

*Mean ± SEM (SEM= Standard Error Mean

Stability studies:

The most satisfactory formulation F8 was stored in sealed sachets of aluminum foil. Then the formulation was exposed to 40±5°C and 75% RH using stability chamber. At the end of one month sample were evaluated for Disintegration time was found to be 3 mins 33 sec and drug content was found to be 93.9%, which confirmed that there was no much differences between the initial values and the result obtained during stability studies, thus indicating stability of prepared formulation is stable. The results were tabulated in Table 5. The formulation F8 After one month of stability study, there was no considerable changes in *in vitro* drug release studies were observed, and showed a drug release of 97.77±1.23% (*n=3 Mean± SD). The results were shown in the Table 7 and Figure 5. Formulation F8 shows better % CDR, disintegration time and drug content, so it was selected. We conclude that formulated Metoclopramide loaded sugar spheres F8, had good stability and better oral solubility of drug which enhances the bioavailability of the drug in systemic circulation, so it is suitable paediatric formulation for the treatment of emesis to enhance better absorption of drug through oral route.

Table 5. Saccharin solution consumption induced by administration of 5 fluorouracil in rats

Saccharin solution consumption (ml)				
Group	n	0-24 h	24-48 h	48-72 h
Blank	2	35.46± 0.34	34.63±0.65	31.28±0.76
Control	3	20.26±10.56	19.32±0.67	26.98±0.65
Metoclopramide	3	28.66±0.65	25.57±0.67	24.63±0.86

*Mean ± SEM (SEM=Standard Error Mean)

Table 7. Evaluation parameters of F8 after 1 month of stability study

Days	Stability condition	Drug content (%)	Disintegration time (sec)*
0	40±2°C,75±5% RH	93.7	193±0.38
15	40±2°C,75±5% RH	92.5	183±0.41
30	40±2°C,75±5% RH	93.9	213±0.34

*n=3 Mean± SD

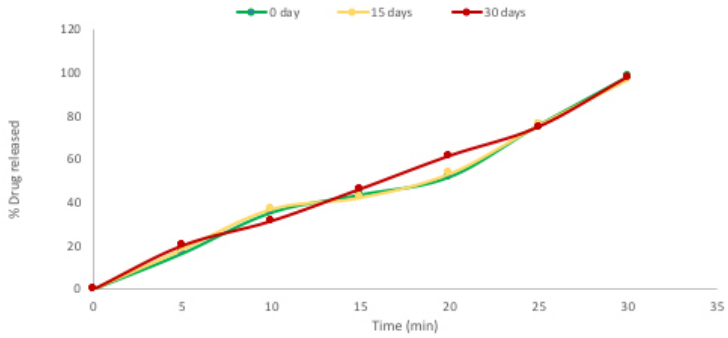

Figure 5. Drug released profiles of F8 formulation after 1 month stability study

DECLARATION OF INTERESTS

Authors declare no conflict of interest.

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Evaluation of anti-diabetic and hypolipidemic effect of ethanolic extract of *Waltheria* indica against streptozotocin induced diabetes in mice

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ABSTRACT

The present study was designed to investigate the anti-diabetic activity of ethanol extract of *Waltheria indica* leaves (EEWI) on streptozotocin induced diabetic mice. Effect of EEWI leaves antidiabetic and hypolipidemic effect was evaluated in streptozotocin (55 mg/ kg, *i.p*) induced hyperglycaemic mice. EEWI (40 and 100 mg/ kg) was administered orally for 15 days. Glibenclamide (1mg/kg, orally for 15 days) was used as reference standard. The diabetic control animals exhibited a significant decrease in body weight compared with control animals. EEWI inhibited streptozotocin-induced weight loss and significantly alter the lipid levels. Administration of the EEWI caused significant dosedependent reduction in serum glucose, cholesterol, triglyceride and glycosylated haemoglobin % levels in streptozotocin induced hyperglycaemic animals. The ethanol extract of *Waltheria indica* leaves exhibited antidiabetic activity might be through increased secretion of insulin and,

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the effect attributed to the presence of flavonoids and phenolic compounds present in extract.

Keywords: Diabetes mellitus, flavonoids, hypolipidemic, hyperglycaemia, streptozotocin

INTRODUCTION

Diabetes mellitus is a social concern. It might even be attributable to inadequate insulin production, or it can be due to insulin's ineffective action, i.e. insulin resistance, or it can be both. Type 1 and type 2 diabetes are the two main forms of diabetes. Insulin Dependent Diabetes Mellitus (IDDM) is another acronym of type 1 diabetes (IDDM). Type 1 diabetes patients have no insulin secretion and must rely on insulin for survival. Type 2 diabetes, also known as Non-Insulin Dependent Diabetes Mellitus (NIDDM), occurs when the pancreas does not produce enough insulin, or when the patient has insulin resistance, or a combination of both. Insulin insufficiency may wreak havoc on a variety of metabolic processes. Diabetes type 2 is more prevalent than diabetes type 1. It is estimated by WHO that an adult suffering from the diabetes mellitus in a low-income family of India, about 25% of the income is being spent.It is studied that 2.8% of world population is affected by diabetes in the year 2000 and it is estimated that it would raise to 366 million in 2030 from 171 million in 2000[1,2]. Sustained diabetes may lead to several consequences like neuropathy, nephropathy, retinopathy and ulcer. The increase in morbid rate due to diabetes is well explained by increase in glycosylated hemoglobin HbA1c[3].

Several approaches have been used to create diabetes mellitus in experimental animals. The pancreatectomy is the most common and successful procedure. Injecting medicines like alloxan or Streptozotocin into animals is the second thing to achieve animal's diabetic[4]. The Langerhans islets beta cells expand and eventually degenerate as a result of these chemicals. Because of its faster inductive rate and lesser toxicity, STZ is recommended over Alloxan for inducing diabetic mice. STZ is a safer alternative to Alloxan. Due to a large reduction in body weight, Alloxan has a greater mortality rate than STZ [5,6]. In rats chemically inoculated with diabetes by Streptozotocin, the last symptoms of insulin insufficiency are easily noticeable [7]. Streptozotocin can trigger an autoimmune process that culminates in the loss of beta cells in the Langerhans islets and can cause beta cell toxicity, resulting in the appearance of clinical diabetes within 2-4 days [8]. We chose the STZ for this research study based on the aforementioned factors.

Waltheria indica (Sterculiaceae.) is a vital medicinal plant distributed throughout tropical forest region in India, and its can be used as sedative, antibacterial, antimalarial, antifungal, anti-anemic, analgesic, antioxidant, anti-convulsant and anti-inflammatory effect [9,10]. The EEWI leaves were found to contain alkaloids, flavonoids, tannins, saponins and cardiac glycosides[11]. These can act as an antioxidant and has potential remedy for diabetes. The project aims at finding out the effect of ethanolic extract of *Waltheria indica* leaves in streptozotocin induced diabetes in Swiss albino mice. As there are no reports specifying this property of this extract in streptozotocin induced diabetic model, hence the project has been carried out.

METHODOLOGY

Plant material and extraction

The leaves of *Waltheria indica* was authenticated and collected from Dr. K.Madhava Chetty, Assistant professor, Department of Botany, Sri Venkateswara University, Tirupati. Cold extraction method using ethanol was used for the extract preparation. The leaves of the plant were sieved and about 100g of the leaves were taken and crushed and later placed in a flask containing 500ml of ethanol.Contents were filtered and filtrate was stored in refrigerator to enhance stability. A second measure of 500 ml of ethanol was run through the residue and left to soak for 24 hrs. Filtrate was collected and stored in the fridge between 0-5°C. The above procedure was repeated for a third time. The filtrates were mixed together and a rotary extractor was used to remove the ethanol from the extract in a 250 ml flask. Flask containing the concentrated extract was stored in a cool dry place until the next stage of experiment.

Animals

Protocols required for performing this study were submitted to the Institutional Animal Ethics Committee (IAEC) during the Animal ethics meeting. After the protocols were approved, healthy animals were procured from the central animal facility for performing the experiments. The CPCSEA approval number for the procured animals is 202/SASTRA/IAEC/RPP. All the animals were maintained in clean polypropylene cages and standard animal diet and water were provided ad libitum. The following table provides the number of mice procured from central animal facility for performing each of the experiments.

Experimental design

A total of 30 Albino mice (25-30g) was used in this study. The mice randomly divided into 5 groups of six animals in each (n=6). Groups are divided as follows:

Group I received with CMC 10ml/kg, *p.o.*; Group II received Streptozotocin at a dose 55 mg / kg, *i.p* ; Group III received Streptozotocin 55 mg/ kg, *i.p* and Glibenclamide 1 mg/kg, *p.o.* ; Group IV & V received Streptozotocin 55 mg/ kg, *i.p* along with EEWI 40 mg/ kg, *p.o.* & EEWI 100 mg/ kg, *p.o.* respectively.

Mice were made diabetic by a single intraperitoneal injection of Streptozotocin, newly dissolved in citrate buffer 0.01 M, pH 4.5 at a dose of 55 mg/kg body weight. The animals were allowed to drink 5% glucose solution overnight after injecting streptozotocin to overcome the drug induced hypoglycemia. Blood glucose levels were measured after 48 hours and animals which had a glucose level greater than 200 mg/dl were considered as diabetic and used for the experiment. After 48hrs of STZ injection the treatment with the plant extract and standard drug was started for 15 days. On 16^{th} day the serum sample were collected for biochemical estimation.

Biochemical estimation

The blood samples were collected from the animals on the 16^{th} day from retro-orbital plexus under mild anaesthesia from overnight fasted animals. Blood samples were centrifuged at 3500 rpm for 15mins to separate the serum from blood. The serum glucose, serum cholesterol and serum triglyceride levels were measured by Bio systems kit and glycosylated hemoglobin % was measured by Monozyme's Glycohemin kit.

Statistical Analysis

The statistical analysis was performed by Graph Pad Prism 5.0 Version (Graph Pad Software, Inc., San Diego, California, USA). All data are presented as Mean ± SEM and comparisons done by one-way ANOVA followed by Tukey's test as a post hoc test values were considered significant at $p<0.05$ or less.

RESULTS and DISCUSSION

Extract yield

The percentage of extract yield was found to be 15.2% when 1500 ml of ethanol was mixed with 100 g of crushed and powdered leaves.

Acute toxicity test

The acute toxicity of plant *of Waltheria indica* was reported that, this plant leaf extract has LD_{50} value of 363 mg/kg of body weight of animal.

Effect of *Waltheria indica* extract on body weight

The EEWI and Glibenclamide did not affected the body weight of the animals,

but significant reduction in body weight was observed in diabetic control animals and are shown in Figure 1. Loss in body weight was observed in streptozotocin induced diabetes mellitus in rats and was controlled by treatment with EEWI. Administration of this extract to hyperglycaemic rats resulted in an increase in body weight compared to streptozotocin induced diabetic rats. The present study findings suggested that EEWI treatment has positive effect on maintaining body weights in diabetic rats. The protective effect of plant extract on body weight of diabetic rats may be due to its ability to reduce hyperglycaemia. A gradual increase in body weights of Glibenclamide treated groups was similar to that of normal control rats. STZ-induced diabetes mellitus was characterized by severe loss of body weight due to increased muscle wasting in diabetes [12].

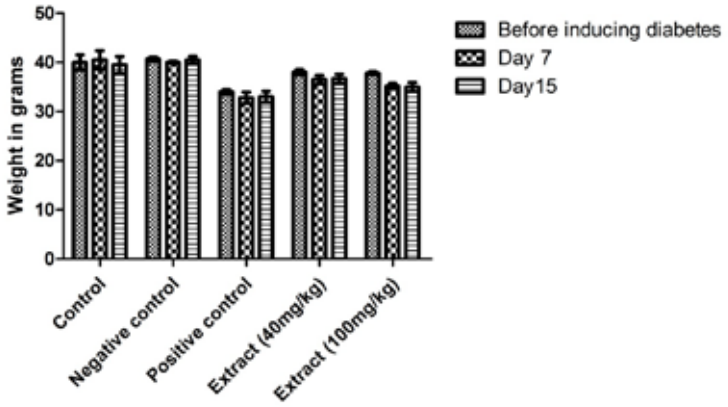

Figure 1. Body weight of the animals before inducing diabetes and on 7th and 15th day of treatment are expressed in grams diabetes using Accu-Check Glucometer.

Effect of *Waltheria indica* extracts on blood glucose levels

The serum glucose level was measured by glucose kit in an auto analyzer[5]. It is evident from the graph that the plant extract is efficient in bringing down the serum glucose level ($p < 0.01$). The blood glucose level of negative control is about 2.5 times greater than that of control Figure 2 & Table 1. The standard drug glibenclamide brought down the serum glucose level by about 58.5 %. The 15th day dose 1 and dose 2 of *Waltheria indica* brought down the serum glucose level by about 49% and 52% respectively and thus can be seen that the extract of *Waltheria indica* provides an effective control in serum glucose level Figure 3.

Table 1. Number of animals procured

S. No	Name of the experiment	No of mice used
1.	Group I: Control group given only CMC (carboxy methyl cellulose) dosage of 0.3ml	6
2.	Group II: Streptozotocin induced diabetic control (55 mg/kg)	6
3.	Group III: Standard drug for diabetes –Glibenclamide (1 mg/kg)	6
4.	Group IV: Effect of Waltheria indica extract on Streptozotocin induced mice at a dose of 40mg/kg	6
5.	Group V: Effect of Waltheria indicaextract on Streptozotocin induced mice at a dose of 100mg/kg	6

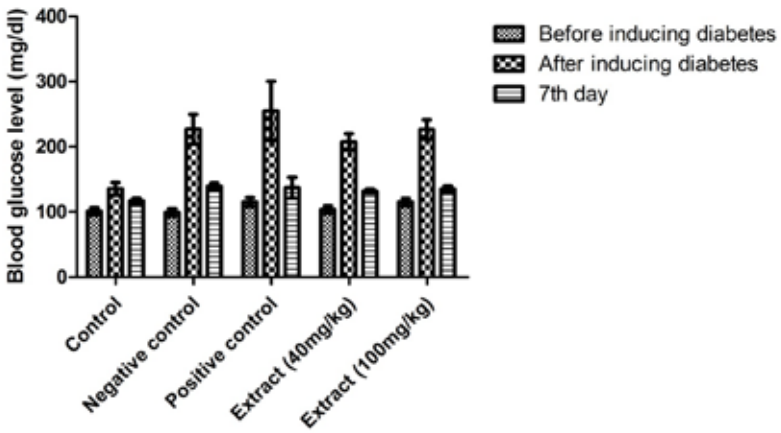

Figure 2. Blood glucose level before and after inducing diabetes and on 7th day of treatment are expressed in mg/dl of *Waltheria indicaextract* on serum glucose levels

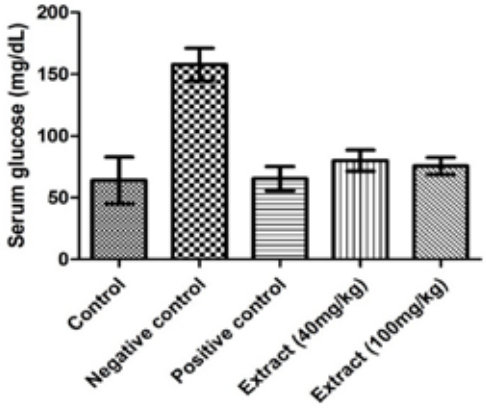

Figure 3. The graph shows comparison between the different groups and their effect on the serum glucose levels of streptozotocin induced diabetic mice.

The antidiabetic effect of *EEWI* may be due to increased release of insulin from the existing β-cells of pancreas. In this context, a number of other plants have also been reported to exert hypoglycaemic activity through insulin release stimulatory effect [13, 14].

Effect of *Waltheria indica* extract on serum cholesterol levels

The serum cholesterol level was measured by cholesterol kit in auto analyzer. It is evident from the graph that the plant extract is not that efficient in bringing down the serum cholesterol level ($P < 0.05$) Figure 4 and Table 1. The induction of diabetes by streptozotocin increased the serum cholesterol level by about 1.5 times. The standard drug brought down the cholesterol level by about 20%. Dose 1 of extract reduced the cholesterol level by 24% and dose 2 of extract reduced cholesterol level by 35%. Thus, the plant extract does not significantly bring down the serum cholesterol but is effective compared with the standard drug glibenclamide. The hypercholesterolemia is associated with Insulin deficiency [15]. The reduction in cholesterol and other lipids in this study was dependent on the concentration of AAWI. It indicates the plant possess the good hypolipidemic effect [16].

Effect of *Waltheria Indica* extract on serum triglyceride levels

The serum triglyceride level was measured by triglyceride kit in auto analyzer. It is evident from the graph that the plant extract is efficient in bringing down the serum cholesterol level ($P < 0.05$) Table 2 & Figure 4. The induction of diabetes by streptozotocin increased the serum triglyceride level by about 2.2 times Figure 5. The standard drug brought down the cholesterol level by about

58%. Dose 1 of extract reduced the cholesterol level by 57.4% and dose 2 of extract reduced cholesterol level by 63%. Thus, the plant extract significantly brings down the serum triglyceride and high dose of extract is more effective than the standard drug glibenclamide.

Table 2. Effect of EEWI on serum glucose, cholesterol, triglycerides and glycosylated in Streptozotocin induced diabetic rat's haemoglobin

Group	Serum glucose(mg/dl)	Serum cholesterol (mg/dl)	Serum triglyceride(mg/dl)	Glycosylated hemoglobin %
Control	64.00 ±19.08	119.00 ±5.29	63.75 ±20.51	6.63 ±0.47
Negative control	157.80 ±13.23	179.00 ±8.00	141.00 ±21.00	10.14 ±0.73
Positive control	65.50 ±9.71	143.30 ±13.49	59.40 ±20.84	6.61 ±0.47
Extract 40mg/kg	80.00 ±8.50	136.00 ±9.38	60.00 ±6.12	7.96 ±0.57
Extract 100mg/kg	75.75 ±7.07	115.50 ±4.66	52.00 ±17.09	6.86 ±0.49

'n = 6' in each group; values are expressed as Mean ± SEM. *p<0.05, ** p<0.01, compared with normal control.. Statistical test employed was one way ANOVA followed by Turkey multiple comparison test.

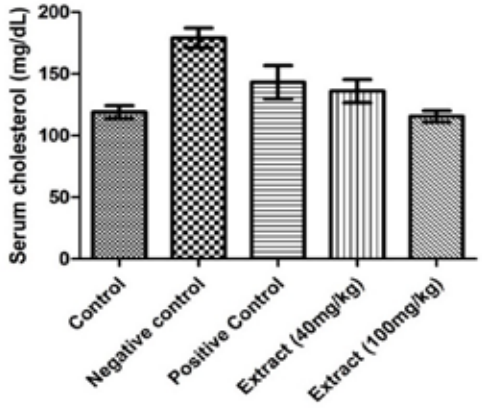

Figure 4. The graph shows comparison between the different groups and their effect on the serum cholesterol levels of streptozotocin induced diabetic mice.

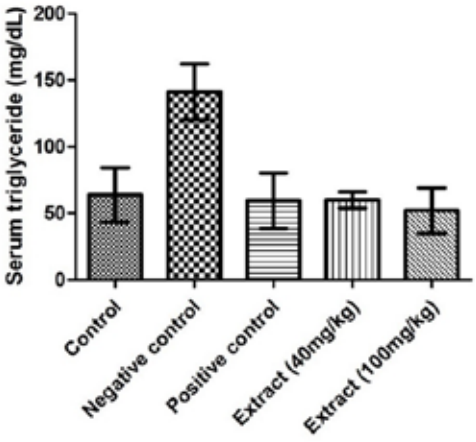

Figure 5. The graph shows comparison between the different groups and their effect on the serum triglyceride levels of streptozotocin induced diabetic mice.

Alterations in lipid concentration, found in 40% of diabetics that similarly were observed in this study in rats with streptozotocin-induced diabetes [17]. Here, the increasing in triglycerides concentrations in diabetic rats it may be due to insulin resistance, an increase in insulin, and glucose intolerance. Treatment with *G. Sylvestre* extract reduced the levels of cholesterol and triglycerides compared with those in their diabetic control groups [18]. The findings of the present work showed that treatment by that *EEWI* caused decreasing in lipid levels like triglycerides in STZ-induced diabetic rats [19].

Effect of *Waltheria indica* extract on glycosylated hemoglobin levels

In diabetic mice, the levels of HbA1C are increased due to the persistent hyperglycemia which results in glycation of hemoglobin. The concentration of HbA1C is related to diabetic retinopathy, nephropathy, and neuropathy and it is considered as a tool for the diagnosis and prognosis of diabetes-associated complications [20]. The synthesis of hemoglobin is reduced in diabetic rats [21]. The present study glycosylated hemoglobin level was measured by glycosylated hemoglobin kit (Monozyme's Glycohemin kit) in auto analyzer. It is evident from the graph that the plant extract is efficient in bringing down the glycosylated hemoglobin% level ($P < 0.05$). The induction of diabetes by streptozotocin increased the glycosylated hemoglobin% level by about 1.5 times Figure 6 & Table1. The standard drug brought down the glycosylated hemoglobin level by about 34%. Dose 1 of extract reduced the glycosylated hemoglobin% level by 32.3% and dose 2 of extract reduced glycosylated hemoglobin level by 21% and restored the glycosylated hemoglobin% to normal level. Thus, the plant extract

significantly bring down the glycosylated hemoglobin and low dose of extract is more effective than the high dose of extract. Plant which has flavonoids, terpenoids, alkaloids, and glycosides have antioxidant activity and claimed to possess antidiabetic effect. Flavonoids present in the plant regenerate the damaged beta cells of pancreases, and the polyphenolic compounds present in the plants produced the hypoglycaemic effects in diabetic rats [22,23]. Antidiabetic effect and hypolipidemic effect of EEWI might be due to presence of flavonoids, polyphenolic compounds in this extract.

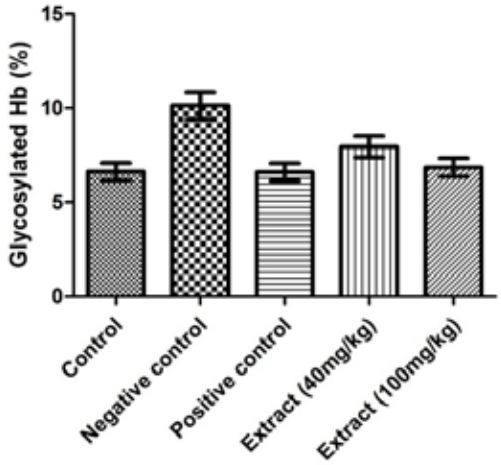

Figure 6. The graph shows comparison between the different groups and their effect on the glycosylated hemoglobin levels of streptozotocin induced diabetic mice.

The present study on the ethanolic extract of *Waltheria indica* leaves possess anti diabetic activity on the streptozotocin induced diabetes in mice by reducing levels of serum glucose, cholesterol, and triglyceride and glycosylated hemoglobin% level. It is also found to be effective in managing the complications associated with diabetes mellitus, such as hypolipidemic, and prevents the defects in lipid metabolism. This might be due free radical scavenging activity through the polyphenols and flavonoids of the plant extract since streptozotocin induces diabetes by increasing hydrogen peroxide. Further studies should be carried out to establish the antidiabetic effect on different animal species, identify the bioactive principle responsible for this effect and also understand the underlying mechanism of cellular actions.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Effect of oral use of various edible oils on wound healing in rats: randomized controlled experimental study

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ABSTRACT

There are various studies showing that oral supplements are shortening or facilitating effects on this process. Therefore, this study investigates the use of edible oils as supplements in the wound healing process. Of the 7 groups (control, *Hypericum perforatum* extracts in olive oil, olive, sesame, fish, black seed, sunflower), each has 8 Wistar Albino rats. In the experimental groups, 1.25 mL/kg oral oil was used for 10 days. Macroscopic images of the wound area were taken. Wound healing was evaluated by histological analysis. Collagen III, IL-6, TNF-α and TGF-ß1 density analyzes were performed on the tissue samples. According to macroscopic analysis, wound narrowing is higher in all groups on the 2nd and 4th days than the control group. Histopathological and immunohistochemical analyses of all experimental groups except sunflower oil group revealed better results than control group.

Keywords: *Hypericum perforatum* olive oil extract, sesame oil, black cumin oil, olive oil, sunflower oil, wound healing

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INTRODUCTION

The skin is the largest organ of the human body[1]. Because many patients endure skin injury, wound healing has a critical role in both daily life and healthcare[2,3]. The wound healing process, which consists of cell to cell and cell to matrix interactions, has 5 important steps; homeostasis and inflammation, granulation tissue formation, neovascularization, re-epithelialization, and remodeling[4].

Various studies have shown that malnutrition negatively affects the wound healing process[5]. Every step in the healing process ultimately depends on the circulating amino acids, lipids and carbohydrates[6]. The basal metabolic rate increases in the presence of a wound, which causes protein (muscle) breakdown and body dehydration[7]. For collagen synthesis, 1 kcal/g (collagen) is required and any change in the presence of precursor amino acids or energy substrate will affect collagen accumulation[8]. Lipids are one of the most important dietary components that contain the essential fatty acids and serve as carriers of lipid soluble vitamins[9]. Some researchers have shown that fatty acids are important substances in the wound healing process[10].

In the wound healing process, many modern and traditional treatment methods have been developed. Traditional treatments are cost effective and easy to access. Numerous nutrients obtained from herbal and animal sources have been used in research to accelerate or regulate wound healing. Some studies have suggested that fish oil may have positive effects on the wound healing process, but its ultimate benefit is still controversial[10-12]. It is also known that the effects of oleic acid on the wound healing process are dubious as well[13,14].

However, studies using *Hypericum perforatum* extract in olive (HPEO) oil for wound healing, which has been used among the folk for many years in order to cure various diseases such as gastritis, burns and bedsore[15-18], has shown that HPEO has huge potential on wound healing. It is stated that the anti-inflammatory effect and collagen accumulation of HP are effective in wound healing[19]. Olive oil is generally used in HP oil extract. Studies on olive oil reported that olive oil had positive effects on wound healing; therefore, the wound healing effect of the HPEO was synergistic[20,21].

Sesame oil has anti-inflammatory, antimicrobial and antioxidant activities[22]. Also, studies have shown that sesame oil can expedite wound healing and tissue regeneration in skin wounds, as it has a stimulating effect on fibroblast production[23]. The seeds of *Nigella sativa*, also known as black cumin, contain essential oils, proteins, alkaloids and saponins[24]. Some studies have evaluated the effects of *Nigella sativa* oil on wound healing, but controversy continues regarding its

use in the wound healing process[25,26]. In one study, it has been found that the oral use of olive oil has more positive effects than sunflower oil. In this study, patients who used olive oil for their burn wounds treatment, have higher levels of serum albumin, shorter hospitalization duration and shorter wound healing time[27]. The studies are limited in the literature showing that sunflower oil has an effect on wound healing. Nonetheless, the effect of sunflower oil has been investigated in the present study with the idea that sunflower oil may have positive effects due to the increasing energy need in the wound healing process.

In the literature, there are not any research that analyze the comparative effects of oral use of HPEO, olive oil, sesame oil, fish oil, *Nigella sativa* oil, and sunflower oil on skin wound healing. Therefore, this study aimed to analyze the effects of oral use of these oils on wound healing in rats with experimental wounds.

METHODOLOGY

Animals and Experimental Design

This animal experiment complied with the ARRIVE guidelines and carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Also, all stages of this research have been approved by Istanbul Medipol University animal experiments ethics committee. This randomized controlled, single blind research was carried out on 56 healthy Wistar Albino male rats, weighing 300-350 ± 5 grams, in the Istanbul Medipol University Experimental Animal Breeding and Research Laboratory in Istanbul. During the experiment, the room temperature is 21 ± 2 °C, relative humidity is 40-60%, having ventilation system (change of 10-15/hour), inside the cage light intensity is 40 lux, the light period is 12 h light / 12 h dark and the noise level is below 85 dB. Throughout the study, all groups of rats were fed ad libitium with standard feed. After anesthesia, rats were placed in separate cages and divided into seven groups, with eight animals per group as follows: Group 1- control group; 1.25 ml/kg/day saline was given with oral gavage, Group 2: 1.25 ml/kg/day HPEO was given with oral gavage, Group 3: 1.25 ml/kg/day olive oil was given with oral gavage, Group 4: 1.25 ml/kg/day sesame oil was given with oral gavage, Group 5: 1.25 ml/kg/day fish oil was given with oral gavage, Group 6: 1.25 ml/kg/day black cumin oil was given with oral gavage, Group 7: 1.25 ml/kg/day sunflower oil was given with oral gavage. All oils and serum were given by physiological gavage and no anesthesia was applied during the gavage procedure.

Natural oils

Olive oil: Produced by the local people in Dikili, Izmir, Turkey

Sunflower oil: Vera® (Izmir, Turkey) brand oil was used.

HPEO: In this study, the flowers of St. John's Wort (*Hypericum perforatum*) plant which grown in the Bergama highlands of Izmir, were used. HP flowers collected from nature and then dried and powdered. Fifty g of HP was added to the glass bottle containing 500 ml of olive oil (olive oil used in the experimental group was used). The bottle was kept under the sun for 4 weeks, with 12 hours a day in the summer. It was observed that the red dye in the plant passes into olive oil[19].

Fish oil: Marincap® Capsules (500 mg) was used.

Black cumin oil: Karden® (Ankara, Turkey) brand oil was used.

Sesame oil: Karden® (Ankara, Turkey) brand oil was used.

It has been observed in the literature that the oils used in the experimental groups can be applied by oral gavage in a single dose of 0.5, 1, 1.25, 1.5, 2, 4 and 5 mL/kg/day [21, 28-31]. In order to determine the amount of fat used, the lowest HPEO dose found in the literature was taken as the basis [29]. 1.25 ml/kg/day was determined as the dose in all groups in order to avoid a difference in energy intake between the experimental groups. Fatty acid components in 100 grams of oils are shown in Table 1[32-34].

Table 1. The Fatty Acid Components in 100 grams of Sesame, Black cumin, Olive, Sunflower and Fish Oil

	Black cumin Oil (g)	Sesame Oil (g)	Olive Oil (g)	Sunflower Oil (g)	Fish Oil (g)
Total SFA (Saturated Fatty Acid)	11.6	15.53	15.583	10.382	
Total MUFA (Monounsaturated Fatty Acid)	27.7	42.31	68.373	30.917	
Total PUFA (Polyunsaturated Fatty Acid)	59.7	42.30	11.434	54.071	30
14:0 (Myristic acid)	0.1	0.01		0.067	
16:0 (Palmitic acid)	7.1	9.57	12.399	5.774	
17:0 (Margaric acid)		0.04	0.096		
18:0 (Stearic acid)	3.2	4.99	2.486	3.308	
20:0 (Arachidic acid)		0.63	0.459	0.249	
22:0 (Behenic acid)	0.6	0.10	0.143	0.717	
24:0 (Lignoceric acid)		0.05		0.268	
16:1 , n-7 (Palmitoleic acid)		0.11	0.755	0.105	
18:1 , n-9 (Oleic acid)	27.7	42.05	67.293	30.649	
20:1 , n-9 (Gadoleic acid)	0.2	0.15	0.325	0.163	
18:2 , n-6 (Linoleic acid)	59.2	42.04	10.812	53.985	
18:3 , n-3 (ὰ- Linolenic Acid)	0.5	0.26	0.621	0.086	
20:5 n-3 (EPA- Eicosapentaenoic Acid)					18
22:6 n-3 (DHA- Docosahexaenoic Acid)					12

Surgery for excisional wound model

For general anesthesia in rats, 100 mg/kg ketamine HCl (Ketasol 10%, Richter Pharma, Austria) and 10 mg / kg Xylazine HCl (2% Rompun, Bayer, Istanbul, Turkey) were used. The stock solution was obtained by mixing 10 mg of xylazine HCl and 100 mg of ketamine into 10 mL of 0.9% isotonic sodium chloride solution. This prepared solution was given intraperitoneally (ip) in μL at 10 times body weight (grams)[35]. After general anesthesia, the back hair of the rats was shaved and washed with providon iodine solution. All procedures were performed aseptically. Full-thickness skin wounds were created using two excisional skin wounds, a punch biopsy tool with a diameter of 1 cm and 5 mm from the midline[36]. Each solution was administered by oral gavage each day until the rats were sacrificed on day 10. Flunixin 2.5 mg/kg/day was applied subcutaneously from the wound creation to the last day in order to reduce the pain of the rats.

Macroscopic evaluation of wound healing

Photographs were taken using a Sony digital camera (Sony Inc., Tokyo, Japan) on days 0, 2, 4, 6, 8 and 10 at a 90° angle to the wound surface in order to measure wound narrowing. Wound surface areas were calculated using an image analyzer (ImageJ.2.0 software, National Institutes of Health, Bethesda, MD) to evaluate the change in wound surface size during healing. This software was then used to analyze photographic results at 32 bit density. The software was used to export data in CSV format for custom analysis.

Histology: Light microscopic evaluation

One of the wound tissues in the back region was placed in neutral buffered formalin for light microscopic evaluation and was left to be fixed for 48 hours. Excess fixatives were removed by washing for 1 hour under running tap water. It was passed through ascending alcohol series (70%, 90%, 96%, 100%) for dehydration and made transparent with xylene. It was kept in paraffin in a 60 °C incubator oven for overnight and embedded in paraffin in ice molds the next day. In order to analyze general morphology with light microscope, microtome (Thermo Scientific, HM340E) sections taken approximately 5 μm thick, and stained with Hematoxylin-Eosin (H&E) (Empire Genomics, BPK 4088-2), Mason trichrome (Bio-optica BPK 2916). Sections were viewed with Nikon Eclipse (Nikon® Instruments Inc., United States).

Wound healing for each group was evaluated using the scoring system described by Geleano et al.[37]. According to this scoring system, the groups are scored between 1 and 4 under three headings: epidermal dermal regeneration, granula-

tion tissue thickness and angiogenesis.

For epidermal and dermal regeneration:

√ Poor epidermal organization in ≥ 60% of the tissue; 1 point
√ Incomplete epidermal organization in ≥ 40% of the tissue; 2
√ Moderate epithelial proliferation in ≥ 60% of the tissue; 3
√ Complete epidermal remodeling in ≥ 80% of the tissue; 4
For thickness of the granulation tissue:
√ Thin granulation layer; 1
√ Moderate granulation layer; 2
√ Thick granulation layer; 3
√ Very thick granulation layer; 4

For angiogenesis (only mature vessels were counted and identified by the presence of erythrocytes in the lumen):

√ ltered angiogenesis (one to two vessels/site) characterized by high degree of edema, hemorrhage, occasional congestion and thrombosis; 1
√ Few newly formed capillary vessels (3–4/site), moderate edema and hemorrhage, occasional congestion, intravascular fibrin deposition and absence of thrombosis; 2
√ Newly formed capillary vessels (5–6/site); 3
√ Newly formed and normal appearing capillary vessels (>7/site); 4

Immunohistochemistry

Sections taken 12 μm thick on positive charged slides with cryomicrotome (CM1950, Leica, Germany) and fixed in 4% PFA for 20 minutes. After washing with PBS and distilled water, they were incubated for 10 minutes at room temperature with 3% H2O2 to stop the endogenous peroxidase activity. Blocking solution (2% BSA containing 10% NGS) was dropped onto the sections and allowed to block for 1 hour at room temperature. Without washing, the sections were incubated with anti-collagen III (1: 100, cat # ab7778; Abcam), anti-IL-6 (1: 1000, cat # ab9324; Abcam), anti-TNFα (1: 1000, cat # ab6671) and anti-TGF-β1 (1:100, cat # sc-146, Santa Cruz) for overnight at +4 °C. The sections were washed 3 times for 5 minutes in PBS and then incubated for 10 minutes in biotinylated secondary antibody. After the sections were washed again 3 times for 5 minutes with PBS, incubated in streptoavidin peroxidase (Mouse and Rabbit Specific HRP (ABC) Detection IHC Kit, ab93677, Abcam) for 10 minutes. Conversion to brown in DAB chromogen (1.5 ml DAB substrate + 30 μl DAB chromogen) after washing with PBS was observed within 3 minutes. Then it was washed with PBS and nucleus staining was performed with Hematoxylin (Bio-

optica 60002). Slices were washed under running tap water for 10 minutes, and then sections were dehydrated in ascending alcohol series and covered with Biomount (Bio-optica 1611). Sections were imaged with Nikon Eclipse (Nikon® Instruments Inc., United States) at 4X magnification for semi-quantitative density analysis. Density analysis was performed using Image J (National Institutes of Health, Bethesda, MD, USA) software.

Statistical Analysis

All statistical analyses were performed using SPSS 24.0 software program. The results were expressed as means ± SD. For macroscopic wound healing assessment, light microscopic evaluation and immunohistological wound healing assessment the differences between the control group and experimental groups were analyzed by using Mann Whitney U test. Values for $p \leq 0.05$ were considered statistically significant.

RESULTS and DISCUSSION

Macroscopic wound healing

The calculated wound surface area values are given in Table 2 as percentage. For Day 0, all groups were considered 100%. In Figure 1, macroscopic images of the wound area which are photographed every two days, are given together.

Table 2. Calculated Wound Surface Areas

	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10
Control	100.00	96.17±1.46	83.88±7.32	50.28±22.18	29.94±21.47	21.36±13.66
HPEO	100.00	78.83±5.99[a]	60.54±9.05[a]	40.46±9.98	24.95±13.49	12.05±8.29
Olive oil	100.00	87.31±2.26[a]	74.71±3.41[b]	42.24±3.16	24.39±5.40	12.87±4.55
Sesame oil	100.00	82.42±4.81[a]	62.05±12.33[b]	39.08±9.58	27.64±9.07	13.77±5.73
Fish oil	100.00	80.57±3.49[b]	70.30±4.05[b]	44.06±12.94	32.77±14.63	16.76±7.29
Black cumin oil	100.00	86.79±6.35[b]	74.22±8.57[c]	44.46±6.86	33.58±5.81	19.89±4.57
Sunflower oil	100.00	93.17±2.15[b]	72.47±8.33[c]	46.10±8.63	28.65±6.21	19.69±6.80

Values are percentage of open wound surface. Data are means ± SD. a : $p < 0.001$. b: $p < 0.005$. c: $p < 0.05$ vs. control group.

On the 2nd day, more wound closure was observed in all experimental groups ($p<0.005$), especially HPEO, Olive oil and Sesame oil groups ($p<0.001$) compared to the control group.

Although there was more wound closure in the control group on the 4th day compared to the 2nd day, even more wound closure was observed in all experimental groups. Highest one is HPEO group ($p < 0.001$) followed by the olive oil, sesame oil and fish oil groups ($p < 0.005$) and finally black cumin oil and sunflower oil groups ($p < 0.05$), compared to the control group.

On day 6, wound closure was higher in all experimental groups than the control group ($p > 0.05$). On the 8th day, wound closure was higher in all experimental groups than the control group except for the fish oil and black cumin oil groups ($p > 0.05$). When the 10th day was reached, wound closure was higher in all experimental groups than in the control group ($p > 0.05$).

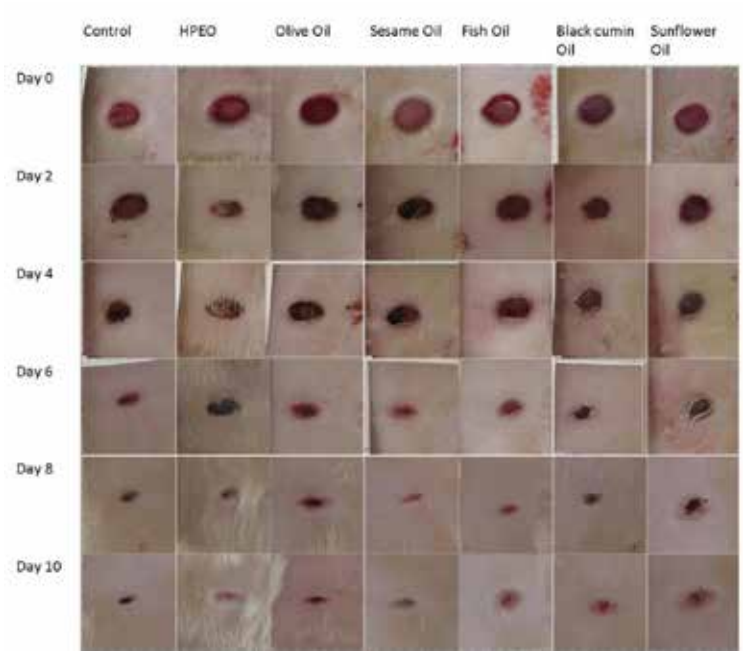

Figure 1. Macroscopic images of all groups on days 0, 2, 4, 6, 8 and 10

Examination of wound healing with Light microscopic evaluation

The effect of oral gavage applied oils on wound healing was stained with Mason trichrome stain for histopathological analysis. Sections were compared for epidermal regeneration, granulation tissue and angiogenesis (Fig. 2). The examination results of the groups, which are scored under three headings by light microscopic examination, are given in Table 3.

Table 3. Histological Wound Healing Scores

	Epidermal and Dermal Regeneration	Granulation Tissue	Angiogenesis
Control	2.2	2.4	2.6
HPEO oil	3.3[a]	3.3[b]	3.8[a]
Olive oil	3.4[a]	3.1[b]	3.3
Sesame oil	3.4[a]	3.6[b]	3.6[b]
Fish oil	3.2	3.0	3.0
Black cumin oil	3.6[b]	3.0	3.4
Sunflower oil	2.8	2.8	2.8

HPEO: *Hypericum perforatum* extract in olive. [a] $p < 0.01$, [b] $p<0.05$ vs. control group.

Considering the results, the control group showed lower scores in epidermal regeneration, granulation tissue and angiogenesis scoring than all experimental groups. Looking at the epidermal regeneration scores, HPEO, olive oil, sesame oil groups ($p <0.01$) and black cumin oil group ($p <0.05$) scored higher than the control. According to the granulation tissue scoring, HPEO, olive oil and sesame oil groups scored higher than the control ($p <0.05$). For angiogenesis scoring, HPEO group ($p <0.01$) and sesame oil group ($p <0.05$) scored higher than the control.

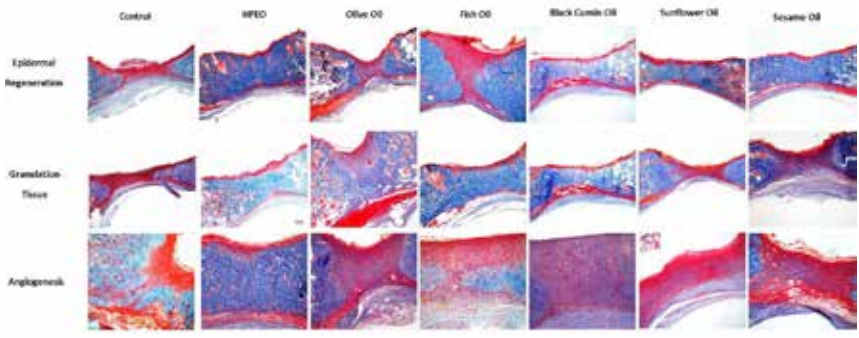

Figure 2. Mason trichrome staining in wound sections

Immunohistological examination results of scar tissue

In the wound healing model, the expression levels of collagen III, proinflammatory (TNF-α and IL-6) and anti-inflammatory (TGF- ß1) factors were compared with immunohistochemical methods (Fig. 3). According to the immunohistological analysis of the groups, the mean and standard deviation of densities measured with Image J program are given in Table 4. In this study, density analyzes of Collogen III, IL-6, TNF-α and TGF-ß1 were performed in the wound area.

According to Collagen III density, all experimental groups, mainly the HPEO group ($p < 0.001$), have higher density than the control ($p < 0.01$). When IL-6 density was examined, the densities of all experimental groups, except sunflower oil group, were lower than the control group. The difference in IL-6 density between control group and all experimental groups, except the olive oil and sunflower oil groups, was statistically significant ($p < 0.01$).

For TGF-ß1 density all experimental groups, except fish oil and sunflower oil groups, have higher density than the control group ($p < 0.01$). Actually, fish oil and sunflower oil groups have higher TGF-ß1 density than control, but this difference is not significant.

When TNF-α density was examined, a lower density than the control was observed in all experimental groups except for black cumin and sunflower oil groups ($p < 0.01$). TNF-α concentration was higher than control in black cumin oil and sunflower oil groups ($p > 0.05$).

Table 4. Immunohistology Density Measurements

	Collagen III		IL-6		TGF-ß1		TNF-α	
	Mean±SD	P	Mean±SD	P	Mean±SD	p	Mean±SD	p
Control	9.67±2.52		28.92±8.20		35.50±1.92		43.78±3.07	
HPEO Oil	48.49±3.33	<0.001	16.45±6.74	0.006	47.52±2.45	0.001	36.21±4.53	0.004
Olive Oil	38.34±7.83	0.001	22.17±3.40	0.051	46.51±3.21	0.001	38.74±1.50	0.001
Sesame Oil	39.36±6.80	0.001	12.34±7.63	0.002	46.33± 4.46	0.001	35.68±8.30	0.004
Fish Oil	18.31±4.67	0.003	11.09±4.92	0.005	38.67±3.81	0.149	35.55±4.05	0.003
Black Cumin Oil	23.41±3.11	0.001	12.89±3.01	0.002	44.18 ± 4.94	0.004	39.95±5.23	0.165
Sunflower Oil	24.45±7.35	0.001	30.49±3.91	0.432	38.60 ±3.66	0.073	40.77±6.39	0.432

HPEO: *Hypericum perforatum* extract in olive

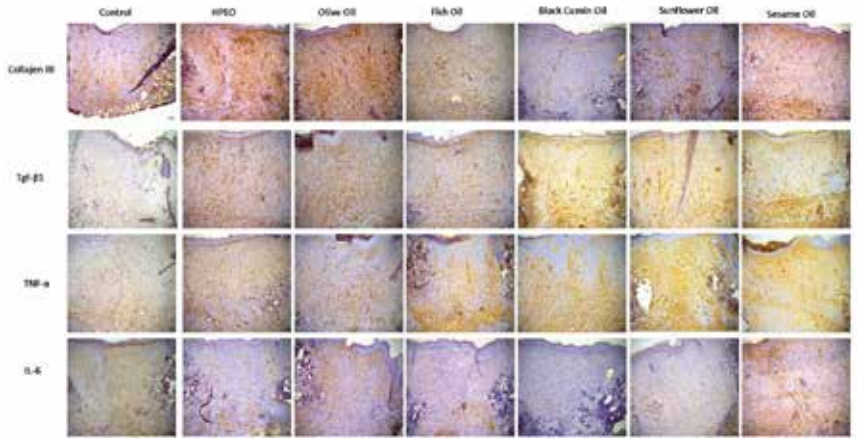

Figure 3. Immunohistochemical analysis of collagen III, proinflammatory factors (TNF-α and IL-6) and anti-inflammatory factor (TGF-β1) in wound healing sections (Magnification 4X)

The success ranking of groups at the end of experiment in analyzes

The success ranking of the experimental and control groups were given in Table 5. Although there was more wound closure in the experimental groups than the control group on days 2 and 4, there was no difference between the groups on the last day of the study (HPEO, olive oil, sesame oil, fish oil, sunflower oil, black cumin oil and control, respectively, from the most effective to the least effective. group).

Table 5. The success ranking of the experimental and control groups in analyzes

Wound healing	Most effective												Less effective
	Min												Max.
Epidermal and Dermal Regeneration	Black cumin	=	Olive	=	Sesame	=	HPEO	=	Fish	=	Sunflower	>	Control
Granulation Tissue	Sesame	=	HPEO	=	Olive	=	Black cumin	=	Fish	≥	Sunflower	>	Control
Angiogenesis	HPEO	=	Sesame	=	Black cumin	=	Olive	=	Fish	=	Sunflower	>	Control
Collogen III	HPEO	>	Sesame	=	Olive	>	Sunflower	=	Black cumin	=	Fish	>	Control
TGF-ß1	HPEO	=	Olive	=	Sesame	≥	Black cumin	≥	Fish	>	Sunflower	=	Control
	Min												Max.
Wound surface area day 10th	HPEO	=	Olive	=	Sesame	=	Fish	=	Sunflower	=	Black cumin	=	Control
IL-6	Fish	=	Sesame	=	Black cumin	≤	HPEO	≤	Olive	≤	Control	≤	Sunflower
TNF- α	Fish	=	Sesame	≤	HPEO	=	Olive	≤	Black cumin	=	Sunflower	≤	Control

HPEO: *Hypericum perforatum* extract in olive
=: Statististically smilar
≤: The previous and next groups are statistically at the same level of impact with each other. The mean of the previous group is smaller than the mean of the next group. However, at least two previous groups and the next group are statistically at different levels of impact.
≥: The previous and next groups are statistically at the same level of impact with each other. The mean of the previous group is bigger than the mean of the next group. However, at least two previous groups and the next group are statistically at different levels of impact.
<: The mean of the previous group is smaller than the mean of the next group.
>: The mean of the previous group is bigger than the mean of the next group.

When we examined the ranking of success level according to the epidermal regeneration score, it was seen that all experimental groups (black cumin oil, olive oil, sesame oil, HPEO, fish oil, sunflower oil, respectively) had similar effects with each other and were more effective than the control group ($p < 0.05$). According to the granulation tissue scoring, the effect levels of sesame oil, HPEO, olive oil, black cumin oil and fish oil groups are higher than the sunflower oil

and control groups ($p < 0.05$), but there is no difference between the effect levels of above-mentioned experimental groups. Fish oil and sunflower oil groups, which have no difference in effect levels, were more effective than the control group ($p < 0.05$). When analyzed in terms of angiogenesis score, while all experimental groups were more effective than the control group (HPEO, sesame oil, black cumin oil, olive oil, fish oil, sunflower oil and control groups, respectively) ($p < 0.05$), there was no difference between the effect levels of the experimental groups.

When the effect levels are examined according to Collagen III density, it is seen that all experimental groups are more effective than the control and also HPEO group is the most effective group ($p < 0.05$). Sesame oil and olive oil groups that come after HPEO are more effective than sunflower oil, black cumin oil and fish oil groups with the same effect level ($p < 0.05$). When TGF-ß1 density is examined, all groups are more effective than sunflower oil and control groups with similar effect levels ($p < 0.05$). The most effective HPEO, olive oil and sesame oil groups have a similar effect level, but sesame oil has a similar effect level with black cumin oil too. Black cumin oil has a similar effect with fish oil.

When IL-6 density was examined, fish oil, sesame oil, black cumin oil groups had a similar effect, but they were more effective than all other groups ($p < 0.05$). Respectively, black cumin oil with HPEO, HPEO with olive oil, olive oil with control and control with sunflower oil groups have similar effects. For TNF- α density success ranking fish oil and sesame oil groups have a similar effect and they are the most effective groups ($p < 0.05$). The least effective group is the control group ($p < 0.05$). HPEO and sesame oil groups have a similar effect. HPEO and olive oil groups show similar effects, and they are more effective than black cumin oil and sunflower oil groups.

In this study, rats with wounds on their backs were given 1.25 ml / kg of various oils (HPEO, olive oil, sesame oil, fish oil, black cumin oil and sunflower oil) for experimental groups and saline for control group for 10 days with oral gavage. To measure the effect of supplementation on wound healing, measurement of wound closure, histological wound healing examination, immunohistological analyzes were performed.

As can be seen in Table 5, there was no difference between the macroscopic images of the groups on the 10th day. As seen in Table 2, the effect levels of the experimental groups that were effective at the beginning (on the 2nd day) decreased over time (on the 4th day) and there was no difference between them and the control group on the last day of the study. Similarly, in a study which lavender oil was used as a treatment, lavender oil was seen to be effective in

wound closure at the beginning, but it was observed that there was no difference between the control group in the last 4 days[38]. In another research[39], while rapid wound closure was observed in the experimental groups at the beginning, no difference was found between the control group on the last day. In the research of Keskin et al.[4], when the histological wound healing score was examined, the Limonene and Fenchone groups were more effective than the control, while for wound surface areas, these groups did not differ from the control. As can be seen from these studies, although macroscopic imaging helps to examine the effect at first, it is not sufficient by itself. These results show that the experimental groups especially HPEO and sesame oil groups, initially accelerated the closure of the wound surface.

A study[40] concluded that sunflower oil provides an increase in granulation tissue and complete healing of the epidermis. In other studies[41-43], it was stated that sunflower oil was more effective in wound healing compared to control, and this may be due to the linoleic acid content. In our study, histological results were compatible with the literature, but when the immunohistological results were examined, it was seen that the sunflower oil group had high IL-6 and TNF-α in the wound tissue.

In the literature, it was reported that increased IL-6 and TNF-α were observed in the tissues of mice given sunflower oil[44]. In this study, although sunflower oil was generally more effective in wound healing than control, it was seen that it was less effective than other experimental groups. Like sunflower oil, the expected effect was not observed in the fish oil group. However, it was observed that the concentration of IL-6 and TNF- α in wound tissue was lower than all groups. Besides, it was determined that collagen-III density was lower than all groups except the control group and TGF-ß1 concentration was lower than all groups except control and sunflower oil groups.

In a study in which various oils were used[45], delays in epithelization and wound closure were observed in the fish oil supplement group. In our study, when the 10th day macroscopic wound closure was examined, there was no statistically significant difference between the groups. When we examined the histological wound healing of wound tissues, it was seen that the fish oil group was more effective than the control, but less effective than all experimental groups except the sunflower oil group. In a wound healing study using omega-3 fatty acids[46], it was found that EPA and DHA were not successful in wound healing contrary to what was expected. A similar result seen in our study was associated with the anti-inflammatory effect of EPA and DHA. Inflammation stimulates and aggravates early wound healing in the healing process so in the absence of inflam-

mation, wound healing may be delayed. In a review prepared by Komprda[47], it was seen in various studies that the wound healing effects of EPA and DHA are mixed (inconsistency): "decreased / increased collagen deposition, lower / higher counts of the inflammatory cells in the healing tissue."

Like fish oil, sesame oil has a high content of unsaturated fatty acids. High levels of polyunsaturated fatty acids can make the oil extremely susceptible to oxidation. However, sesame oil is extremely stable due to its antioxidant content (like polyphenols, carotene). Also, sesame oil contains 15.24 mg / kg of carotene which is the precursor of vitamin A. For healthy regeneration of the skin structure, vitamin A is essential. Sesame oil also shows antibacterial activity, which is an important feature in wound healing[48]. Sesame contains the most sesamin and sesamoline as fat soluble lignans compounds. These compounds have antioxidant and anticancer properties. Sesamin is protective against oxidative stress[49]. In a study[50] comparing the FRAP (Ferric Reducing / Antioxidant Power) values of sesame oil, olive oil and sunflower oil, it was concluded that 803 μmol /l, 153 μmol/l and 108 μmol/l, respectively. On the 15th and 31st days, while sesame and olive oil had the lowest oxidant value, it was observed that sunflower oil had the highest value. Sesame oil contains sesaminol, sesamolinol, sesamolin and sesamol which all have an antioxidant effect. This content reduces lipid oxidation and has a wound healing effect[51]. In a study, sesame ointment accelerated wound healing and stimulated fibroblast proliferation. In addition, sesame ointment has been found to be effective in angiogenesis. Also, it has been concluded that sesame ointment shortened the inflammation phase and promoted the proliferation phase[23]. As seen in our study, there were no difference between the sesame oil group and the fish oil group regarding TNF-α and IL-6 in scar tissue (Table 5). In addition, TGF-ß1 content was similar to HPEO group and olive oil group, probably due to the antibacterial properties of sesame oil. Also the collagen accumulation of this group was similar to the olive oil group and higher than all groups except HPEO group. In our study, it was also seen in the histological examination of the oils that Sesame oil was strikingly effective in wound healing among all oils. Olive oil has antioxidant properties due to its oleic acid, vitamin E and phenol compounds (tyrosol, hydroxytyrosol, oleuropein, 1-acetoxypinoresinol and flavonoid) content[52-54]. Donato-Trancoso et al.[54] have observed that olive oil application reduces the oxidative damage by reducing ROS (reactive oxygen products) and NO (Nitric Oxide) formation, thus reducing inflammation and necrosis, increasing wound closure and improving collagen deposition. In one study, supplementing the diet with olive oil instead of sunflower oil improved the healing of burns and reduced hospitalization in burn patients[27]. According to the study of Edraki et al.[52], infection

was seen in the control group but not in the olive oil group. This is thought to be due to the phenolic content of olive oil, such as secoiridoids (oleuropein and derivatives). In our study, we found that the collagen deposition in the wound tissue of the olive oil group was similar to the sesame oil group. Regarding the TGF-ß1 deposition, olive oil group was one of the highest in the experimental groups, and this result was consistent with the literature[53]. In addition, studies show that olive oil improves angiogenesis. In our study, the angiogenesis scoring of olive oil was better than the control group. In a study comparing olive oil and fish oil[53], it was stated that fish oil caused inflammation by increasing norepinephrine levels, but no inflammation was observed in the olive oil group due to the anti-inflammatory properties of it. This shows that olive oil is more effective in wound healing than fish oil. It can be a good wound healing supplement due to its content.

It is known that olive oil extract of *Hypericum perforatum* is traditionally used in wound healing[55]. Various studies have proven this effect[55-58]. This effect is thought to be the biological properties of the compounds in HP and the synergetic effect of the oil from which HP was extracted. HP contains Hypericin, which has antimicrobial, antiviral and anti-inflammatory properties[59-62]. The Hyperforin contained in HP contributes to wound healing by having antibacterial and antimalarial effects[63-66]. In addition, since quercetin is involved in the inhibition of monoamine oxidase A, it reduces the formation of free radicals and helps in wound healing[67]. HP contains glavanoids such as quarcitrin hyperosidei, rutin, kaempferol, biopigenin and amentoflavone[68]. As seen in our study, HPEO increased collagen and TGF-ß1 deposition. TNF-α level was similar to olive oil and IL-6 level was lower than olive oil. Only in the epidermal examination part of the histological wound healing examination, the HPEO group had a lower mean than the olive oil group. However, there was no difference between olive oil group and HPEO group in any histological wound healing score.

When black cumin oil was examined, it was seen that the results were more effective than the control, but not as successful as the sesame oil, HPEO and olive oil groups. In a study[69], HP oil cream and black cumin oil cream were used in wound healing. In this study[69], while the antioxidant properties of the black cumin group were higher, improvements were found in epithelization and granulation in the HP group. The benefits of black cumin oil actually come from its fatty acid and essential oil content. Black cumin oil has more than 30% fixed oil and 0.4%-0.45% volatile oil content. The biggest compound of this volatile oil content is thymokine (27.8-57.0%). Thymokine is followed by p-cymene, varvacrol, 4-terpineol, longifoline and t-anethole[26,32]. In addition, black cumin oil contains about 60% essential fatty acids[32]. Thymoquinone is stated to be

responsible for antioxidant, analgesic and anti inflamatory actions[26,32]. As seen in Table 1, black cumin oil contains 59.2% linoleic acid and 27.7% oleic acid[32]. It was stated that oleic acid prevented the formation of free radicals. However, the oleic acid content of black cumin oil is less than half of the content of olive oil. Therefore, black cumin oil may not be as effective as olive oil in the wound healing process. In addition, it is noteworthy that the linoleic acid content of sunflower oil and black seed oil are similar and the results we have found that they have similar affect levels on wound healing.

As a result of the present study clearly showed that orally used oil extract (HPEO) and oils (sesame oil, olive oil, black cumin oil, fish oil and sunflower oil) have wound healing effects. With the help of future studies, it will be acknowledged that the use of various oils as supplements in wound healing is important in terms of shortening the current treatment period and hospitalization.

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CONFLICT OF INTEREST

The authors declare no competing interest.

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The Effect of Chamomile Extract on Blood Sugar Level, Lipid Profile and Body Weight in High-Fat Diet Fed Rats

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ABSTRACT

The addition of herbs to the diet is one of the methods for the treatment of obesity. It is aimed to examine the extracts of chamomile to use in the treatment of obesity, against high-fat diet. In this study, 6-8 weeks old fifteen female Sprague-Dawley rats were used. Five of the rats separated randomly for the control group and fed with a standard pellet for ten weeks. Ten rats were fed with a high-fat diet for four weeks. After this period, the rats were randomly divided into two groups (high-fat control and chamomile). Blood samples were taken for lipid profiles and glucose. With the high-fat diet, body weights and total cholesterol had increased significantly ($p<0.05$). Chamomile had a 39.2% decrease in body weight and a 38.8% decrease in blood glucose ($p<0.05$). There was no difference between groups in other lipid parameters. Chamomile may be used in the treatment of obesity.

Keywords: Chamomile, high-fat diet, obesity, weight loss

INTRODUCTION

Obesity is one of the most common diseases all over the world. It is a risk factor for early mortality and comorbidities such as hypertension, type 2 diabetes, dyslipidemia, coronary heart disease, stroke, sleep apnea, and some cancers [1]. Therefore, obesity is one of the major problems in public health. In the World

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Health Organisation (WHO) obesity fact sheet, it was stated that in 2016, more than 1.9 billion adults aged eighteen years and older were overweight; of these, over 650 million adults were obese [2]. Therefore, the underlying mechanism of obesity has been investigated, and several approaches gain importance for preventing or treating obesity [3–5]. There are a lot of reasons for obesity. One of them is the disruption of energy balance[6]. A high-fat diet, in particular, has been associated with obesity and comorbid diseases [7,8]. Chamomile (*Matricaria chamomilla* L.*)* is a member of the Asteraceae family and contains various phenolic compounds (a polyphenol group member) and flavonoids. Chamomile is the most common herb in the diet as a herbal tea. It is thought that chamomile extract acts as a pain killer. In addition to that, it has antiseptic, antibacterial, antioxidant, and anti-inflammatory activity also protects from some diseases like high-fat liver, Type 2 diabetes, and cancer[9,10]. Although chamomile is well studied in some diseases like diabetes and maintaining blood glucose levels, the anti-obesity effect on body weight has not been examined[11-13].

Herbs rich in phytochemicals have been used to maintain well-being, prevent and treat several diseases for decades[14,15]. Polyphenol, naturally occurring in plants, effectively regulates physiological and molecular pathways, including energy metabolism, adiposity, and obesity[16,17]. Chamomile has been traditionally used for weight loss in Turkey. In addition to weight loss, chamomile is also used for health benefits in cosmetics, the food industry, and medical applications[18].

Although herbs are commonly used as a tea in daily diet, the effect of most of the herbs on health has not been well established. Therefore, if there was not known the effects of the herbs on health, using herbs for the treatment of some diseases may have negative consequences. With all of the knowledge, this study planned to examine the effects of chamomile on blood glucose level, lipid profile, and body weight in high-fat diet-fed rats.

METHODOLOGY

Research Protocol

A high-fat diet was applied for ten weeks in all rats except the control group. After four weeks, the high-fat diet-fed rats were separated randomly into equal two groups. One group was chosen for the chamomile group, and the other was selected for the high-fat diet control group. Chamomile applied for six weeks via gavage. The high-fat and control groups have taken placebo (water) via gavage from the beginning of the fourth week to the end of the tenth week. Blood samples were collected at the beginning, end of the fourth week, and end of the study. Animals with net weight change greater than 50 g were considered obese.

Sample Selection

In this study, 15 Sprague-Dawley albino female rats (83-202g) aged 6-8 weeks were used. All of the rats were fed a standard diet ad libitum for ten days. The rats were kept in 21.8 ° C and 60% humidity, twelve hours of light, and twelve hours of darkness. The rats were randomly allocated to cages with a maximum of five rats in each cage in the Istanbul Medipol University Regenerative and Restorative Medical Center (MEDITAM).

Initially, five of the rats were randomly separated and determined as a control group; they were fed with a standard diet and 2 mL water via gavage throughout the study. Then the remaining rats formed the high-fat diet group. These rats were fed with a high-fat diet for four weeks. After high-fat diet-fed rats (n=10) were randomly separated into two equal groups. The three groups comprised:

(i) Control Group (**n=5)-** Standard diet

(ii) High-Fat Control Group (n=5)- High-fat diet

(iii) Chamomile Group (n=5)- High-fat diet + 100 mg/ kg chamomile extract

Standard and High-Fat Diet

The standard diet consisted of corn, full-fat soy (obtained from genetically modified soybean), sunflower seed meal, wheat ram, wheat flour, alfalfa flour, sugar beet molasses, beef meat-bone-chicken flour, dicalcium phosphate (inorganic), calcium carbonate, vitamin premix, and mineral premix.

High-fat diet applied for according to Ari et al.[19] protocol. The high-fat diet contained 25 g butter for each 100 g standard pellet. The butter was melted and added to the standard pellet until the pellets had absorbed all the melted butter. The high-fat diet was given to all groups except the control group until the end of the study.

Preparation of Chamomile Extract

Chamomile flowers were obtained from the local market in May 2016. The flowers were controlled by Tuğba İduğ (Ph.D. lecturer) and İrem Atay Balkan (Ph.D. lecturer). A hydroalcoholic solution (100 g Chamomile in a 1 L solution containing 37% ethyl alcohol and 63% distilled water) of chamomile was extracted in a Soxhlet apparatus. After the alcohol was evaporated, the solution was lyophilized and kept at -20°C until use. The extract was weighed 100 mg for each kg of rat weight. It was dissolved in water with a maximum of 2 mL of gavage per rat. The dosage of chamomile extract was decreased according to negative weight changes in Chamomile groups.

Weight Measurement

Rats were taken one by one from their cages, and their weights were taken using sensitive scales (0.0001 g/ 200 g) every Monday until the end of the study.

Blood Samples

One ml of blood was collected from each rat at the beginning (day 0), middle (day 28), and at the end of the study (day 70) from the subclavian vein. The blood samples were centrifuged at 3000 rpm for ten minutes at +4^{0}C. The blood serum samples were analyzed at Istanbul Medipol University MEGA Hospitals Complex Biochemistry laboratory. The values of serum Low-Density Lipoprotein (LDL), High-Density Lipoprotein (HDL), triglyceride (TG), total cholesterol (TC), and blood glucose were analyzed utilizing Cobas 6000 (Roche, Tokyo) biochemistry auto-analysis.

Statistical Analyses

The SPSS 18.0 statistics program (IBM, NY, USA) was used for the statistical analysis. The values were mean and ± standard deviation (SD), differences in comparisons of multiple groups were made using the One-Way ANOVA test. Independent t-tests were used to compare the difference between day 28 and day 70 data. The general linear analysis was made for repeated weight measures. The changes were tested using the paired-sample t-test method and were considered significant when $p < 0.05$ and $p < 0.01$.

RESULTS and DISCUSSION

Control and high-fat diet-fed rats' weight changes were calculated from day 0 to day 28. Average weight changes were 46.00±18.52 g for the high-fat intervention group and 29.00±9.24 g for the control group. In comparison, the average weights increases were 43.7% in high-fat diet-fed rats, 15.9% in the control group. This increase was found statistically significant ($p<0.05$). The average weight changes of control and high-fat diet-fed rats can be shown in Figure 1. It was found that the chamomile group has weight loss from day 28 to day 70 when the average weight change compared with the high-fat control group. The average weight change was 18.06 ± 8.16 g for the high-fat diet control and 13.37±6.75 g for the chamomile group. Also, it was shown that on day 70 chamomile group has a lower weight than the high-fat diet-fed rats in Figure 2 ($p<0.05$).

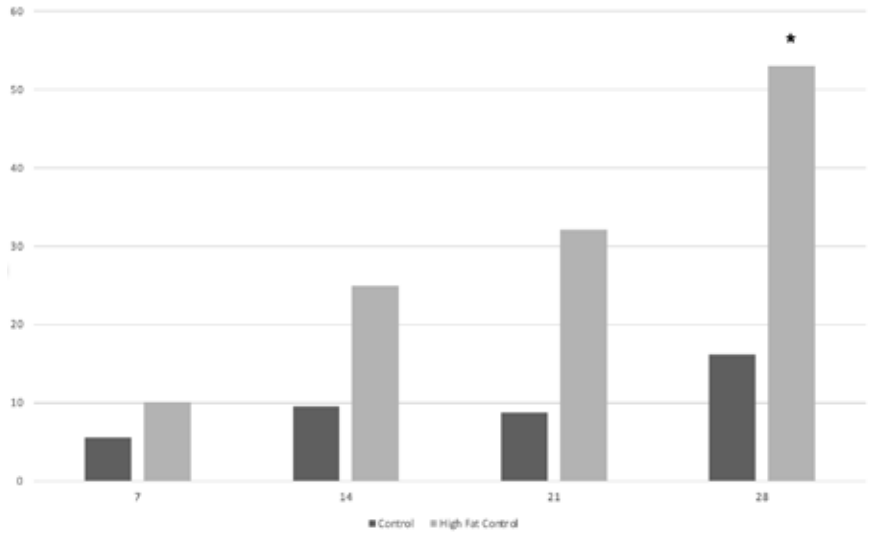

Figure 1. High-fat diet fed rats and control group average weight change for 4 weeks. *p<0.05

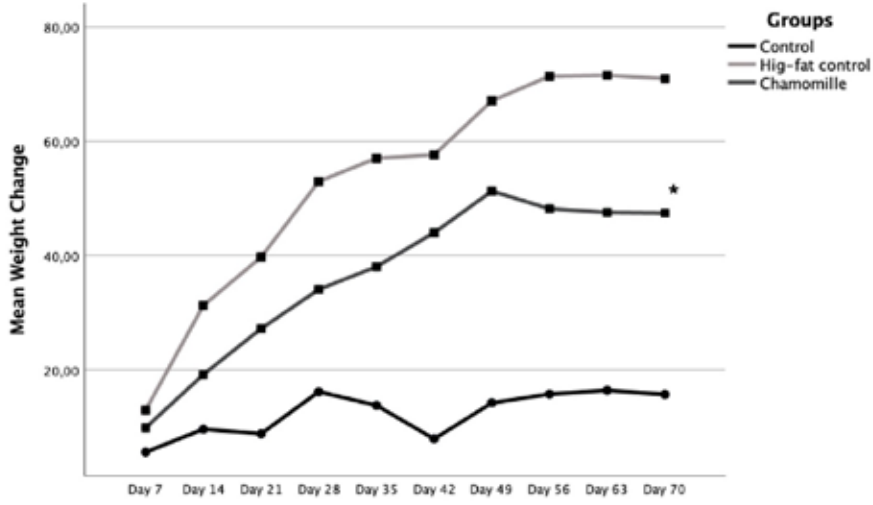

Figure 2. The difference in the High-Fat-Control, Control, and Chamomile group average weight change for ten weeks. (*p<0.05)

This study found a significant difference between the weight change of the control group and the high-fat control group. It was found that rats' weight was increased with a high-fat diet compared to the standard diet—bodyweight was affected differently by the quantity of food and a variety of nutrients[-7,8]. In one study, the metabolic syndrome rat model developed with a high-fat and high-

carbohydrate diet. As a result of a high-carbohydrate (8 weeks) and high-fat diet (16 weeks), found an increase in body weight, energy intake, and BMI, compared with the control group [21]. In another study, male rats were fed a high-fat diet for 16 weeks. It was stated that beta-cell dysfunction was observed in the female offspring of these rats. Consumption of a high-fat diet leads to increased body weight, energy intake, higher adiposity values and leptin levels, and fatty liver masses [22]. Despite that, few studies showed no significant difference between a high-fat diet and weight gain in rats [19,23]. One study found that no effect on weight changes of consumption of a high-fat diet between a high-fat and a low-fat diet for sixteen weeks [23].

Day 28 and day 70 biochemical analyses were compared for all groups. It was found that TC was increased in the high-fat control group and blood glucose decreased in the Chamomile group ($p<0.05$). Examined to change of TC and glucose within groups, it was shown that the control group has the lowest level of TC, and the chamomile group has the lowest level of glucose in Table 1.

Table 1. Biochemical Analysis Results of Control, High-fat Control, and Chamomile Group.

		TC mg/dL n±SD	TG mg/dL n±SD	LDL mg/dL n±SD	HDL mg/dL n±SD	Glucose mg/dL n±SD
Control						
n=5	**Baseline**	45.12±9.39	69.68±15.18	-10.13±3.38	41.32±7.62[a]	307.80±26.33[a]
n=5	**Day 28**	57.64±6.72	60.0±8.80	2.60±2.40	47.20±4.47[a]	123.04±15.87
n=5	**Day 70**	57.70±6.72[a]	82.92±36.31	0.00±0.00	51.40±6.62	99.08±4.83[a]
High-Fat Control						
n=5	**Baseline**	55.50± 6.46[Å]	61.83±1 6.51	-6.50±2.32	49.63± 3.45[aÅ]	223.86± 40.98[a]
n=5	**Day 28**	65,30±4,75	50.70± 10.95	3.33±1.52	58.23±7.19[a]	130.0±12.21
n=3	**Day 70**	65.80±8.17[aÅ]	94.70±16.78	0.00±0.00	55.13±7.02[Å]	106.80± 9.44[a]
Chamomile Group						
n=5	**Baseline**	53.32± 11.09	69.70±27.72	-5.26±8.99	44.64± 4.67[a]	206.34± 44.85[a]
n=5	**Day 28**	74.64± 14.20[Å]	65.54± 17.50	4.40± 4.61	59.24± 8.24[a]	201.48± 37.58[Å]
n=5	**Day 70**	68.78± 10.89[aÅ]	87.64±53.10	0.00± 0.00	60.84± 9.82	123.34±12.92[aÅ]
Referans levels						
		77.33±5.30	56.00±4.12	15.06±1.22	54.59±5.08	71.11±23.58

TC:total cholesterol, TG: tryglyseride, LDL: low density lipoprotein, HDL: high density lipoprotein

⊕ Independent T-test statistically significant difference was found at $p<0.05$., day 28 and day 70 was analyzed.

a One-way ANOVA, $p<0.05$ within-group

People have used herbs for decades for the treatment of various diseases. Chamomile is a herb used to treat and prevent obesity with different properties, but there are only a few studies about this effect [9,12,18,24]. This study found that the chamomile effect reduces body weight compared to a high-fat diet in rats. Some studies have shown that chamomile's anti-obesity effect is linked to apigenin content [25,26]. In another study, the effect of chamomile extract on blood glucose levels and body weight were examined in rats. It was found that the chamomile group has a lower body weight than the control group [27]. According to these results, chamomile extract can be used to reduce body weight against a high-fat diet, and it was thought to prevent obesity. However, which molecules are responsible for these effects and how they work will be investigated further.

Although chamomile affects body weight, blood sugar level, and lipid profile in this study, few limitations exist. Rats number is one of the limitations. In the beginning, each group had five rats, but some rats have died during blood collection. In addition to that LDL, cholesterol results were found different from the literature. LDL cholesterol was calculated with the Friedewald low-density lipoprotein cholesterol method (F-LDL-C) [28]. Therefore, it was thought that the cause of differences in different formulations. Despite the limitations, the effects of chamomile on body weight, blood sugar, and lipid profile have been shown in this study. Therefore, it was thought that this herb might be used for the treatment of obesity. Primarily chamomile tea may be used in the diet for reducing body weight. But further investigation is needed to understand the mechanisms and which phytochemicals are responsible for these effects.

The importance of macronutrient distribution has been long known in the development of obesity and many diseases. A high-fat diet is mainly associated with some metabolic diseases such as cardiovascular disease, Type 2 diabetes, fatty liver, neurological disease, kidney dysfunction, and obesity. Various medical and traditional methods have long been used in the treatment of obesity. The use of plants has always been widespread by people with no specialized or medical knowledge of how they function. Chamomile is an example of the plants used in the treatment of obesity. In this study, the effects of chamomile on body weight, blood lipid level, and blood sugar level in rats were investigated. It is found that a high-fat diet caused an increase in body weight. In addition, chamomile against a high-fat diet affects limiting weight gain. In the chamomile group, blood glucose levels significantly decreased. The use of chamomile could be an effective strategy for restricting weight gain, but further studies are needed to understand the anti-obesity effects of chamomile and other herbs.

STATEMENT OF ETHICS

This experiment was approved by the Istanbul Medipol University Animal Experiments Local Ethics Committee (IMU-HADYEK, decision no: 84) before the study.

CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

AUTHOR CONTRIBUTION

HKBG and NB have planned and conducted to study. Tİ has helped for extraction of chamomile and laboratory use. HKBG has prepared the first draft of the research. NB and Tİ have checked for the final document.

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