ACTA PHARMACEUTICA SCIENCIA

International Journal in Pharmaceutical Sciences, Published Quarterly

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

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Contents

Aims and Scope of Acta Pharmaceutica Sciencia

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

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ORIGINAL ARTICLES

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Total phenolic contents, *in vitro* antioxidant activity, enzymes inhibition and anti-inflammatory effect of the selective extracts from the Algerian *Lavandula multifida*

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ABSTRACT

The present research aimed to carry out the phytochemical analysis, *in vitro* antioxidant activity, enzymes inhibition and anti-inflammatory effect of the selective extracts of the Algerian *L. multifida* from two regions; Msila region and Constantine region. The total phenolic and flavonoids contents of crude extract (CE) and its solvent partition fractions: dichloromethane (DME), ethyl acetate (EAE) and n-butanol (BUE) were determined spectrophotometriclly. The antioxidant activity of extracts was achieved by the use of seven methods and the enzyme inhibitory activity of extracts was evaluated against α-amylase and

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butyrylcholinesterase. Moreover, the *in vivo* anti-inflammatory activity of the aqueous extract of *L. multifida* from Constantine region was evaluated using paw edema model. The M.EAE present the highest TPC and TFC, which were about 462.23±11.74µg GAE/mg extract and 125.90±0.16µg QE/mg extract, respectively. In addition, the M.EAE exhibited an excellent antioxidant activity, as it has a great ability to scavenging the DPPH, ABTS and galvinoxyl free radicals, as well as reducing power and metal chelating. However, the M.CE and C.CE showed the best inhibitory activity of the α-amylase butyrylcholinesterase (IC50=64.17±1.81µg/ml and 83.55±1.97µg/ml, respectively. The preliminary investigation reveals that the EAE has a good bio-pharmacological activity, which it possesses an interesting potential for pharmaceutical/nutraceutical applications.

Key words: Antioxidant activity, anti-inflammatory effect, enzymes inhibition, *Lavandula*.

INTRODUCTION

Since the beginning of human existence, human has become acquainted with plants and has used them in various ways and in different fields. Medicinal plants play an important role in the prevention and treatment of human diseases. This relationship between plants and human has developed and many plants have been used as a natural traditional remedy. In recent years, the pharmacological effects of medicinal plants have been considered as a potential source of future medicaments [1]. In biological systems, the reactive oxygen species (ROS) and reactive nitrogen species (NERs) can damage DNA and cause oxidation of lipids and proteins in cells. Naturally, an antioxidant system present in the human body can eliminate these free radicals, which would help maintain the balance between the production of reactive oxygen species and the antioxidant capacities of the body [2]. Studies on the toxicity of synthetic antioxidants have increased the demand for natural antioxidants, particularly from a plant source, in the food, pharmaceutical and cosmetic sectors, since they can be used as natural substituents for these synthetic antioxidants[3]. The presence of natural antioxidant such as phenolic compounds, flavonoids and tannins in plants may provide protection against a number of diseases, and their use has been inversely associated with mortality and morbidity due to degenerative disorders [4]. Lamiaceae, or Labiatae, is a family of angiosperms with 236 genera and more than 7000 species [5]. Lamiaceae are widely used in traditional and modern medicine worldwide, thanks to the many beneficial pharmacological effects they can exert [6]. The genus Lavandula is an important member of Lamiaceae, it comprises 39 species. *Lavandula multifida* is a small, semi-evergreen perennial shrub composed of several small leaves and aromatic

flowers. It grows commonly in the Mediterranean region and North Africa, where it is mainly distributed in pre-Saharan areas, more and more on rocky outcrops and on more or less drained limestone soils at the edge of temporarily drained rivers, between 800 and 2000 meters of altitude 7-[8]. The main aim of this work is the total phenolic contents estimation and of biological activities study of *L. multifida* from two Algerian geographical areas.

METHODOLOGY

Biological material

L. multifida aerial parts were collected in 2016 from the Maadid region of Msila wilaya (Algeria) in April 2018. The second region *L. multifida* plant was collected from Jebel El Ouahch region of Constantine Province (Algeria) in June 2019 (**Figure 1**). After drying in the shade, the aerial parts of the plant are crushed, the vegetable powder thus obtained was used for extraction.

Figure 1. The Geographic position of Msila (**1**) and Constantine (**2**) in Algeria.

The *in vivo* study was carried out on female Wistar rats, whose weight varies between 130 g and 170 g. These animals were obtained from the animal department of the natural sciences and life of the University of Mentouri Constantine Brothers. The rats were placed in polypropylene cages at room temperature (20-25 °C) where they had free access to water and food. These animals benefited from an adaptation period of one week before their use.

Extraction

The hydromethanolic extraction: The hydromethanolic extraction was carried out by maceration of 100 g powdered dry plant (of each region) in 1 L of methanol (80%) for 72 h at room temperature. After 72 hours of contact, the methanol mixture was filtered and concentrated at 40 °C under reduced pressure using a rotary evaporator (BUCHI) to give crude extract (M.CE: crude extract of the plant from Msila region; C.CE: crude extract of the plant from Constantine region). The crude extract of etch plant was subjected to liquid-liquid extraction (fractionation) using organic solvents with increasing polarity using dichloromethane giving dichloromethane extract labeled (DME), ethyl acetate giving ethyl acetate extract labeled (EAE) and *n*-butanol giving butanol extract labeled (BUE). All solvents were removed using rotary evaporator to give the respectively extracts: M.CE and their fraction M.DME; M.EAE and M.BUE; C.CE and their fractions C.DME; C.EAE and C.BUE.

The aqueous extraction: For the evaluation of the anti-inflammatory activity *in vivo*, the 10 g dried and powdered aerial parts of *L. multifida* from Constantine region were extracted by maceration with 500 ml of distilled water. After 24 hours of contact, the whole is filtered with filter paper in order to recover the filtrate; the latter has been dried in an oven to obtain aqueous extract [8].

Total phenolic contents (TPC)

The total phenolic content of the extracts was determined using the Folin-Ciocalteu reagent (FCR) according to the method described by [9]. In the 96-well microplate, a volume of 20 µl of plant extract (1 mg/ml) was added to 100 µl of diluted FCR (1:10). Then 75 µl of sodium carbonate (7.5%) was added. The mixture was left in the dark for 2 hours at room temperature. The absorbance of different intensities of the resulting blue color was measured at 765 nm using a PerkinElmer 96-well microplate reader (USA). The TPC was expressed as µg gallic acid equivalent per mg of extract (µg AGE/mg E), using the linear regression equation of the calibration curve plotted by gallic acid (y=0.0034x+0, 1044) with R^2 = 0.9972.

Total flavonoid contents (TFC)

The total flavonoid content of the extracts was determined using aluminium nitrate ($Al(NO_3)_3$) reagent according to the method described by [10]. In the 96-well microplate, a volume of 50 µl of the extract was mixed with 130 µl of methanol, 10 µl of potassium acetate and 10 µl of aluminum nitrate. The mixture was incubated for 40 minutes and then the absorbance was measured at 415 nm using a PerkinElmer 96-well microplate reader (USA). The TFC was

expressed as μg quercetin equivalent per mg of extract (μg QE/mg E), using the linear regression equation of the calibration curve plotted by quercetin (y=0.0071x+0.0274; R^2 = 0.9985).

In vitro antioxidant activity

The antioxidant capacity of extracts was achieved through seven methods: DPPH free radical scavenging assay, galvinoxyl free radical scavenging test, ABTS assay, reducing power, cupric reducing antioxidant capacity (CUPRAC), Fe^{+2}-phenanthroline reduction and metal ions chelation.

DPPH radical scavenging activity

The DPPH antiradical activity of extracts was determined spectrophotometrically according to the method described by [11]. Briefly, a 160 μl of DPPH solution (0.6 mg/100 ml methanol) was mixed with 40 μl of the extract prepared in several concentrations. The mixture was kept in dark at room temperature. After 30 min of incubation, the absorbance was measured at 517 nm. The antiradical activity was expressed as EC_{50} the values, i.e., concentration of the studied extracts, which causes a 50% decrease in the absorbance at 517 nm as compared to the control. The percent inhibition was calculated according to the following equation: DPPH scavenging (%) = ((*A* of control – *A* of sample)/ *A* of control) × 100

Galvinoxyl radical-scavenging assay

The antiradical activity of the extracts against galvinoxyl radical was determined according to the method described by [12]. Briefly, 160 μl of galvinoxyl radical (10 μM) was mixed with 40 μl of extract at several concentrations. The reaction was carried out for 2 h at room temperature. The decrease in galvinoxyl radical concentration was determined spectrophotometrically by measuring the absorbance at 432 nm, the antiradical activity was expressed as EC_{50} the values, i.e., concentration of the studied extracts, which causes a 50% decrease in the absorbance at 432 nm as compared to the control sample. The percent inhibition was calculated according to the following equation: Galvinoxyl radical-scavenging (%) = ((*A* of control – *A* of sample)/ *A* of control) ×100

$ABTS^{\bullet+}$-scavenging assay

$ABTS^{\bullet+}$-scavenging capacity was determined according to the method described by [13]. The $ABTS^{+}$ radical was generated by the mixture of ABTS solution (7 mM) with potassium persulfate ($K_2S_2O_8$) solution (2.45 mM). The mixture was incubated in the dark at room temperature for 16-24 h. The standard solution of ABTS was diluted by the addition of methanol to have an absorbance of

0.700 (±0.02) at 734 nm. An aliquot of 40 µL of extract was mixed with 160 µL of ABTS and absorbance was recorded after 30 minutes.

The antiradical activity was expressed as EC_{50} the values, i.e., concentration of the studied extracts, which causes a 50% decrease in the absorbance at 432 nm as compared to the control. The percent inhibition was calculated according to the following equation: Galvinoxyl radical-scavenging (%) = ((*A* of control – *A* of sample)/ *A* of control) × 100.

The data were the concentration required for an effect of 50% (EC_{50}).

Cupric reducing antioxidant capacity

The cupric ion reducing antioxidant effect of the extracts was estimated according to the method of [14]. 40 µL of extract soultion was mixed with 50 µL of copper chloride solution (10 mM), 50 µL of neocuproine alcoholic solution (7.5 mM) and 60 µL of ammonium acetate buffer solution (1 M, pH 7.0) to make final volume of 200 µL. After one hour of incubation, the absorbance was measured at 450 nm against the reagent blank. Standard curve was prepared using different concentrations of Trolox and the results were expressed as $A_{0.50}$ (µg/ml) corresponding to the concentration giving an absorbance of 0.50.

Reducing power

The reducing power of the extracts was determined according to the methods of [15]. A volume of 10 µl of the extract at different concentrations was mixed with 40 µl of phosphate buffer solution (0.2M, pH=6.6) and 50 µl of a potassium ferricyanide [$K_3Fe(CN)6$] solution (1%), the mixture was incubated was incubated at 50 °C for 20 min. Then, 50 µl of trichloroacetic acid (10%) were added to stop the reaction and the whole was centrifuged at 3000 r/min for 10 min. Finally, 50 µl of the supernatant solution was mixed with 50 µl of distilled water and 10 µl of $FeCl_3$ (0.1%) and the absorbance was recorded at 700 nm after an incubation for 10 min. Increased absorbance of the reaction mixture. The positive control was represented by two standard antioxidant solutions; ascorbic acid and α-tocopherol, the absorbance of which was measured under the same conditions as the samples. The results were expressed as µg ascorbic acid equivalent/mg extract.

Reduction activity by phenanthroline method

Reduction activity by phenanthroline method was determined according to [16]. A volume of 10 µl of the extract at different concentrations was mixed with 50 µl (0.2%) of ferric chloride ($FeCl_3$), 30 µl of phenanthroline (0.5%) and 110 µl of methanol. The obtained solution was left at room temperature in a dark

for 20 min. After the incubation, the absorbance of an orangered solution was measured at 510 nm. BHA and BHT wre used as standards. The results were calculated as $A_{0.5}$ (µg/ml) corresponding to the concentration indicating an absorbance of 0.50.

Metal ions chelation activity

Iron (Fe^{+2}) chelating property of the extracts was determined using the method of [17]. A volume of 40 µl of extract solution at various concentrations was mixed with 40µl of $FeCl_2$ (0.2 mM) and of 40 µl of methanol. The reaction was initiated by the addition of 80 µl of the ferrozine (5 mM) to the whole. After an incubated in the dark for 10 min, the absorbance of the solution was measured at 562 nm against a similarly prepared blank.

The metal chelating activity of ethylenediamine tetra-acetic acid (EDTA) was also determined and the metal chelating activity was expressed as µg equivalent EDTA /mg extract.

Enzymes inhibition activity

Alpha-amylase inhibition

The α-amylase inhibitory activity of the extracts was assayed according to the procedure described by [18]. The extract solution (25 µL) was mixed with 25 µL of phosphate-sodium buffer (5 mM, pH 6.9) and 50 µL α-amylase solution (1U), the mixture was incubated at 37 °C for 10 min. Then, 50 µl of soluble starch (1%) were added and the mixture re-incubated for 10 min at 37 °C. After 10 min of incubation, the reaction was stopped by the addition of 25 µl of HCl (1M) and 100 µl of iodine-potassium iodide solution. The absorbance was measured at 630 nm and the percentage inhibition of α-amylase was calculated as follows:

α-amylase inhibition % = [(A of control –A of sample)/ A of control] × 100

The IC_{50} concentration required for inhibition of 50% of α-amylase was determined graphically and Acarbose was used as a positive control.

Butyrylcholinesterase (BuChE) inhibitory activity

Butyrylcholinesterase (BuChE) inhibitory activity was measured using Ellman's method as previously reported by [19]. Briefly, the plant extract (10 µL) was mixed with dithiobisnitro-benzoate (DTNB) (10 µL) and BuChE (10 µL) in 150 µl of sodium phosphate buffer (100 mM, pH 8.0) in a 96-well microplate. The reaction was initiated by the addition of 20 µl butyrylthiocholine chloride (0.2 mM). The solution mixture was maintained at 30 °C for 15 min using water bath. The hydrolysis of these substrates was followed spectrophoto-metrically

at 412 nm with the formation of a yellow color. The BuChE inhibitory activity was calculated as follows:

$$\text{BuChE inhibition \%} = [(\text{A of control} - \text{A of sample}) / \text{A of control}] \times 100$$

The IC_{50}, concentration required for inhibition of 50% of BuChE was determined graphically and Galantamine was used as a positive control.

Evaluation of anti-inflammatory activity *in vivo*

The *in vivo* study was carried out on female Wistar rats, whose weight varies between 130 g and 180 g. These animals were obtained from the animal department of the natural sciences and life of the University of Mentouri Constantine Brothers. The rats were placed in polypropylene cages at room temperature (20-25 °C) where they had free access to water and food. These animals benefited from an adaptation period of one week before their use. The experimental protocols were approved by Institutional Animal Ethics Committee (N°: 35/2017) in accordance with the guideline formulated by Committee for Purpose of Control and Supervision of Experiments on Animals, Algeria.

The anti-inflammatory activity of extracts of *L. multifida* (from Constantine region) was evaluated using formaldehyde induced paw edema method according to [20]. The rats were randomly divided into 3 groups based on their body weight; each group contains six animals. One hour before the formaldehyde injection, each group receives via intraperitoneal injection the following experimental solutions:

Group I: Negative control, received 200 µl of saline water (0.9%).

Group II: Positive control, received 200 µl of anti-inflammatory drug Diclofenac (CLOFENAL®) at dose of 25 mg/Kg dissolved saline water.

Group III: Treated group, received 200 µl of aqueous extract of *L. multifida* (200 mg/Kg) dissolved saline water.

One hour after the extract (drug) administration, 0.1 ml of 1% v/v formaldehyde solution was injected into the subplantar aponeurosis of the left hind limb of the rat. The follow-up of the evolution of the inflammation was done by measurement of the paw volumes before and after induction of the edema at 0, 30, 60, 180 and 360 minutes. The paw volumes measured using a graduated tube and were expressed as a percentage change in paw volume at various time intervals in comparison to the initial values. The anti-inflammatory activity was expressed as paw volume reduction, which was given by the following formula:

The anti-inflammatory activity %= [1-(paw volume of treated group at time n - paw volume of treated group control at time 0)/(paw volume of control group at time n - paw volume of control group at time 0] ×100.

Statistical analyses

The experiments of the *in vitro* study were done in triplicates and all data were shown as mean±standard error of the mean (SD). The IC_{50} (50% inhibition concentration) and A0.50 (the concentration indicating 0.50 absorbance) values are calculated by the linear regression method.

The data were recorded as mean±SD in *in vitro* study, while as mean±SEM in *in vivo* study. The Graphpad Prism 7 was used to analyzed the data. The statistical differences between the experimental groups were analyzed using two-way analysis of variance (ANOVA) followed by a Tukey post-test. The differences are considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Extraction, TPC and TFC

The TPC and TFC of the different extracts were analyzed and presented in **Table 1**. As shown, the results indicate that the M.EAE exhibited higher TPC and TFC comparatively to the other extract, which were about 462.23±11.74 µg GAE/mg E and 125.90±0.16 µg QE/mg E, respectively. However, the C.DME had the lowest TPC and TFC, with 29.2±1.11 µg GAE/mg E and 3.98±0.08 µg QE/mg E, respectively.

Extraction is the main step for recovering and isolating phytochemicals from plant materials, extraction yield can be affected by the chemical nature of phytochemicals, the method used, the solvent used, as well as the presence of interfering substances [21].

The difference in the polyphenol and flavonoid content of the crude extracts and their fractions results from the difference in polarity of the organic solvents, the extraction time and temperature, the solid-liquid extraction ratio as well as the chemical and the physical characteristics of the samples [22]. By comparing the results obtained, we note that the *L. multifida* plant from the Msila region contains more polyphenols and flavonoids than that of Constantine region with an amount up to 462.23±11.74 µg EAG/mg extract of polyphenols and (125.90±0.16 µg EQ/mg extract) of flavonoids in the case of EAE. This variability in the polyphenol and flavonoid contents of the two *L. multifida* plants is probably due to the phenolic composition of the extracts, the nature of the soil and the type of microclimate, and the bioclimatic stages

where this plant grows [23].

Other studies on the phytochemical hydromethanolic extracts of *L. multifida* collected from different regions of Morocco, demonstrated that the quantity of polyphenols and flavonoids of the plant from south-west of Morocco are 29.87±0.57 µg EAG/mg of extract and 5.51±0.19 µg EQ/mg of extract, respectively. In other hand, the extracts of the plant from the south of Morocco are less rich of polyphenols flavonoids (16.38 µg EAG/mg extract and 14.31 µg EQ/mg extract) [24; 25].

In vitro antioxidant activity

DPPH radical scavenging activity

In this study, the free radical scavenging activity of the different extracts of *L. multifida* harvested from two regions was evaluated by DPPH radical scavenging test. The obtained results showed that the EAE has the highest free radical scavenging activity for the two plants. Moreover, the C.EAE free radical scavenging activity compared to all extracts with an effective concentration (EC_{50}) (EC_{50}=12.32±0.82 µg/ml), this activity is two times lower than that of BHA (EC_{50}=5.73±0.41 µg/ml). While, the M.DME and C.DME showed the lowest activity with an EC_{50}>100 µg/ml (**Table 1**). Having analyzed the obtained results, *L. multififida* from the Msila region showed better free radical scavenging activity compared to that of Constantine region. The activity of extracts its poverty in polyphenols can be justified according to [26], by the fact that the inhibiting activity of the DPPH radical is not dependent on the total content polyphenols but polyphenols which have specific chemical structures. In other words, by the high selectivity of DPPH because it only reacts with flavonoids containing hydroxyl groups in the B ring, as well as aromatic acids containing more than one hydroxyl group [27].

The study of the antioxidant activity of the hydromethanolic extract of the *L. multifida* plant collected from different regions of Morocco has found a percentage of inhibition of the order of (74.10±0.14%) in the south-west of the country, while in the south of the country this extract gave a percentage inhibition equal to 90.35% [11; 12]. For their part, Messaoud *et al*., (2012) [28] found a great capacity of methanolic extract of Tunisian *L. multififida* to scavenge the DPPH radical with an EC_{50} value of 19.3±1.2 µg/ml.

Galvinoxyl radical scavenging capacity (GOR)

The galvinoxyl radical scavenging activity of extracts was determined, and the results were expressed as $EC_{50.}$ However, the results showed that the M.EAE

exhibited the highest activity (EC_{50}=9.60±0.06 µg/ml), this result is eight times greater than the activity of C.EAE (EC_{50} =72.04±4.33 µg/ml) and three times lower than that of BHT (EC_{50}=3.32±0.18 µg/ml) (**Table 1**).

From these results, it can be said that the *L. multifida* plant from the Msila region showed a good scavenging activity for the Galvinoxyl radical compared to that from the Constantine region. This variability in extract activity is due to the types of polyphenols contained in the extract. Indeed, it is well known that the hydrogen-donating antioxidant reacts quickly with the Galvinoxyl radical. The latter can be used to measure the stoichiometric number of phenolic hydrogens in an antioxidant. So, this method can be used for the determination and comparison of the antioxidant activity of hydrogen-donating compounds, whether in pure substances or in mixtures [29]. This study is the first in evaluation of the scavenging activity of the Galvinoxyl radical of the *L. multifida* plant. On the other hand, a study on the methanolic extract of *L. stoechas* from Algeria, a species of the same genus as *L. multififida*, gave a scavenging activity of the Galvinoxyl radical equal to (227 µg of Trolox equivalent/mg of extract) [30].

ABTS radical cation scavenging activity

The antiradical activity of extracts was measured using ABTS scavenging assay. In this test, the antioxidant reacts with $ABTS^{\bullet+}$ blue/green in color by electron transfer to restore the colorless $ABTS^{\bullet+}$. This transformation was followed by measuring the absorbance and determining the EC_{50} of different extracts in comparison with the BHA and BHT standards. The results obtained were showed that the M.EAE exhibited the highest antiradical activity (EC_{50}=4.89±0.20 µg/ml), this activity was five times lower than the BHA standards (EC_{50}= 1.03±0.01 µg/ml) and three times than the BHT (EC_{50}=1.59±0.03 µg/ml) (**Table 1**). These results of the free radical scavenging activity of the *L. multifida* plant from the Msila region confirm the strong antioxidant capacity of these extracts compared to those of *L. multifida* from the Constantine region. In this test, the extracts showed a better free radical scavenging effect than in the DPPH test. This difference can be justified by the ability of the cation radical ABTS to be more versatile than DPPH, which is soluble in water and organic solvents and allows an evaluation of free radical scavenging activity for hydrophilic and lipophilic compounds [31]. Our study is the first carried out using the ABTS method to evaluate the free radical scavenging of *L. multifida* extracts plant. However, a study on the essential oils of three plants of *L. stoechas*, a species of the same genus as *L. multifida*, collected in different regions of Spain revealed a scavenging activity ranging between 175.3±3.3 and 14.8±0.6 µmol Trolox equivalent/ml of essential oils against ABTS radical [32]. In addition, Amira *et*

al. (2012) [30] noted that the methanolic extract of *L. stoechas* from Algeria gave a scavenging activity equal to 457 µg of Trolox equivalent/mg of extract. In study released by Nikolic *et al.* (2019) [33] on the hydromethanolic extract of *L. angififolia* from Serbia gave a scavenging activity equal to 2.54±0.2 µg of Trolox equivalent/mg of extract.

Reduction activity of the copper-neocuproin complex (CUPRAC)

The reducing activity of extracts was determined using CUPRAC method, which used to measure the ability of antioxidant to reduce ferric Fe (III) and cupric Cu (II) ions to their respective lower valency state [34]. The results showed that the M.EAE has an excellent reducing activity ($A_{0.5}$ =5.8±0.50 µg/ml), compared to the BHA ($A_{0.5}$= 3.6±0.19 µg/ml).

The reducing activity of M.EAE was eight times stronger than the activity of C.EAE ($A_{0.5}$ =49.65±5.42 µg/ml) (**Table 1**). On the other hand, the extracts M.DME, C.CE and C.DME showed a low reduction activity ($A_{0.50}$> 100 µg/ml). This study allowed us to confirm the high activity of the *L. multifida* plant from the Msila region in which the EAE gave an excellent reduction of the copper-neocuproin complex compared to BHA standard and compared to extracts of *L. multififida* from the Constantine region. The difference in the reducing activity of the extracts may be due to several criteria, which polyphenols have such as the number and the position of hydroxyl groups as well as the degree of conjugation of the entire molecule (double bond) [35]. The carbonyl group in position 4 (oxo) and the ortho-dihydroxy structure on the B ring (catechol group) which are important for the easy transfer of electrons [36]. The evaluation of the antioxidant activity by CUPRAC method is made for the first time for *L. multifida* plant. A study on the hydromethanolic extract of *L. angififolia* from Serbia, a species of the same genus as *L. multifida* gave a reduction activity of the copper-neocuproin complex equal to 0.07±0.00 mg of Trolox equivalent/g of extract [33]. Another study on the methanolic extract of *L. stoechas* from Turkey gave a reduction activity of the copper-neocuproin complex equal to 369.66±6.73 mg of ascorbic acid equivalent/g of extract [37].

Ferric reducing/antioxidant power (FRAP)

The reducing power of the extracts was determined. The results obtained showed that the M.EAE have the strongest reducing capacity with values 1181.50±8.64 µg AA equ/mg E. Then, this activity was followed by the activity of M.BUE with 626.27±7.29 µg AA equ/mg E (**Table 1**). While the extracts M.DME, C.CE, C.DME and C.BUE showed the lowest reducing power compared to the other extracts with an activity lower than 50 µg AA equ/mg E. For this activity, the

presence of reducers compounds in plant extracts makes it possible to reduce the Fe^{+3} to Fe^{+2}. The reducing power of plant extracts was dependent on their concentration, where polyphenol-rich extracts have a higher reducing power [38; 39]. The reducing power of *L. multifida* extracts (Msila) is probably due to the presence of phenolic compounds containing hydroxyl groups which can serve as electron donor, which can react with free radicals and convert them into more stable products, thus putting an end to chain reactions of free radicals [40; 41]. The study carried out by Ramchoun *et al.* (2009) [42], on the aqueous extract of *L. multififida*, harvested in Morocco recorded a reducing power with a value of (12.76±0.48 mmol of Trolox equivalent/g of extract), this activity is low compared to our results. In addition, the work carried out by Amri *et al.* (2015) [24] on the hydromethanolic extract of the *L. multifida* collected from different regions of Morocco gave the value of (325.65±3.20 µmol/g extract), this value is similar to our results.

Reducing activity using Fe(II)-phenanthroline complex

According to the **Table 1**, the results demonstrated that the extracts; M.EAE, M.CE and M.BUE showed the greatest reducing power ($A_{0.5}$=10.92±3.31; 13.85±1.16 and 15.91±2.11 µg/ml; respectively). However, the two standards, BHA and BHT, have the strongest reducing activities ($A_{0.50}$=0.93±0.07 and 2.24±0.17 µg/ml, respectively). In addition, the extracts of *L. multifida* from Constantine region showed the lowest reducing power compared to the extracts of *L. multifida* from Msila region. In this activity, the presence of reducers in the plant extracts makes it possible to reduce Fe^{+3} to Fe^{+2}. Consequently, the latter forms a stable complex with phenanthroline, which is orange-red in color. However, to the best of our knowledge, there was no reference on application of the reaction between ferrous ions and 1,10-phenanthroline for determination of antioxidant capacity of edible oils and the other foodstuffs. Only, Berker *et al.* (2010) [43] used 1,10-phenanthroline method for assay of antioxidant capacities of different antioxidants and their mixtures. Besides that, Phen method was applied for measuring the total antioxidant capacity of plasma, pleural effusion and antioxidants defense system.

This difference in the reducing power may be due to the reducing capacity of polyphenols as antioxidants which depends on the degree of hydroxylation and the degree of conjugation of phenolic compounds [44]. Specifically for flavonoids, it has been suggested that the capacity for eliminating free radicals increases when the following conditions are present: the presence of a 3′, 4′-dihydroxy structure in the B cycle, the presence of a double bond (C2-C3) in conjunction with the 4-oxo group in the heterocycle and the presence of 3- and 5-hydroxyl

groups in ring A with a 4-oxo function in rings A and C [45]. Our study of the iron reduction activity by the formation of the Fe^{+2}-phenantroline complex was the first carried out on the *L. multifida* plant. A study on the extract of *L. pedunculata* collected from the south of Portugal, gave percentages of the Fe^{+2}-phenantroline complex of the order of 5.9±1.27% for the aqueous extract and 50.1±0.14% for the hydroethanolic extract. The activity is low compared to our results [46].

Metal ion chelation activity

Bivalent ferrous ions play an important role as catalysts of oxidative processes, leading to the formation of superoxide anion radicals and hydroxyl radicals via Fenton reactions. It was reported that the generated free radicals would cause the production of oxyradicals, lipid peroxidation and DNA damage [47]. These processes can be delayed by iron chelation or an antioxidant. In this assay, ferrozine can quantitatively form complexes with Fe^{+2}. In the presence of other chelating agents or antioxidants, the complex formation is disrupted with the result that the purple color of the complexes decreases. Monitoring this activity by determining the EC_{50} values and in comparison, with the EDTA standard. As shown in **Table 1**, all the extracts of *L. multifida* from Msila and Constantine showed a very low chelating power (EC_{50}> 800 µg/ml) compared to that of the EDTA standard (EC_{50}=8.80±0.47 µg/ml). However, the weak chelation activity of *L. multififida* extracts despite the great richness of that harvested from Msila in polyphenols can be justified according to [48], by the fact that the chelation capacity is not dependent on the total polyphenol content but on the type of polyphenols having hydroxyl groups in the vicinity or in the ortho-position of the benzene ring.

Among the phenolic compounds which have a good chelating capacity are the flavonoids, specifically those containing a ring B catechol nucleus, 3-hydroxyl and 4-oxo groups of the C ring, and 4-oxo and 5-hydroxyl groups between cycles A and C [49; 50]. A study carried out by Messaoud *et al.*, (2012) [28], showed that the methanolic extract of *L. multifida* exerted a capacity to chelate iron of EC_{50} of the order of (0.8±0.1 mg/ml).

Table 1. Total phenolic and flavonoids content and antioxidant activity of *L. multifida* extracts

	L. multifida from Msila region				*L. multifida* from Constantine region				Reference
	M.CE	M.DME	M.EAE	M.BUE	C.CE	C.DME	C.EAE	C.BUE	
TPC (µg GAE/ mg E)	204.29±6.57	52.72±7.94	462.23±11.74	291.94±11.22	60.66±26.05	29.2±1.11	178.21±47.45	43.60±13.45	
TFC (µg QE/mg E)	50.31±3.39	21.58±1.30	125.90±0.16	87.17±4.97	33.61±5.74	3.98±0.08	34.16±3.83	32.22±0.44	
Antioxidant assays									
DPPH (EC_{50} µg/mL	21.29±0.50	>100	16.46±0.35	34.73±0.38	42.74±1.22	>100	12.32±0.82	43.48±3.36	
BHA									5.73±0.41
Galvinoxyl (EC_{50} µg/mL)	14.37±0.29	>100	9.60±0.06	12.02±0.06	34.65±0.58	>100	72.04±4.33	36.29±2.34	
BHT									3.32±0.18
BHA									5.38 ±0.06
ABTS (EC_{50} µg/mL)	13.80±0.33	>100	4.89±0.20	9.88±0.60	33.71±0.24	>100	9.28±0.20	28.59±2.33	
BHT									1.59±0.03
BHA									1.03±0.01
CUPRAC ($A_{0.5}$, µg/mL)	16.59±0.26	>100	5.87±0.50	11.16±1.03	>100	>100	49.65±5.42	55.48±1.88	
BHT									9.62±0.87
BHA									3.64±0.19
RP (µg AA equ/ mg E)	356.50±9.64	<50	1181.50 ±8.64	626.27±7.29	<50	<50	77.75±5.97	<50	
RP phenanthroline ($A_{0.5}$ µg/mL)	13.85±1.16	>100	10.92±3.31	15.91±2.11	>100	>200	26.82±1.82	85.95±4.28	
BHT									0.93±0.07
BHA									2.24±0.17
Metal chelation (EC_{50} µg/mL)	>800	>800	>800	>800	>800	>800	>800	>800	
EDTA									8.80±0.47

RP: Reducing power; AA: Ascorbic acid; E: Extract

Enzymes inhibitory activity

Alpha-amylase inhibition

The anti-diabetic activity of extracts of *L. multififida* from the two regions was evaluated by measuring their capacities to inhibit the alpha-amylase enzyme.

In this study, the inhibitory activity of extracts on the alpha-amylase was estumated by determining the inhibitory concentrations IC_{50} compared to the standard acarbose based on the ability of a substance to inhibit this enzyme.

From the results obtained (**Table 2**), it was noted that the alpha-amylase inhibitory activity has been recorded only at the level of the M.CE with an (IC_{50}=64.17±1.81 µg/ml). This activity is fifty-six times higher than that of the standard acarbose (IC_{50}=3650.93±10.70 µg/ml), the latter reacts *in vivo* better than *in vitro*. By the comparison, we can see that *L. multififida* from the Msila region showed excellent anti-diabetic activity compared to that from the Constantine region. This difference in alpha-amylase inhibitory activity can be justified by the presence in the M.CE of a class other than flavonoids

because the latter only account for 24.62% of the total polyphenol content. This class was probably tannins, one of the main classes of phenolic compounds characterized by the presence of non-specific inhibitory molecules of various hydrolytic enzymes such as α-amylases, α-glucosidases and lipases. This inhibition is perhaps associated with their ability to bind strongly to proteins and carbohydrates whose interaction between tannins and proteins is the result of multiple hydrogen bonds and hydrophobic associations. As a result of this interaction, the catalytic sites of enzymes are blocked and their activity is therefore inhibited [51]. The inhibitory activity of the alpha-amylase enzyme of the *L. multifida* plant is the first carried out on this species and its genus. In addition, a study on the aqueous extract of *Ocimum basilicum* from Saudi Arabia (a species of the Lamiaceae family) gave an alpha-amylase inhibiting activity equal to 42.50 mg/ml [52], this result is similar to our result.

BuChE inhibitory activity

Butyrylcholinesterase (BuChE) is an enzyme which has been shown to be involved in the patho-genesis, treatment and prognosis of Alzheimer's disease [53]. The BuChE inhibition activity of the various extracts of the *L. multifida* plant was determined according to the method of [54]. Ellman's test was based on the cleavage of butyrylthiocholine by the BChE to produce thiocholine. The latter will react with 5,5'-dithiobisnitrobenzoate (DTNB) to form a yellow anion. In the presence of an enzyme inhibitor the yellow color will decrease, which makes it possible to evaluate the reaction, and subsequently to quantify the inhibition of the enzyme. This inhibition was followed spectrophotometrically by the measurement of the absorbance at 412 nm, to determine the inhibitory concentration (IC_{50}) of the different extracts in comparison with the standard galantamine.

In this study, it was shown that the C.CE exhibited the strongest inhibitory activity (IC_{50}=83.55±1.97 µg/ml), when the BuChE inhibitory activities of the extracts were compared among themselves. The activity of C.CE is twice lower than that of the standard galantamine (IC_{50}=34.75±1.99 µg/ml). In contrast, the C.DME slightly inhibited the activity of BuChE (IC_{50}=152.44±0.63 µg/ml). While, the extracts M.CE, M.DME, M.EAE, M.BUE and C.EAE have shown a weak inhibitory activity against BuChE (IC_{50}>200 µg/ml) and they are far from being compared to galantamine (**Table 2**). However, C.BUE was inactive for the different concentrations. From these results, it can be said that *L. multififida* from the Constantine region exhibited a moderate activity of inhibition of BuChE compared to that of the Msila region.

Table 2. Inhibition of α-amylase and BuChE by extracts. The results were presented as IC_{50} values in μg/mL.

Sample		α-amylase inhibition	BuChE inhibition
L. multifida from Msila	M.CE	64.17±1.81	>200
	M.DME	>400	>200
	M.EAE	>400	>200
	M.BUE	>400	>200
L. multifida from Constantine	C.CE	>25	83.55±1.97
	C.DME	>25	152.44±0.63
	C.EAE	>25	>200
	C.BUE	>25	Inactive
References	Acarbose	3650.93±10.70	/
	Galantamine	/	34.75±1.99

The BuChE inhibitory activity by the *L. multifida* plant may be due to the presence of polyphenols. It is also due to alkaloids, terpenes, and coumarins, which are compounds with anticholinesterase properties [55]. A study on three extracts of *L. viridi* from Portugal, gave percentages of inhibition of BuChE of around 32.34±3.03%, 63.01±1.84%, and 51.19±1.52% for the aqueous extract, the hydroethanolic extract and the ethanolic extract respectively [56], these results are similar to those recorded in our study. In the present study, fraction method and methanol solvent were the most suitable solvent and method to get the strongest anticholinesterase activity. To the best of our knowledge, there have been no reports in literature on the anticholinesterase activity for this species. Therefore, in this study, the anticholinesterase activity of plant's extracts was examined for the first time.

In vivo anti-inflammatory activity

According to the results obtained, which represents the evolution of the edema after the intraperitoneal injection of formaldehyde, the inflammation was more pronounced in the control group who not received the treatment.

Moreover, the results showed that, the administration of aqueous extract of *L. multififida* (200 mg/kg) prevented formaldehyde-induced paw edema in rats, with an anti-inflammatory activity of 10.02%, 21.83%, 37.20%, and 43.71% at 30, 60, 180 and 360 min, respectively. While the Diclofenac showed a good anti-inflammatory effect, and the activity was as follows: 27.22%, 36.83%, 53.26%, and 80.28% at 30, 60, 180 and 360 min, respectively (**Figure 2**).

This moderate capacity of the aqueous extract of *L. multififida* to inhibit edema and therefore to inhibit the synthesis of pro-inflammatory substances such as cytokines and prostaglandins can be justified by the low content of our extract in flavonoids, the latter have a capacity to inhibit cyclooxygenase and therefore causing inhibition of inflammation [57].

Formaldehyde-induced paw edema model is a suitable experimental animal model for evaluating or screening the anti-inflammatory effects from natural products. We can also observe two intervals of evolution of the edema explained by the two-phase nature of the inflammatory response by formaldehyde, the first phase results mainly from the concomitant release of inflammation mediators such as serotonin, histamine and kinin. The second phase is characterized by the release of prostaglandins produced by macrophages [58].

In study of anti-inflammatory activity of *L. multifida* collected from southern of Morocco, showed that the ethanolic extract has an edema reduction capacity of up to 62%, while the aqueous extract has shown a weak anti-inflammatory activity with an edema reduction capacity equal to 33% in mice [47; 59].

It has been said that presence of certain flavonoids exerts profound anti-inflammatory activity by stabilizing the lysosomal membrane [60]. The outcome of our study of and from the previous database on this plant, it can be predictable that the anti-inflammatory effect exerted is because of flavonoid content. Sometimes it happens that the crude plant extracts are extra pharmacologically lively than their isolated active compounds [61]. The targeted mechanism of action for the anti-inflammatory activity of studied specimen just is not identified, but the extract may be intercepting the construction of inflammatory mediators dependable for inflammation, either COX pathway or different specific enzymatic mechanism.

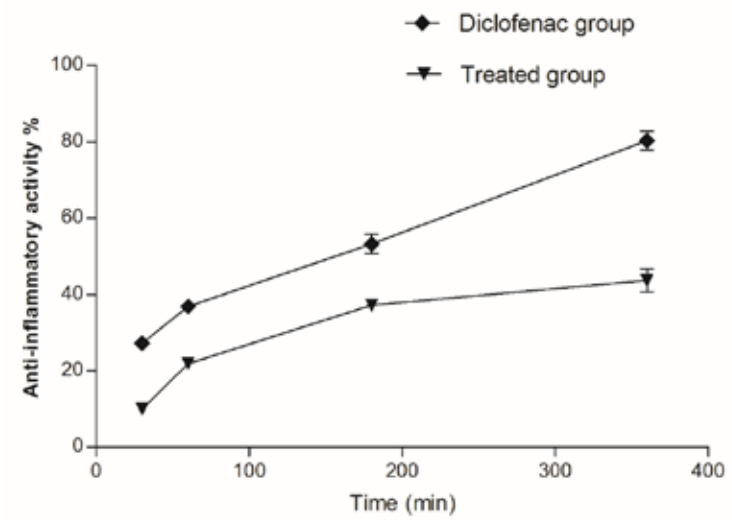

Figure 2: Anti-inflammatorty activity of the aqueous extract of *L. multifida* from Msila

Herbal remedies have been used therapeutically for thousands of years to naturally treat a variety of diseases. This property has been confirmed by modern scientific research, which has ensured the effectiveness of these plants thanks to their richness in secondary metabolites which give them diverse biological properties. In this context, in this study, we have extracted and phytochemical analyzed the phenolic components of the medicinal plant *L. multifida* from two different regions. When different extracts were obtained, we examined the *in-vitro* antioxidant, enzymes inhibition and anti-inflammatory effect the extracts. We examined the effect of the region and extraction methods on the biological activity. In addition, the evaluation of the biological activities of the *L. multifida* from Msila region showed an interesting *in-vitro* antioxidant potential, a remarkable *in-vitro* anti-diabetic activity, and moderate *in-vivo* anti-inflammatory activity and a weak anticholinesterase activity. This biological activity will be probably linked to the richness of the species, in particular that of Msila, in secondary metabolites, in particular in flavonoids.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication and dissemination of the information provided here in.

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Application of Near-infrared Spectroscopy and Multivariate Methods for the Estimation of Isopropyl Alcohol Content in Hand Sanitizer Formulation

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ABSTRACT

To address the need of alcohol-based hand sanitizers during COVID-19, U.S. FDA has issued a guidance for the preparation of hand sanitizers that recommends 80% v/v ethanol or 75%v/v isopropyl alcohol (IPA) along with other ingredients. The aim of this study was to develop a new method to estimate IPA content in hand sanitizers by using Near-infrared (NIR) spectroscopy with a multivariate chemometric approach. Calibration samples containing 10-90% of IPA were used for model development. NIR data was mathematically pretreated with multiple scattering correction before development of partial least squares (PLSR) and principal component regressions (PCR) model. Both models showed good linearity over the selected range of IPA content with high R2 (>0.993), low root mean squared error (<2.163), minimum difference between standard errors between calibration and validation models (0.0009). The proposed NIR with multivariate methods provide rapid analysis of IPA content in the hand sanitizer.

Keywords- Isopropyl alcohol, disinfectant, near infrared, partial least square, calibration validation

INTRODUCTION

Hand sanitizer, also called hand rub or hand antiseptic, is applied to hands to protect from common pathogens when washing hands using soap is not an

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available option [1, 2]. These products are made available in different forms such as foam, gel, or liquid, and majority of them are alcohol-based preparations. Though less effective, non-alcohol-based sanitizers are also available, and have triclosan or benzalkonium chloride as active ingredients [3, 4]. Centers for Disease Control and Prevention recommends use of alcohol-based hand sanitizers with greater than 60% ethanol or 70% isopropanol [5]. As a broad-spectrum bactericidal agent alcohol acts by either breaking proteins, splitting cells or interfering with a cell's metabolism [6-9]. The virucidal activity of alcohol is proportional to their concentration. Higher concentrations of ethanol (95%) generally have better virucidal activity than do lower concentrations, such as 60 to 80% and especially against naked viruses [10-13]. Likewise, the bactericidal activity of isopropanol begins at a concentration of 30% [14] and increases parallelly with increasing concentrations till 90% where after it shows a slight decline [15].

Due to the outbreak of novel coronavirus disease 2019 (COVID-19), hand sanitizers are flying off the shelves from grocery stores worldwide. Purchases of these products have skyrocketed in the U.S. from last week of February 2020, a period that saw the first American death from COVID-19. From March 2020 hand sanitizer market in the U.S. shot up by 470% with annual sales of more than $200 million compared to last year. In the early March 2020, an 8-ounce bottle sanitizer that would normally cost $2.50 was briefly on sale for $90 online [16]. An acute insufficiency was observed throughout the USA. To address shortage of sanitizers during this public emergency, the Food and Drug Administration (FDA) has issued a guidance for the preparation of alcohol or isopropyl alcohol (IPA) based hand sanitizers [17]. According to this guidance, the hand sanitizer product should contain 80%v/v ethanol or 75%v/v IPA in the formulation [17]. As most of the healthcare professionals and general public are relying on hand sanitizer as one of the preventing means, it is crucial to have a good quality control test to estimate the IPA or ethanol concentration in the final product. World Health Organization (WHO) recommends the use of alcohol based sanitizers with ethanol effective at 75%-85% (± 5%) and IPA at 77% (± 1%) and suggests alcoholmeter for quality control evaluation [18]. On the other hand, ethanol (60-95% v/v) and IPA (70-91.3% v/v) specifications are broad in FDA guidance document compared to WHO guidance. The agency recommended method for IPA and ethanol quantification are gas chromatography, alcoholmeter, hydrometer, or other equivalent method in terms of accuracy [17].

The burgeoning demand for sanitizers combined with the paucity of hydrometers makes it indispensable to develop an alternate analytical method to quantify alcohol content in hand sanitizer. Alternate analytical method could be ba-

sed on vibration spectroscopic methods. Near-infrared (NIR), a convenient and rapid vibration spectroscopic method, is becoming vital pharmaceutical tool of choice for nondestructive analysis where practically no sample preparation is required over a traditional wet chemistry method [19-24]. Unlike chromatographic methods, NIR peaks are not sharp due to higher order overtones and combination bands. Furthermore, the spectra may also be interfered by excipients present in the formulations. This results in a complex spectrum with overlapping and multiple bands of varying intensity/height, which required multivariate methods for quantitative estimation. Generally used multivariate analytical tools are principal component analysis (PCA) and projection to latent structures or partial least squares (PLS) [25]. The objective of this work is to combine NIR method with multivariate tools to build and validate chemometric models for quantification of IPA in FDA recommended hand sanitizer. This research work has not been reported in the literature to the best of our knowledge.

METHODOLOGY

Materials

IPA (USP grade 99%) was obtained from VWR International, LLC, Radnor, PA. Glycerol (USP/FCC grade,) and hydrogen peroxide (35% solution) were obtained from Fisher chemicals, Fair Lawn, NJ. Millipore water collected from Milli Q water system.

Manufacturing of hand sanitizer

Hand sanitizer was prepared as per FDA guidelines. It contained 75% v/v IPA, glycerin 1.45% v/v, hydrogen peroxide 0.125% v/v and water quantity sufficient to make 100% v/v. Briefly, glycerin and hydrogen peroxide were added to measured quantity of IPA. Volume was made up with water. The batch size was four liters, and twenty six batches were prepared. The batches were stored for 72 hours before complimentary distribution to various colleges of the university campus. Quarantine of 72 hours allow destruction of microbial spores, which may have formed during preparation steps [18].

Preparation of calibration samples

Calibration samples were prepared by the method described above. Glycerin and hydrogen peroxide content in the samples were identical to hand sanitizer formulations but contained varying percentage of IPA and water. IPA and water content varied 10-90%. 10 ml quantity was prepared for each sample in scintillation vials. All samples were characterized by NIR spectroscopy before developing multivariate models.

Near-Infrared spectroscopy

The NIR data of the samples was generated by using modular Nicolet™ iS™ 50 system (Thermo Fisher Scientific, Austin, TX). The instrument was equipped with a scanning grating monochromator and a diffuse reflectance apparatus (rapid content analyzer). NIR spectra ranging from 4000 to 10,000 cm^{-1} with a data resolution of 8 cm^{-1} and 100 scans were collected after conducting the diagnostic and reflectance tests. Prior to scanning, samples in a 20 ml borosilicate glass vial were mixed homogeneously by shaking, then placed on the sample window and centered with an iris. All samples were scanned in 6 replicates. Spectral acquisition was performed with OMNIC software, version 9.0.

Statistical analysis

Multivariate analysis of NIR data was performed using Unscrambler® X software (version 10.5; CAMO Software Inc., Woodbridge, NJ). Cross-validation approach was used to validate the models. The predictability of the models was further tested on independent samples. The performance of the chemometric models was evaluated in terms of correlation coefficient (R), determination coefficient (R^2), root-mean-squared error of calibration (RMSEC), root-mean-squared error of prediction (RMSEP), standard error of calibration (SEC), standard error of prediction (SEP) and bias.

RESULTS AND DISCUSSION

Spectral characterization

The NIR spectra of samples demonstrated broad bands due to vibrations of fundamental functional groups such as C-H, O-H, C-O and C-C [26, 27]. The spectra of IPA displayed characteristic bands at 8176, 8415, 8716, 9846 cm^{-1} and a trough at 7590 cm^{-1}. On the other hand, liquid water showed absorption band at 9330 cm^{-1}, and hydrogen peroxide exhibited absorption band at 9300 cm^{-1} with broader trough. Glycerol peak was characterized by a shoulder at 8269 cm^{-1} with troughs on both sides at 8874 and 7625 cm^{-1}. However, the IPA bands were not interfered by other components present in the formulation (**Figure 1**) making it an amenable method for its qualitative and quantitative estimation.

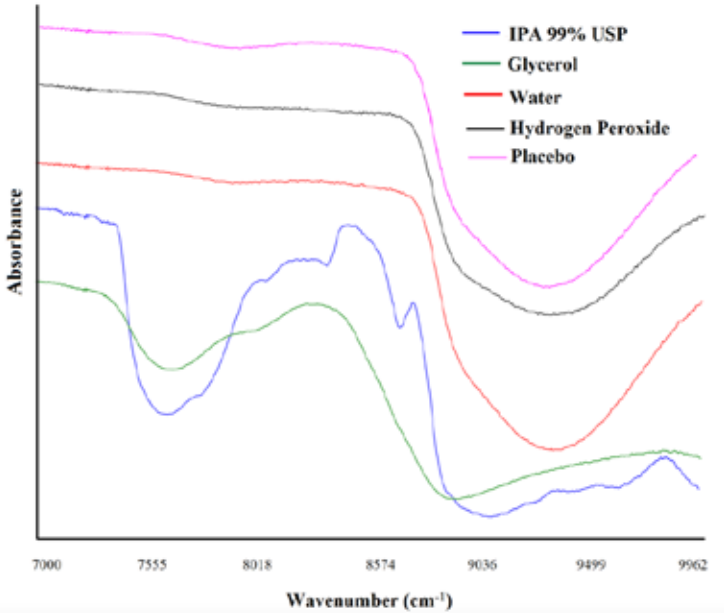

Figure 1. NIR spectra of IPA, water, glycerol, hydrogen peroxide and placebo sample

Chemometric analysis

Data processing

Truncated data of 7000-10000 cm^{-1} range was used for model development as it showed major bands of IPA. The data was mathematically pretreated with scatter correction methods such as extended multiple scattering correction (MSC) and standard normal variate (SNV), and spectral derivative method like Savitzky-Golay (SG), second derivative third-order polynomial with 9 smoothing points. The criterion for selection of mathematical method was based on values of R, R^2, standard errors (SEC and SEP), and root mean square errors (RMSEC and RMSEP) [23]. The pre-treatment methods are applied to individual spectra while mean centering and auto-scaling methods are applied to each individual variable of the samples [19, 20]. Single (MSC, SNV or SG), and combining two or more pretreatment methods (MSC-SNV, MSC-SG and MSC-SNV-SG) were explored to improve the quality of the data. Based on the values of statistical parameters (R, R^2, RMSEC, RMSEP, SEC and SEP) and spectral features, MSC method was selected for data treatment before models development (**Figure 2**).

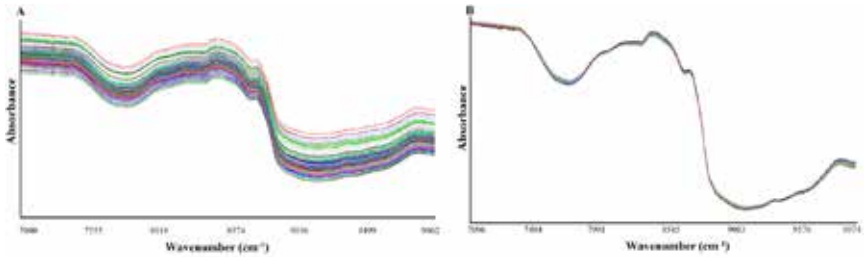

Figure 2. A) Truncated NIR, B) truncated NIR spectra pretreated with MSC

Regression models

PCA and PLS are data dimensionality techniques. PLS combine features of PCA and multiple linear regressions [28-30]. Both methods construct new predictor variables known as components PC (principle component) or least squares which is a linear combination of original predictor variables, but they construct those components in different ways. These components are also called as latent variables (LV). Principle component regression (PCR) generates components to describe the observed variability in the predictor variables, without considering the response variables. On the other hand, partial least squares regression (PLSR) does take the response variable into account, and hence frequently leads to models that are able to fit the response variable with fewer components. The model development starts with selection of number of LVs or PCs that would explain the variation in the data [31-33]. Number of LVs used in this model were optimized based on statistical parameters determination coefficient (R^2CV) and root-mean-square error of cross validation (RMSECV) [34, 35]. Two LVs were selected for model development with R^2_{CV} 0.994 and RMSEcv 2.053. These values were similar with three and four LVs, hence two LVs were used for model's development. The next steps in model development were detection and removal of the outliers from the dataset that has significant influence over the model prediction capability. Outlier detection was carried out using Hotelling's T^2 test at $p<0.01$, leverage and score plots. **Figure 3** showed Hotelling's plots of PCR and PLSR models. Hotelling's T^2 threshold limits at $p<0.01$ were 7.39 and 10.7 for first and second PC/LVs for both models, respectively. Hotelling's T^2 values of the samples were well below threshold limit (**Figure 3**).

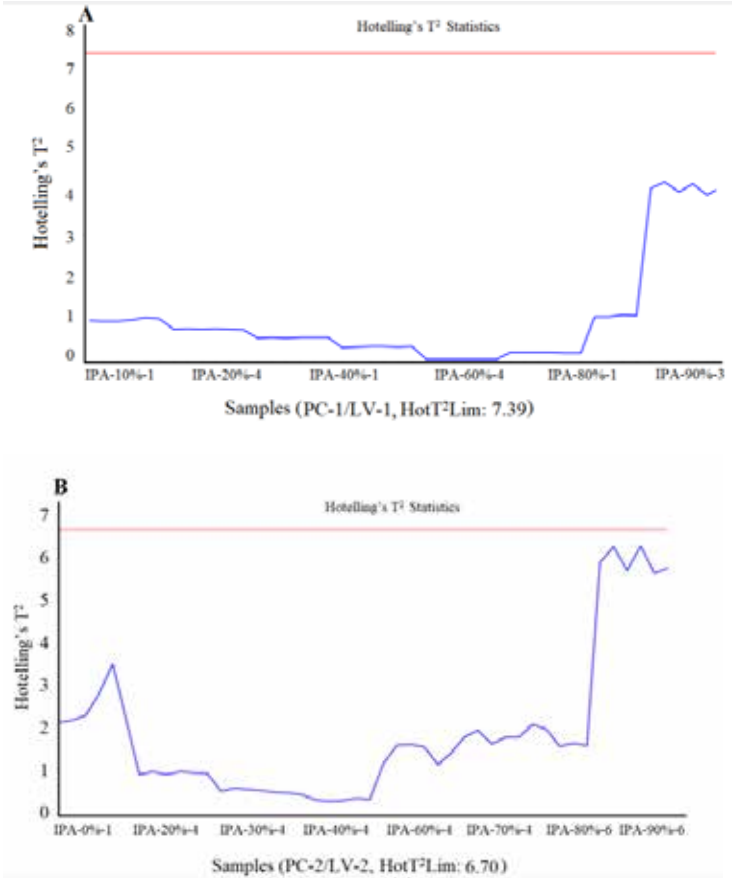

Figure 3. Hotelling T^2 curves for A) PC-1/LV-1 and B) PC-2/LV-2.

Leverage limit is defined by the formula 2A/I or 3A/I, where A is the number of LV and I is the number of samples. The leverage limit is used for quantifying the influence of sample on the model (**Figure 4**).

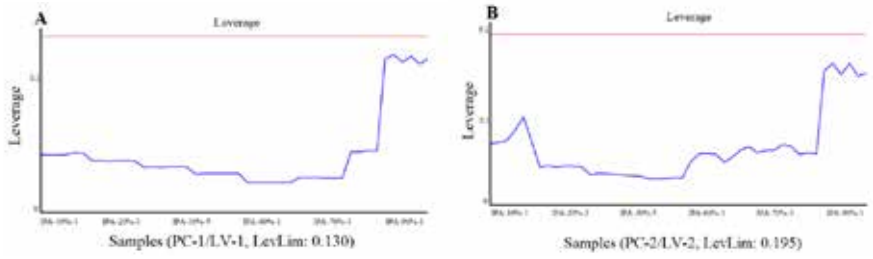

Figure 4. Leverage plots for A) PC-1/LV-1 and B) PC-2/LV-2.

The leverage limits were 0.130 and 0.195 for the first and second PCs/LVs for both models and samples were below the limits. Score plots of the samples between and first and second PC/LV are shown in Figure 5. Samples of identical concentration were clustered together that indicated samples belong to that particular group. Furthermore, score plots showed an increase in first PC/LV values with an increase in IPA concentration in the samples which indicated that first PC/LV was related to IPA in the samples. No such trend was observed in second PC/LV.

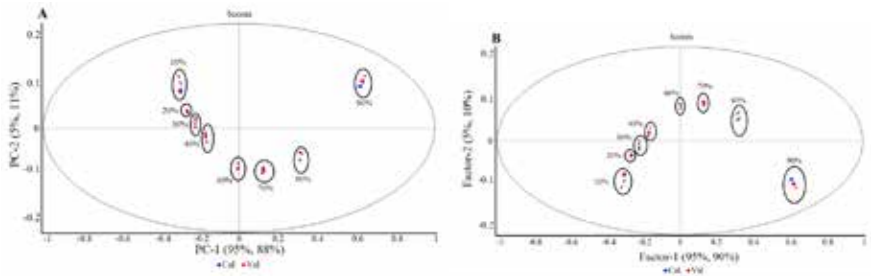

Figure 5. Score plots between A) PC-1 and PC-2 and B) LV-1 and LV-2.

Calibration and Internal validation

Initially, samples containing 0%-100% of IPA was used for model development with full cross validation wherein the same sample set of calibration models was used to validate the model. This is called internal validation. In this study, NIPALS algorithm was used for PLS and PCR regression models development [36]. Statistical parameters used to assess the calibration model are slope, offset, R, R^2, RMSEC, and SEC. Due to outlier detection in both PLSR and PCR models, samples containing 10-90% IPA was used in the final calibration set. The slope was close to 1 in both MSC treated PCR and PLS models, but the offset and RMSEC values were slightly higher in PCR model. The offset and RMSEC values of PCR and PLSR models were 0.306 and 0.276, and 2.162 and 2.052, respectively. However, the performance of calibration models was assessed by statistical parameters of the prediction model (**Figure 6**). The RMSEP and SEP values of PCR and PLSR models were 2.163 and 2.187, and 2.053 and 2.076, respectively. As the statistical parameters of both calibration and prediction were close to each other, the developed models would be considered a good fit models [19] (**Table 1**).

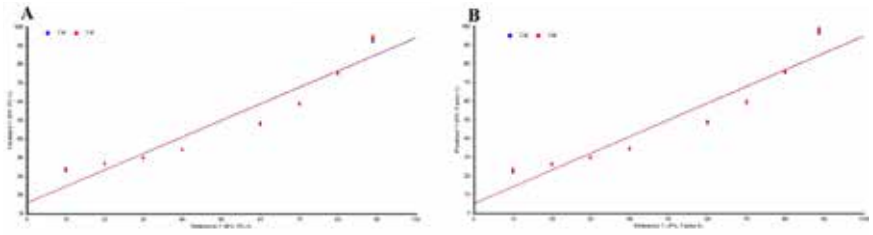

Figure 6. Calibration and validation plots for A) PCR and B) PLSR

FDA guidance document states that significant difference between SEC and SEP determines inadequacy of the model. The difference between SEP and SEC was less than 0.0009 for both PCR and PLSR models. The internal validation results showed good correlation between predicted and actual values for both PCR and PLSR models. Error in the model was estimated by residual values. Residual values between reference and model predicted values were low which indicated low error in the models.

Table 1. Statistical parameters of the model (pretreated with MSC)

Regression model	Model	Sample No.	Slope	Offset	Correlation	R^2	RMSEC (P)	SEC (P)	Bias
PCR	Calibration Validation	46 46	0.993 0.991	0.306 0.435	0.996 0.996	0.993 0.992	2.162 2.281	2.186 2.306	0 0.009
PLSR	Calibration Validation	46 46	0.994 0.992	0.276 0.394	0.997 0.996	0.994 0.993	2.052 2.174	2.075 2.198	0 0.008

RMSEC (P) – Root mean square error of calibration or prediction;

SEC (P) – Standard error of calibration or prediction.

Likewise, LV/PC in the loading plots of PLS/PCA regression model may provide the physical and chemical information of the samples by comparing spectra of individual components as well as formulations. The PLS1 showed all characteristic bands of IPA except the inversion of a trough at 9368 cm^{-1}. The PLS2 showed inverted peaks/valleys at 7536, 8369, 8709, 8805 and 9368 cm^{-1}. These bands were related to all the components of hand sanitizer formulations (**Figure 7**). Similarly, PC1 exhibited all the peaks of IPA, and PC2 exhibited peaks of all other components.

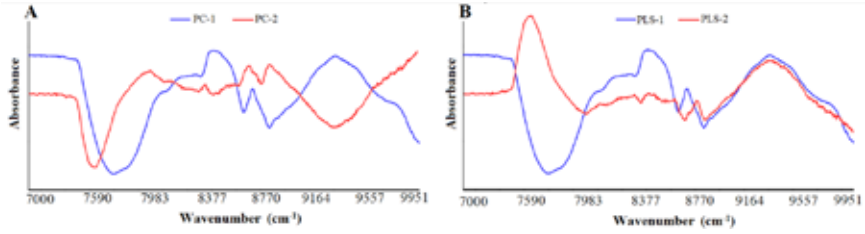

Figure 7. Loading plots of A) PCR and B) PLSR models

External validation of sanitizer batches

The PLS and PCR models were externally validated with independent samples of sanitizer batches which were not used in the model development. 10 mL of samples were collected in 20 mL scintillation vials from each batch. The NIR data of all batches was treated in the same way as was done on the samples used for model development. The data was plugged into the developed models after mathematical treatment. The amount of IPA predicted in the samples was close to the actual amount of 75%. The accuracy of the data was measured by residual values and the range was -3.74 to 1.78% for PCR, and -3.68 to 1.66% for PLSR. In general, residual value was lower in PLS models compared to PCR models. The minimum deviation was detected with batch #22 (1.81% for PCR and 1.73% for PLS) and maximum with batch #19 (3.67% for PCR and 3.49% for PLS) from the target values of IPA. All batches showed IPA content from 73.2±3.2% to 78.7±2.0% with PCR and 73.3±3.2% to 78.7±1.91% with PLS models (**Figure 8**). Batch #6 and #13 exhibited minimum and maximum IPA content for both models. Furthermore, predicted values of IPA by PCR and PLSR models overlapped for all the batches except batch #25 and #26. The PCR predicted values for batch #25 and #26 were 75.73±2.6 and 75.84±2.1%, respectively. Similarly, the PLS predicted values for batch #25 and 26 were 76.65±2.5 and 77.15±2, respectively. FDA guidelines dictates hand sanitizer formulation should contain 70-91.3% (v/v) IPA. Thus, the prepared formulation batches met IPA content criteria.

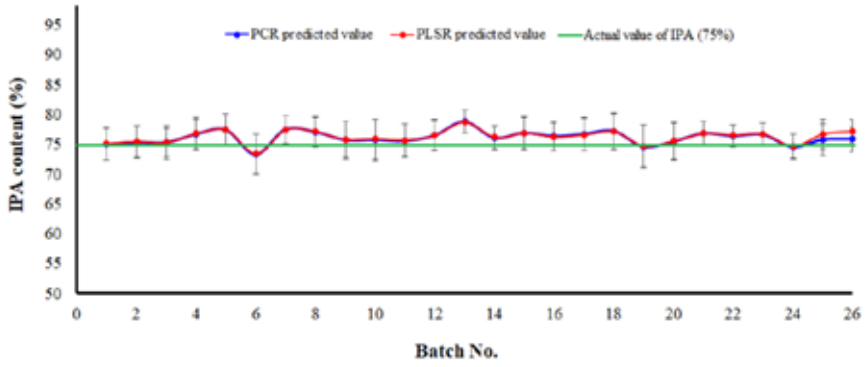

Figure 8. Comparison of PCR and PLSR predicted values with actual values of all batches.

The NIR spectroscopy methodology was developed for determination of IPA content in the hand sanitizer formulation. The peaks of IPA were distinct, not interfered by the other components of the formulation. A chemometric approach was combined with spectroscopy to achieve the goal of IPA prediction. Data was mathematically treated by various methods to improve its quality, detect and eliminate the outlier before development of PCR and PLSR models. MSC treated data set was used in PCR and PLS models development. The models showed high R^2 (>0.993), low RMSE (<2.163), and minimum difference between SEP and SEC (0.0009). The models were independently verified with unknown samples. The predicted values were in close concurrence with actual values with low residual (<3.76). The proposed analytical method is rapid and fast and, provide convenient way to measure IPA in the hand sanitizer. Thus NIR spectra can be used for qualitative and quantitative analysis in conjunction with multivariate method of IPA in the hand sanitizer formulation.

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CONFLICT OF INTEREST: Authors have no conflict of interest

AUTHOR CONTRIBUTIONS:

Sathish Dharani: Design of experiment, Drafting manuscript and statistical analysis

Tahir Khuroo: Preparation of sanitizer and Acquisition of data

Sogra F. Barakh Ali: Analysis of the data and statistical analysis

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Histological effect of traditional rose ointment application in the excisional wound model

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ABSTRACT

The aim of our study was to evaluate the histological effects of traditional rose ointment application on artificially induced skin wound treatments. 18 adult BALB/c mice were artificially wounded by the formation of a puncture-generated exhaled wound model. Wound tissues of mice were analyzed histologically with light microscopy after hematoxylin eosin and Masson trichrome stainings were performed. Vascular endothelial growth factor (VEGF) expressions were evaluated by immunohistochemical analysis in order to demonstrate the angiogenesis throughout the tissue. Tissue regeneration rate was significantly increased in traditional rose ointment treated group although there was no significant difference in granulation and angiogenesis between the groups. Traditional rose ointment treatment seems to have a positive effect in the treatment of skin wound by inducing the regeneration capacity in the tissue. Further studies are needed to confirm this finding and to evaluate its potential to be used in wound healing.

Key words: Phytotherapy, Wound Healing, *Rosa damascena,* Histology, Rats.

INTRODUCTION

Phytotherapy includes the treatment methods in which herbal compounds are used to assist medical treatment. Fragrant roses are widely used in phytotherapy and the rose oil is one of the most widely used among the rose deriva-

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tives[1]. *Rosa damascena* Mill. (Damask rose, Oil-bearing rose, Pink rose) is the most important species, producing a high-value aromatic oil, which is used in the pharmaceutical, flavourings and fragrance industries[2]. Turkey is one of the countries leading the production of *Rosa damascena* in the world with a production capacity of approximately 10.000 tonnes of rose flowers and rose oil annually[3].

The process of wound healing is a dynamic, complex interplay of cytokines, involving many different cell types. The skin has important immune and protective characteristics and has an amazing ability to heal, invariably with scarring. Scarring is quite variable and is based on many factors, dependent on patient characteristics and overall health (intrinsic) as well as the healing environment (extrinsic). All epithelial tissues in the body, except for bone, heal by scar formation rather than regeneration. The skin is not spared by this. It is important to identify wound-healing problems early to minimize scarring. To understand the effects of injury and potential for scarring, one must first look at the layered histology and physiology of the largest organ in the body. The skin is separated into an epidermis, dermis, and hypodermis. The epidermis itself has 5 layers or strata from superficial to deep: corneum, lucidum, granulosum, spinosum and basale [4,5,6]. The skin has two tissue layers: a keratinized stratified epidermis and an underlying thick layer of collagen-rich dermal connective tissue providing support and nourishment. Adjunct like glands and hairs are derived from and linked to, the epidermis but project deep into the dermal layer. As the skin serves as a protective barrier against the outside, any break in it must be immediately and efficiently repaired. A temporary mend is achieved in the form of a clot that plugs the defect, and over following days steps to regenerate the missing parts are began. Inflammatory cells and then fibroblasts and capillaries invade the clot to form a contractile granulation tissue that draws the wound margins together; meanwhile, the cut epidermal edges migrate forward to cover the denuded wound surface[7].

We aimed to investigate the efficacy of the Rosa damascena ointment treatment by comparing the outcome with Madecassol® (Bayer, 00001199) which is used as a pharmacologically reference drug on the studies dealing with excisional wound healing which contains 1% *Centella asiatica* extract[8].

METHODOLOGY

Traditional *Rose damascena ointment* was prepared by Ayten Altıntaş as following: beewax (1g) + rose solution (rose water + pure olive oil) (3 g) + 50µl of rose oil. *Rosa damascena* were obtained from Isparta, Turkey.

Experimental Model

Healthy adult male BALB/c mice (25-30 g, n = 18) obtained from Istanbul Medipol University Medical Research Center (Istanbul, Turkey) were fed ad libitum and kept in a controlled room at 24°C temperature and humidity under 12 h long light/dark period.

The mices were anesthetized intraperitoneally (i.p.) with ketamine (10 mg/kg) and xylazine (80-100 mg/kg), both anterior-dorsal side of each mouse was shaved and washed with povidone-iodine solution. Two full thickness excisional skin wounds were created 5 mm in diameter by punch biopsy as described previously[9].

Mices were randomly divided into 3 different groups as, untreated control group (n: 6), Topical Madecassol applied group (n: 6), and Topical Rose ointment group (n:6). Madecassol and rose ointment was applied once a day topically onto the wounds sufficient to cover the surface of the wounds completely until the day rats were sacrificed at day 7th. All procedures were performed aseptically on dorsal sides of the mice.

Histological and Immunohistochemistry Studies

All animals were sacrificed by decapitation and the skin of the back including the wound area was removed at 7th day. Full-thickness biopsy samples extended from the outside margin to the center of the treated area were collected. The skin covering the wounds was fixed in 10% neutral buffered formalin solution for histological and immunohistochemistry analysis. All wound tissue parts were embedded into paraffin and 3 mm thick sections were sliced with microtome (Thermo, Microm HM 340E). Sections were stained with hematoxylin and eosin (H&E) and Masson trichrome staining for histologic evaluation and and vascular endothelial growth factor (VEGF) expressions were evaluated by immunohistochemistry to demonstrate the angiogenesis throughout the tissue.

Hematoxylin Eosin and Masson Trichrome Stainings

The first step in histological staining is deparaffinization. Paraffin sections were deparaffinized with toluene for 30 minutes. Then tissue sections were rehydrated through descending grades of alcohol (100%, 90 %, 70 %) to water for 5 minutes. The sections were stained with hematoxylin and eosin and Masson trichrome staining for histologic evaluations.

For hematoxylin and eosin staining; tissue sections were incubated in Mayer hematoxylin staining solution for ten minutes and then in running tap water for 30 minutes. After rinsing twice with distilled water, tissue sections were put into eosin staining solution for 30 seconds and were again rinsed with distilled water twice.

For Masson trichrome staining; tissue sections were differentiated with 1% acid alcohol and washed well in tap water. Then the sections were stained in acid fuchsin solution for 5 minutes, rinsed in distilled water and treated with phosphomolybdic acid solution b for 5 minutes. After drain, the sections were stained with methyl blue solution c for 4 minutes and rinsed in distilled water. Finally, the sections were treated with 1% acetic acid for 2 minutes and dehydrated through ascending grades of alcohol.

All tissue sections were dehydrated in alcohols and clarified with toluene and subsequently coverslipped in mounting medium.

Wound healing for each group was evaluated using the scoring system described by Geleano et al. for epidermal and dermal regeneration: 0 represents the absence of epithelial proliferation in 70%; 1 represents poor epidermal structure in ≥ 60%; 2 represents deficient epidermal structure in ≥ 40%; 3 represents limited epithelial proliferation ≥ 60%; 4 represents full epidermal remodeling in ≥ 80% of the tissue. For thickness of the granulation tissue scoring was; 0 represents immature and inflammatory tissue in ≥ 70%; 1 represents thin granulation layer; 2 represents limited granulation layer 3 represents thick granulation layer; 4 represents very thick granulation layer. For the evaluation of angiogenesis, intact vessels were counted and identified by the presence of erythrocytes in the lumen. To distinguish well-formed from poorly formed capillary vessels, we evaluated the presence or absence of edema, congestion, hemorrhage, thrombosis and intravascular or intervascular fibrin formation as: 1 represents altered angiogenesis (few vessels/site) characterized by high degree of edema, hemorrhage, occasional congestion and thrombosis; 2 represents few newly formed capillary vessels (3–4/site), moderate edema and hemorrhage, occasional congestion, intravascular fibrin deposition and absence of thrombosis; 3 represents recently formed capillary vessels (5–6/site); 4 represents recently formed and normal visible capillary vessels (>7/site).

The results obtained from histological observation of skin tissue sections were evaluated by analyzing epidermal and dermal regeneration, granulation tissue thickness and angiogenesis, as shown in Figures 1-3. Increasing epidermal and dermal regeneration is the first concern used for measuring the effect of treatment on wounds studies. Epidermal regeneration is characterized by well-structured epithelial layers with no evidence of crusting or intra-epithelial inflammatory cells [10].

Statistical analysis

All statistical analyzes were performed using GraphPad Instat for Windows

(Ver. 3.06) program. The results were expressed as means ± SD. For histological wound healing assessment, the differences between groups were analyzed by ANOVA followed by Tukey analysis of variance. Values for $p \leq 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

Histological results

All groups showed complete wound healing closure. Re-epithelialization and granulation tissue organization were significantly better in Rose ointment group. There were also significantly higher dermal and epidermal regeneration in the Rose ointment ($p<0.01$) compared with the control and Madecassol groups, (Figure 1).

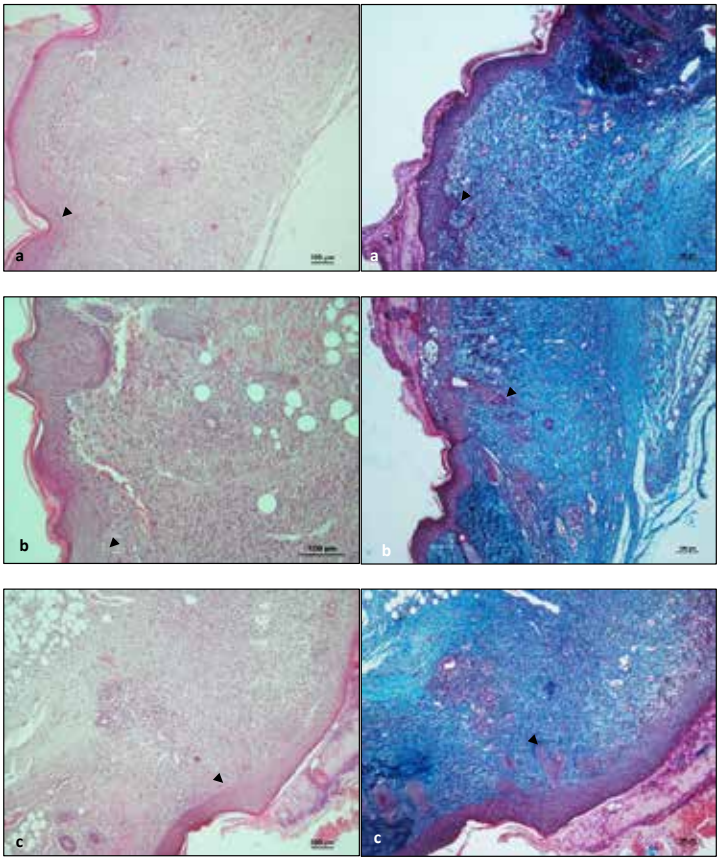

Figure 1. Light micrographs of wound healing area. Control (a), Madecassol (b), Rose ointment(c) groups. Hematoxylin eosin and Masson trichrome staining; bar = 100 mm (a, b, c). Arrowheads represent re-epithelialization.

Immunostaining results

An increase VEGF expression were demonstrated in the wound area in rose and Madecassol groups compared with the control group, although the difference was not statistically significant (Figure 2).

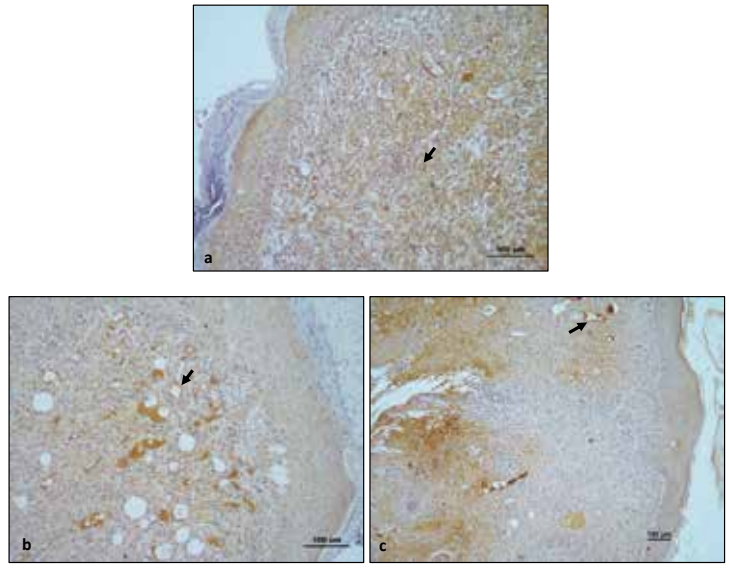

Figure 2. Light micrographs of wound healing area. Control (a), Madecassol (b), Rose ointment (c) groups. Vascular endothelial growth factor (VEGF) immunohistochemistry; bar = 100 mm (a, b, c). Arrows indicates endothelial cells positive for VEGF (VEGF+).

Tissue regeneration was found to be statistically significant in rose ointment group, although there was no significant difference in granulation and angiogenesis between the groups (Figure 3).

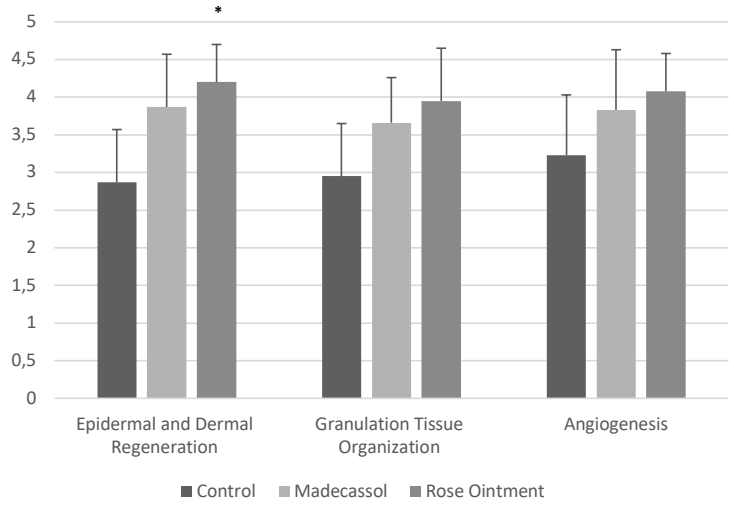

Figure 3. Histologic scores in wounds from mice given control, Madecassol, Rose ointment groups. Each point represents the mean SD of experiments. *p<0.01 vs. control.

Rose has been an important plant used traditionally to treat many diseases for many years. It is reported to contain carboxylic acid, myrcene, vitamin C, kaempferol and quercetin and its flowers also contain a bitter principle, tanning matter, fatty oil and organic acids [11, 12, 13, 14, 15]. It has been reported to be a promising plant for anti-HIV, antibacterial, antioxidant, hyptonic, anti-aging effect [16, 17, 18, 19, 20]. Rose oil is reported to be used in many different diseases including depression, grief, nervous stress and tension. It is also reported to help reduction of thirst, healing old cough, wound healing, and skin health. Vapor therapy of rose oil is helpful for some allergies, headaches, and migraine [21, 22]. Besides all these known effects, we showed the beneficial effects of *Rosa damascena* ointment on wound healing for the first time. Previous studies in the literature reported the effects of other natural products sequestered from medicinal plants for skin regeneration. In present study an in vivo animal wound healing model was employed to represent the effects of rose ointment.

Rose treated group showed significantly increased Re-epithelialization and granulation tissue organization. There were also significantly higher dermal and epidermal regeneration in the Rose ointment compared with the control and Madecassol groups indicating the positive effect of the Rose on wound healing. In addition, although statistically insignificant, an increase in VEGF expression were demonstrated in the wound area in Rose treated group indicating the increased angiogenesis which is an important parameter in wound healing.

According to the recent results it may be concluded that Rose damascana positively affect tissue regeneration during wound healing and may be used as a supportive treatment strategy for medical treatment in cases of serious wound treatment. Further studies with different doses and different applications are needed to confirm the findings.

STATEMENT OF ETHICS

Our study was approved by Medipol University local ethics committee with the number 38828770-604.01.01-E.3412 and all experiments were carried out according to the internationally accepted principles for laboratory, animal use and care as found in European Community Guidelines.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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There are no any funding sources.

AUTHOR CONTRIBUTIONS

These authors contributed equally.

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Phytochemical profiling and antioxidant activities of *Monodora myristica* and *Dennettia tripetala* against lipid peroxidation in rat heart

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ABSTRACT

This study was carried out to investigate the phytochemical profiling and *in vitro* antioxidant activities of methanol extract of *Monodora myristica* (MM) and *Dennettia tripetala* (DT) against lipid peroxidation in rat's heart. The antioxidant activities and lipid peroxidation inhibition were evaluated with spectrophotometric methods. Thereafter, the extracts were profiled using high performance liquid chromatography (HPLC) method. The results showed that MM and DT possessed polyphenol content which culminated in antioxidant activities. However, MM exhibited significantly higher ($p < 0.05$) antioxidant activities than DT. Also both extracts were able to inhibit lipid peroxidation in rat's heart *in vitro*. The extracts profiling showed the abundance presence of myristyl chloride, linalool and nerolidal in MM extract and elemicin, myristicin, eugenol and pinene in DT extract. Therefore, it can be concluded that MM and DT may be used against oxidant related diseases of the heart. Also, there might be need to isolate these compounds and evaluate them for probable drug lead

Key words: Antioxidant, *Dennettia tripetala,* lipid peroxidation, *Monodora myristica*, HPLC

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INTRODUCTION

The contribution of free radicals in the aetiology of cardiovascular diseases such as hypertension and myocardial infarction has been well established [1,2]. These diseases are mediated via reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase–driven production of reactive oxygen species (ROS) [2]. Atherosclerosis, the hardening of arteries under oxidative stress is a chronic process initiated from the deposition of oxidized low density lipoprotein (LDL) underneath the artery wall. The polyunsaturated fatty acids in LDL are the primary target of this free radical-induced lipid peroxidation in the artery [3,4]. Recent studies have highlighted lipid peroxidation products (oxidized phospholipids) as biomarker and therapeutic target in cardiovascular diseases[5]. The elevation in free radicals generation which cause an increase in the lipid peroxidation can be averted or minimized by antioxidants which protect the body against free radicals induced damage [6, 7]. However, plants have been shown as natural reservoir of many phytochemicals with vast antioxidant properties [8, 9].

Monodora myristica Dunal (MM) is a member of Annonaceae, popularly known as African nutmeg. It is one of the most important trees in the evergreen forest of southern Nigeria as almost every part has economic importance but the seed is the most economically important part because of its spicy aroma [10]. Traditionally, the seed is usually used as condiment to prepare pepper soup, to treat constipation and to control intra-uterine bleeding in women immediately after child birth [11]. Apart from this, it is use in the treatment of hypertension and diabetes mellitus [12, 13]. Previously, different extracts of MM have been shown to possess numerous phytochemicals and antioxidant potentials [14, 15].

Dennettia tripetala G. Baker (DT), also a member of the family Annonaceae is popularly called Pepperfruit because of its spicy taste. It is well eaten among the people of southern Nigeria not only for its spicy taste but it is believed to increase alertness in the community and also medicinal [16,17]. Folklorically, it is used in the prevention and management of sore throat, cough, nausea, hypertension, diabetes and as purgative [16, 17]. Like MM seeds, the seeds of DT are important in the diet of postpartum women because it is believed to aid contraction [18]. Previously, Omage et al (2018) have reported the presence of phytochemicals like phenols, flavonoids, saponins, tannins, alkaloids in extracts of DT fruits [16].

Despite the wide traditional uses of these plant products for treatment and management of diseases especially hypertension, there is paucity of information on their mechanisms of action in heart-related diseases. Therefore, this study is designed to investigate the *in vitro* antioxidant activity of *Monodora myristica* and *Dennettia tripetala* against lipid peroxidation in rat's heart.

METHODOLOGY

Plant materials

Seeds of *Monodora myristica* (African nutmeg) and *Dennettia tripetala* (Pepper fruit) used in the study were purchased from the Okitipupa local market, Ondo State, Nigeria. Identification and authentication were carried out at the Herbarium of the Department of Biological Sciences, Olusegun Agagu University of Science and Technology, Okitipupa, Ondo State, Nigeria. The herbarium numbers (OSUSTECH/568 and OSUSTECH/560) respectively were deposited in the herbarium. Seeds of African *Monodora myristica* and *Dennettia tripetala* were dehulled, shade dried at ambient temperature and pulverized into powdery form using a laboratory blender.

Preparation of *Monodora myristica* and *Dennettia tripetala* extracts

One hundred gram of powdered *Monodora myristica* and *Dennettia tripetala* seeds were soaked separately in 2 L of methanol for 72 hours. Thereafter the extracts were filtered using clean cheese cloth and concentrated with rotary evaporator. The percentage yield of MM and DT concentrated extracts were 15.66 % and 13.40 % respectively and stored in the freezer until use[19, 20].

Polyphenols content

The total phenolic content (TP) of the extracts was evaluated by the Folin-Ciocalteu phenol reagent method of Kim et al (2003). The TP was calculated from gallic acid calibration curve and expressed as mg per 100g gallic acid equivalent (mg GAE/100g) [21].

The Total flavonoid content (TF) of the extracts was evaluated using the method of Park et al (2008). The flavonoid content was calculated from quercetin standard curve and expressed as mg per 100g quercetin equivalent (mg QUE/100g) [22].

In vitro antioxidant assays

The total antioxidant capacity (TAC) of the extracts was determined using the phosphomolybdate method of Prieto et al (1999). The total antioxidant capacity was calculated from ascorbic acid standard curve and expressed as mg per 100g ascorbic acid equivalent (mg AAE/100g) [23].

The 2,2-Diphenyl-1-picrylhydrazyl scavenging activity (DPPH) activity of extracts was determined by the method of Gyamfi et al (1999) and expressed as percentage inhibition[24].

The reducing power (RP) of extracts was evaluated using the method of Oyaizu (1986). The reducing power was calculated from ascorbic acid standard curve and expressed as mg per 100g ascorbic acid equivalent (mg AAE/100g) [25].

The 2, 2-azobis-3-ethylbenzothiazoline-6-sulfonate radical scavenging ability (ABTS) of the extracts was evaluated using the method of Re et al (1999) [26]. Trolox was used as standard and trolox equivalent was subsequently calculated as mg TEAC/100g

The Ferric reducing antioxidant power (FRAP) of the extracts was evaluated using the method of Benzie and Strain (1996). The FRAP was evaluated from ferrous sulfate calibration curve and expressed as mg Fe^{2+}/100g [27].

Nitric oxide scavenging ability of the extracts was determined by the method of Modal et al (2006) and expressed as percentage inhibition [28].

Experimental animals

Six healthy male Wistar rats (120-140) g were obtained from the Department of Physiology animal holding facility, University of Ibadan. The animals were acclimatized for 2 weeks before used for the experiment. They were given standard pellet diet and water *ad libitum*.

Statement of ethics

The experimental protocols were conducted according to the guidelines of National Institute of Health on the handling and use of laboratory animals (NIH Publication No. 80-23) revised in 2011 [29]. This protocol was approved by the Research Ethics Committee of Olusegun Agagu University of Science and Technology (OAUSTECH/ETHC-BCH/2020/01)

Lipid peroxidation (LPO)

The heart homogenate was prepared according to the method described by Akinyemi et al (2013). The experimental rats were anesthetized and decapitated using sodium pentobarbitone. The heart tissue was removed and weighed on ice. The tissue was homogenized with cold normal saline (1:4 w/v) on ice. The homogenate was centrifuged at 3,000 rpm for 10 min and the supernatant was used for determination of lipid peroxidation[30].

The Lipid peroxidation inhibition capacity of the extracts was determined using the method of Ohkawa et al (1973) and expressed as percentage inhibition [31].

Phytochemical profiling by HPLC-DAD

The phytochemical profiling was done using the method [32]. Twenty grammes of

samples were extracted with 15 ml acetonitrile and stabilized with ethyl acetate and made up to 25ml; filtered through 0.45 mm membrane filter and then degassed by ultrasonic bath prior to use. Stock solutions of standards were prepared in the HPLC mobile phase in the concentration range of 0.030–0.500 mg/ml. The flow rate was maintained at 500 µl/min. Before injecting the sample, standard (cayophyllene, phytol, myristyl chloride, linalool, nerodial, copaene, squalene, estragole, camphene, elemicin, cineol, sabinene, myristicin, eugenol, pinene, methyleugenol, limonene and terpene) form of analytes were first injected to generated a chromatogram of given peak area and peak profile that was used to create a window in the HPLC for the test sample analysis. Briefly, an aliquot (5 µl) of the extracted sample (0.8mg/ml) was injected into the HPLC to obtain a corresponding peak area and peak profile. Identification of the compound was achieved by comparing the peak area of the sample to the registered standard spectra. The concentration of the sample was calculated using the formula:

Concentration= {Peak area of the analyte in sample × Analyte (standard)}/ Peak area of Standard

Statistical analysis

Results are presented as the mean ± SD of three measurements. The results were analysed using analysis of variance (ANOVA) and significance was established with least significant difference (LSD) post hoc treatment at $p < 0.05$. Correlation was calculated using Pearson correlation test and significance was determined at $p < 0.05$

RESULTS AND DISCUSSION

The results of total phenolic (TP), total flavonoid (TF), total antioxidant capacity (TAC), ferric reducing antioxidant power (FRAP), 2, 2-azobis-3-ethylbenzothiazoline-6-sulfonate (ABTS) and reducing power (RP) are presented in Table 1. Total phenolic is one of the secondary metabolites vastly found in plants. They are synthesized from tyrosine and phenylalanine and they exhibit different biological activities such as antioxidant, anti-inflammation and antimicrobial [33]. In this study, the TP of MM (25.97 mg GAE/100g) was significantly higher ($p < 0.05$) than that of DT 14.23 mg (GAE/100g). Some studies have reported a strong positive correlation between total phenolics and scavenging capacities [34, 35]. The TP of our extracts was higher when compared with the result reported for different varieties of spice (*Curcuma longa*), which ranged from 4.52-16.07 mg GAE/100g [36]. Flavonoids are one of the polyphenols widely found in human diets with many antioxidants and health benefits

[37]. Also like the total phenolics, MM (14.41 mg QUE/100g) possessed significantly higher ($p < 0.05$) flavonoid content than DT (10.73 mg QUE/100g). The flavonoid content of both samples ranked well when compared with flavonoid content of other spicy plants reported by Do et al., (2014) [38]. The contents of TP and TF in this study were higher than the ones reported for the leaves and bark of MM by Moukette et al (2015) [39]

Table 1. Polyphenols and antioxidant capacity of methanol extracts of MM and DT

	MM	DT
TP (mg GAE/100g)	25.97± 1.13[a]	14.23± 0.91[b]
TF (mg QUE/100g)	14.41 ± 0.32[a]	10.73± 0.47[b]
TAC (mg AAE/100g)	90.67 ± 6.79[a]	75.60 ± 3.92[b]
FRAP (mg Fe^{2+}E/100g)	18.03 ± 1.04[a]	16.59 ± 1.52[a]
ABTS (mg TEAC/100g)	1.09 ± 0.02[a]	1.01 ± 0.03[a]
RP (mg AAE/100g)	14.80± 1.71[a]	13.18 ± 1.22[a]

Data were presented as Mean ± SD. Values with same alphabet across the row are not significantly different ($p < 0.05$). TP: Total phenolics, TF: Total flavonoid, TAC: Total antioxidant capacity, FRAP: Ferric reducing antioxidant potential, ABTS: 2, 2-azobis-3-ethylbenzothiazoline-6-sulfonate, RP: Reducing power

Antioxidants are substances that scavenge or slow down the activities of free radicals thereby inhibiting oxidative mechanisms that lead to chronic and degenerative diseases in the body [40]. Antioxidant ability assays can be divided into two; hydrogen atom transfer (HAT) and single electron transfer (SET) assays. Most of the HAT assays are kinetics based and involve a competitive reaction scheme while SET assays measure the ability of an antioxidant to reduce an oxidant with corresponding colour change when reduced [40,7]. Examples of SET assay include DPPH, FRAP, TAC, RP ABTS, NO and LPO. Many SET assays were employed in this study because of their wide uses, accuracy and precision in *invitro* studies. In the present study, MM exhibited higher antioxidant activities in all antioxidant models than DT, though, the differences were not significant ($p < 0.05$) in some models (FRAP, RP and ABTS).

The result for DPPH assay is presented in Figure 1, a concentration-dependent relationship was found in the DPPH scavenging ability of MM and DT. Their IC_{50} (Table 2) showed that MM (0.34 mg/ml) had a significantly ($p < 0.05$) lowered IC_{50} than DT (0.55 mg/ml), which is an indication of higher DPPH scavenging ability of MM. The higher ability of MM corroborated with the results of total phenolic and total flavonoid as many studies have reported strong posi-

tive correlation between these polyphenolic compounds and DPPH scavenging effect [42, 43, 44, 45, 46]. The IC_{50} reported for MM and DT in this is lower than the one reported for aqueous and ethanol extracts of DT by Josiah et al., (2016) [47].

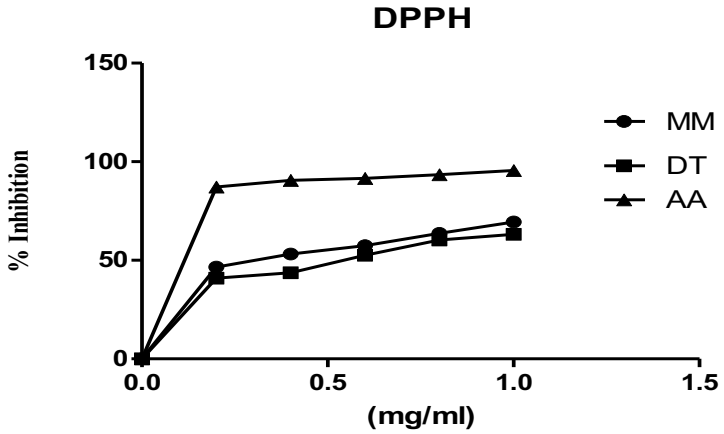

Figure 1. DPPH scavenging activity of methanol extracts of MM and DT

Table 2. IC_{50} (mg/ml) of DPPH, NO and LPO

	MM	DT	AA
DPPH	0.34 ± 0.02[a]	0.55 ± 0.03[b]	0.13 ± 0.01[c]
NO	0.55 ± 0.04[a]	1.01 ± 0.08[b]	0.18 ± 0.01[c]
LPO	0.78 ± 0.02[a]	1.69 ± 0.14[b]	0.11 ± 0.01[c]

Data were presented as Mean ± SD. Values with same alphabet across the row are not significantly different ($p < 0.05$). DPPH: 2,2-diphenyl-1-picrylhydrazyl scavenging activity, NO: Nitric oxide scavenging ability, LPO: Lipid peroxidation inhibition. AA= Ascorbic acid

Nitric oxide radical and lipid peroxidation have been two of the prominent sources of free radicals in cardiovascular diseases causing atherosclerosis [48]. Nitric oxide (NO•) is a reactive radical that has been implicated in many physiological processes which include neurotransmission, blood pressure regulation, immune regulation, defence mechanisms, and smooth muscle relaxation [48]. However, overproduction of this reactive species leads to a condition known as nitrosative stress (RNS) in the body [49]. The result of NO-radical scavenging ability is presented in Figure 2. The extracts of DT and MM were able to inhibit (dose dependent) the generation of nitric oxide at physiological pH from sodium nitroprusside (SNP). The IC_{50} of MM (0.55 mg/ml) was significantly ($P < 0.05$) lower than that of DT (1.01 mg/ml). The IC_{50} of MM was lower while DT was higher than the one reported for aqueous extract of DT[46]. The capacity

of these extracts to inhibit the generation of this radical might be due to their antioxidant activities. This study is in agreement with the studies of some researchers that reported extract(s) rich in phenolics and flavonoid to possess NO scavenging ability [50, 51]. Also, correlation analysis (Table 5) showed a positive relationship between polyphenol and NO scavenging ability.

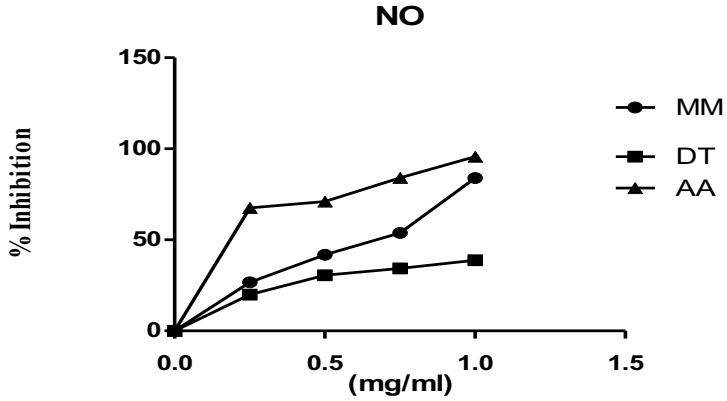

Figure 2. NO scavenging ability of methanol extracts of MM and DT

In this study, the lipid peroxidation on rat's heart homogenate was initiated by the pro-oxidant effect of transition metal, which produced lipid peroxides by stimulation of the oxidative machinery (OH.) through haber-weiss reaction [52]. The extent of lipid peroxidation is determined by the thiobarbituric acid reactive species (TBARS) formed. In this study, incubation of rat's heart homogenate with ferrous sulphate led to increased production of TBARS in heart homogenate. However, MM and DT were able to significantly ($p < 0.05$) inhibit the production of TBARS in a dose dependent manner but inhibition was significantly higher in MM than DT (Figure 3). The IC_{50} of MM (0.78 mg/ml) is significantly ($p < 0.05$) lowered than that of DT (1.69 mg/ml). The capacity of the extracts to inhibit the generation of lipid peroxides could be due to their phenolic contents as correlation analysis (Table 5) showed a significant ($p < 0.05$) positive relationship ($r=0.95, 0.94$) between them. The IC_{50} obtained in this study is higher when compare with the one reported by Assadpour et al., (2016) for *Allium rotundum L* [53]. This study corroborated the previous study of Oyetayo and Ojo (2017), where DT seeds inhibited lipid peroxidation in liver and brain homogenates [54].

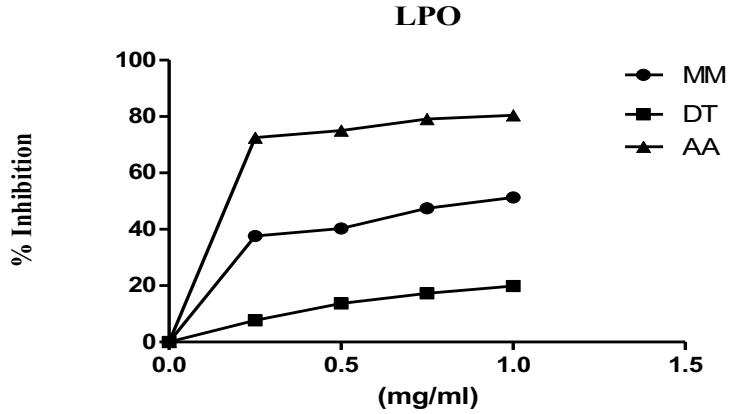

Figure 3. Lipid peroxidation inhibitory activity of methanol extracts of MM and DT

Table 5: Correlation among the polyphenols, antioxidant abilities and lipid peroxidation in rat's heart

	TP	TF	TAC	DPPH	RP	ABTS	FRAP	NO	LPO
TP	1								
TF	0.99*	1							
TAC	0.92*	0.91*	1						
DPPH	0.66	0.62	0.48	1					
RP	0.51	0.56	0.26	0.06	1				
ABTS	0.82*	0.84*	0.56	0.36	0.73	1			
FRAP	0.66	0.69	0.78	0.07	0.24	0.49	1		
NO	0.78	0.78	0.77	0.86*	0.09	0.56	0.42	1	
LPO	0.95*	0.94*	0.84*	0.76	0.37	0.80	0.50	0.90*	1

Significant is established at ($p < 0.05$). TP: Total phenolics, TF: Total flavonoid, TAC: Total antioxidant capacity, DPPH: 2,2 diphenyl-1-picrylhydrazyl scavenging activity, RP: Reducing power, ABTS: 2, 2-azobis-3-ethylbenzothiazoline-6-sulfonate scavenging activity, FRAP: Ferric reducing antioxidant potential, NO= Nitric oxide scavenging ability, LPO= Lipid peroxidation inhibition

The extracts profiling by HPLC (Figures 4 and 5) showed ample presence of myristyl chloride, linalool and nerolidal in MM (Table 3) and elemicin, myristicin, eugenol and pinene in DT (Table 4). The ability to inhibit lipid peroxidation by these extracts might not be unconnected to the presence of these compounds; linalool has be shown to reduce lipid peroxidation in uremia induced vascular calcification and acrylamide-induced neurotoxicity in rats [55,56], Pinene has also reduced lipid peroxidation in the brain of rats following induced focal ischaemic stroke in rat[57]. Also, myristicin has been proven to protect against ulcerative colitis induced by acetic acid in mice by mitigating lipid peroxidation[58]. Also, there might need in future to isolate these compounds from the extracts (MM and DT) and evaluate them for probable drug lead. Conclusively,

the present study has shown that MM and DT has considerable polyphenolic content which culminated into antioxidant activity and ability to inhibit nitric oxide radical and lipid peroxidation in rat's heart homogenate *invitro*. Therefore, the extracts can serve as natural antioxidants against free radical-induced cardiovascular injury.

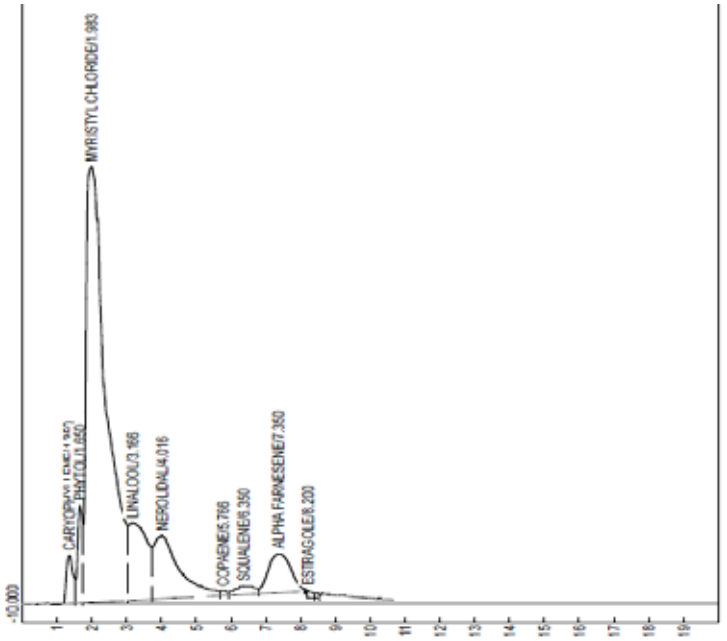

Figure 4. Chromatogram of MM using HPLC

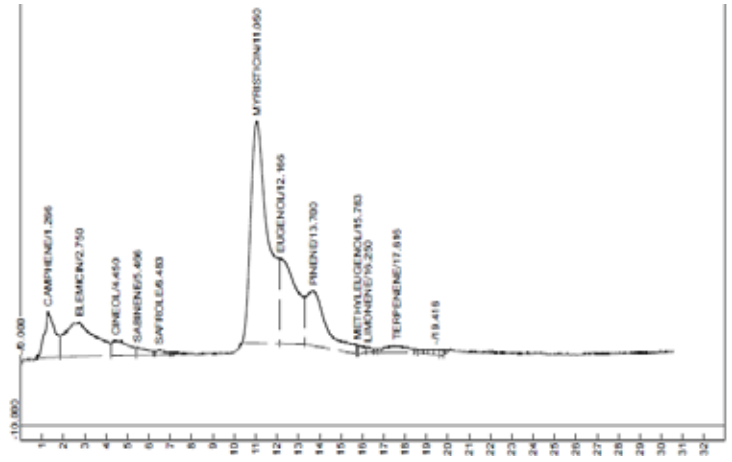

Figure 5. Chromatogram of DT using HPLC

Table 3. Phytochemicals found in MM using HPLC

	Compound	mg/g
1	Caryophyllene	0.04 ±0.00
2	Phytol	0.06 ±0.01
3	Myristyl chloride	1.12 ±0.05
4	Linalool	0.18 ±0.01
5	Nerolidal	0.21 ±0.02
6	Copaene	0.01 ±0.00
7	Squalene	0.02 ±0.00
8	Alpha farnesene	0.11 ±0.01
9	Estragole	0.01 ±0.00

Values were presented as Mean ± SD.

Table 4. Phytochemicals found in DT using HPLC

	Compound	mg/g
1	Camphene	0.10 ±0.01
2	Elemicin	0.22 ±0.04
3	Cineol	0.05 ±0.00
4	Sabinene	0.02 ±0.00
5	Safrole	0.01 ±0.00
6	Myristicin	0.78 ±0.05
7	Eugenol	0.30 ±0.03
8	Pinene	0.23 ±0.03
9	Methyl Eugenol	0.01 ±0.00
10	Limonene	0.01 ±0.00
11	Terpenene	0.03 ±0.00

Values were presented as Mean ± SD.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest

AUTHOR CONTRIBUTIONS

KOK and FOA designed the study; KOK, ESA and DAO accumulated the data; KOK, ESA, DAO and FOA drafted the manuscript.

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Anticancer potentials of Morinda lucida and Annona muricata on Ki67 and Multidrug resistance1 genes expressions in Sodium arsenite-induced hepato-toxicity in rats

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ABSTRACT

This study evaluated anticancer potentials of *Morinda lucida* (ML) and *Annona muricata* (AM) on Ki67 and Multi-drug resistance1 (MDR1) concentrations in livers of rats in Sodium arsenite (SA)-induced hepato-toxicity. 60 adult female rats were randomly divided into 12 groups (n= 5). Groups 1 and 2 received physiological saline and 10mg/kg bodyweight of SA respectively. Groups 3-6 received SA followed by treatments with ML and AM doses. Groups 7-10 received extracts only. Groups 11-12 received co-administrations of SA with extracts. Drugs/extracts were administered orally. Experimental procedure was 5 weeks. Consequently, Liver histo-pathology and ELISA concentrations of Ki67 and MDR1 were evaluated. Data were statistically analyzed ($P \leq 0.05$). Results showed decreased levels of Ki67 (Groups 11-12) and MDR1 (Groups 3-4 and 11) compared with Group 2, indicating that ML ameliorated SA-induced hyperplasia and drug resistance, while AM ameliorated SA-induced hyperplasia. Therefore, ML possesses anti-proliferation and

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anti-drug resistance potentials, while AM possesses anti-proliferation potentials.
Keywords: *Morinda lucida*, *Annona muricata,* Sodium arsenite, Ki67 and Multidrug resistance1.

INTRODUCTION

Morinda lucida (ML) is a medium size tree with short crooked branches. Although it is very bitter, different parts of ML have been reported to possess medicinal properties. The leaf extract of the plant was reported to possess trypanocidal, antimalarial activities and aortic vaso-relaxant effect. The use of a weak decoction of the stem bark has been documented to treat severe jaundice. ML leaf extract has also been reported to have a strong oral hypoglycemic property. In Southern Nigeria, numerous people treat malaria by drinking aqueous leaf extract of ML. It is well documented that ML leaf extract has various therapeutic benefits with no known adverse effect among the users[1,2].

Annona muricata (AM) is a member of the Annonaceae family and is a fruit tree with a long history of traditional use. It's also known as soursop, graviola and guyabano. It is an evergreen plant that is mostly distributed in tropical and subtropical regions of the world. The fruits of AM are used to prepare syrups, beverages. A wide array of ethno-medicinal activities has been attributed to different parts of AM, and indigenous communities in Africa extensively use this plant in their folk medicine. Numerous researches have substantiated these activities, including anticancer, anticonvulsant, anti-arthritic, antiparasitic, antimalarial, hepato-protective and antidiabetic activities[3,4].

Arsenic is an established human clastogen that exists naturally either in organic or inorganic forms in the environment with attending potential for neurotoxicity, cardiac dysfunction and hepatotoxicity. Arsenic-induced cytotoxicity is via increased generation of free radicals and further confinement of oxidative stress in body organs resulting in damages to DNA, proteins and lipids[5], as well as resulting in increased micronuclei frequency and chromosomal aberrations[6]. Arsenic-induced toxicity can lead to cancers of the skin, lung, bladder, liver and kidney in exposed organisms[5,6].

Furthermore, cancers comprise of cancer stem cells (CSCs), macrophages and vascular endothelial cells, with CSCs having tumourigenic capacity while others do not[7,8]. Cancer treatment regimens kill most cancer cells, but do not eliminate CSCs, which have protective and resistance mechanisms[7,8] via upregulation of biomarkers of proliferation (Ki67) and drug resistance (multidrug resistance1 (MDR1) gene or P-glycoprotein and Aldehyde dehydrogenase

1)[9]. CSCs are, therefore, able to regenerate other cancer cells well after completion of treatment regimens. Hence, the characteristic survival of CSCs provides explanations for failures of cancer treatments, as well as informed directions for the development of more potent anticancer drugs from plants or other sources.

Ki-67 protein is detected during all the active phases of the cell cycle and it is usually used as a complement to grading systems that include mitotic counting as a sign of proliferation[10,11]. It is one of the five genes (out of 16 cancer-associated genes) of proliferation that is of important weight to the Oncotype score. Ki-67 is not expressed by quiescent or resting cells in the G0phase, hence it is an excellent operational marker for evaluation of the proliferation of a given cell population and the aggressiveness of malignancies[10-12]. The MDR1 gene or P-glycoprotein is localized in the cell-membrane and it functions pharmacologically as an active drug efflux transporter protein of various substances including drugs and toxins[9,13,14]. The MDR1 protein is physiologically expressed at the bile canalicular membrane of the liver functioning in biliary excretion of lipophilic drugs[15]. The MDR1 protein has affinity for hydrophobic compounds and efforts have been made to by-pass its efflux effect using reversal agents such as R-verapamil, Tween-80 and Cremophor EL. These reversal agents have, however, been reported to induce significant toxicity at required doses for MDR1's inhibition[9,13,14].

The characteristic abnormal cellular proliferation (hyperplasia) with accompanied increased expressions of Ki67 and MDR1 by CSCs makes the treatment of cancers a very challenging task. It is, therefore, very relevant to evaluate plants sources towards the isolation of drugs compounds that can specifically target CSCs and reduce or eliminate drug resistance. Arsenic-induced toxicity is of global health concerns; hence it is relevant to search for edible plants' sources which can prevent or counteract the adverse effects of Arsenic-induced toxicity. Therefore, this study evaluated the effects of *Morinda lucida* and *Annona muricata* on immunomodulations of Ki67 and MDR1 protein expressions in the liver tissues of rats in Sodium arsenite-induced hepato-toxicity in-order to further determine which plant fractions possess hepato-protective, anti-proliferation, anti-drug resistance and anticancer potentials.

METHODOLOGY

Collection, Authentication and Deposition of *Morinda Lucida* (ML) and *Annona muricata* (AM) Leaves

Freshly cut leaves of ML and AM were obtained locally from forest reserves in Ilorin and samples identified and authenticated by a Pharmaceutical Botanist of the Department of Botany, Faculty of Life Sciences, University of Ilorin, Ilorin, Nigeria. ML and AM leaves were deposited at the herbarium of the Department of Botany, Faculty of Life Sciences, University of Ilorin, and assigned Herbarium Identification Numbers UITH/004/1103 and UITH/003/1106 respectively.

Preparations and Ethanolic extractions of *Morinda Lucida* (ML) and *Annona muricata* (AM) Leaves

ML and AM leaves were air-dried at the laboratory unit of the Department of Chemistry, University of Ilorin, Ilorin, Nigeria. The dried leaves of ML and AM were grinded to powder form to enable proper absorption of solvent and weighed using the electronic compact scale. Extraction was carried out using distilled ethanol in-order to remove impurities, and the resultant product was put in a conical flask and heated. Liquid ethanol flowed from the condenser into a container and was continuously recycled to keep the process running. Boiling chips/anti-bumping granules were put in the conical flask to prevent liquid ethanol from 'bumping' into the condenser[16].

The mixture was decanted and then sieved after 24 hours. After decantation, another distilled ethanol was added to the sieved ML and AM and left for another 24 hours. When the colour quality and texture of the dissolved ML and AM in ethanol became evidently low (compared to previous solutions decanted), the procedure was halted. Ethanol was separated from ML and AM and Column chromatography was done to get different fractions of ML and AM[16]. Column Chromatography Fractionation of Ethanol Extract of *Morinda Lucida* (ML)

The ethanol extract of ML was were fractionated in a silica gel open column, using n-hexane, dichloromethane, ethyl acetate and ethanol in an increasing order of polarity (N-hexane: Dichloromethane [3;1,3:2,1:1,1:2,1:3]; Dichloromethane, Dichloromethane: Ethylacetate [3:1,3;2, 1:1, 1:2, 1;3]; Ethylacetate; Ethylacetate: Methanol [3:1, 3:2, 1:1, 1:2, 1:3] and Methanol, to afford thirty-six eluents of 250ml each. The resulting eluents were pooled based on the colour of the solvents that elute them to give a total of nine combined fractions[16]. The fraction MLF1 which had the best preliminary antioxidant potential out of the 9 fractions was used in this study to evaluate the effects of ML on Sodium arsenite-induced hepato-toxicity in rats.

Column Chromatography Fractionation of Ethanol Extract of *Annona muricata* (AM)

The ethanol extract of AM was fractionated in a silica gel open column, using n-hexane, dichloromethane, ethyl acetate and ethanol in an increasing order of polarity (N-hexane: Dichloromethane [3;1,3:2,1:1,1:2,1:3]; Dichloromethane, Dichloromethane: Ethylacetate [3:1,3;2, 1:1, 1:2, 1;3]; Ethylacetate, Ethylacetate: Methanol [3:1, 3:2, 1:1, 1:2, 1:3] and Methanol, to afford thirteen eluents of 250ml each. The resulting eluents were pooled based on the colour of the solvents that elute them to give a total of five combined fractions[16]. The fraction AMF1 which had the best preliminary antioxidant potential out of the 5 fractions was used in this study to evaluate the effects of ML on Sodium arsenite-induced hepato-toxicity in rats.

Animal Care and Feeding

A total number of sixty (60) female Wistar rats with an average weight of 156g and 2 months of age were used in this study. The rats were of different initial bodyweights (Kg) depending on time of birth as available from the same colony bred. Male rats were used in our previous study which evaluated the effects of plants' extracts on Ki67 and MDR1 levels in 7,12-Dimethylbenz[a]anthracene-induced cancer model [16]. Hence, female rats were used in this study in-order to provide comparative gender analyses on the ameliorative effects of plants' extracts on Ki67 and MDR1 levels. The rats were acclimatized for 5 days, received water ad libitum and kept in the animal house located in the Faculty of Basic Medical Sciences, College of Health Sciences, University of Ilorin. The animals were fed daily with pelletized grower feed from Ogo-Oluwa Livestock and Aqua Feed enterprise, Kwara State, Ilorin. The grower feed contains 15% crude protein, 7% fat, 10% crude fibre, 1% calcium, 0.35% phosphorus and 2.55% kcal/kg of metabolized energy as indicated on the pack. The animals were grouped into ten with five animals each in a wire gauzed cage. The animals were kept under a normal room temperature of 37°C and double-crossed ventilation.

Chemicals and Reagents

Sodium arsenite (SA) was a product of Sigma–Aldrich Japan Co. (Tokyo, Japan), and was purchased from Emed Ejeson enterprises in Ilorin, Kwara State, Nigeria. Normal Saline was obtained from MOMROTA pharmaceutical company in Ilorin, Kwara State, Nigeria.

Experimental Procedures and Drugs Administration

The experimental procedures and drugs administration are as detailed in Table 1. The number of rats employed in this study was determined based on the guidelines and approval of the University Ethical Review Committee (UERC) of University of Ilorin, Nigeria. The dose of Sodium Arsenite was determined from a previous study[17], while the doses of ML and AM were determined from previous study on anticancer potentials of ML and AM in Lead acetate-induced toxicity in rats[18]. In addition, bodyweights (g) of all rats were measured on Day 1 of experimental procedure and at the end of each week.

The Experimental treatments and toxicological profiling Groups were in six categories as detailed below.

Anticancer potentials of ML: Groups 3 and 4.

Anticancer potentials of AM: Groups 5 and 6.

Toxicological profiling of ML: Groups 7 and 8.

Toxicological Profiling of AM: Groups 9 and 10.

Chemo-preventive potentials of ML: Group 11 and

Chemo-preventive potentials of AM: Group 12.

Table 1. Doses of Drug/Extract administered and Period of Administration.

Groups of rats	Doses of drug/extract administered
1	Physiological saline (5 weeks)
2	10mg/Kg bodyweight Sodium arsenite (SA) (5 weeks)
3	10mg/Kg bodyweight SA (2 weeks) + 7.5mg/Kg bodyweight *Morinda lucida* (3 weeks)
4	10mg/Kg bodyweight SA (2 weeks) + 15mg/Kg Bodyweight *Morinda lucida* (3 weeks)
5	10mg/Kg SA (2 weeks) + 7.5mg/Kg bodyweight *Annona muricata* (3 weeks)
6	10mg/Kg bodyweight SA (2 weeks) + 10mg/Kg bodyweight *Annona muricata* (3 weeks)
7	7.5mg/Kg bodyweight *Morinda lucida* (5 weeks)
8	15mg/Kg bodyweight *Morinda lucida* (5 weeks)
9	7.5mg/Kg bodyweight *Annona muricata* (5 weeks)
10	10mg/Kg bodyweight *Annona muricata* (5 weeks)
11	Co-administration of 15mg/Kg bodyweight *Morinda lucida* + 10mg/Kg bodyweight SA (5 weeks)
12	Co-administration of 10mg/Kg bodyweight *Annona muricata* + 10mg/Kg bodyweight SA (5 weeks)

Animal Sacrifice

At the end of experimental procedures, all rats were sacrificed by cervical dislocation.

Histo-pathological Evaluations of the Liver

The liver tissues of all rats were excised and fixed in 10% formal saline of at least five times of its volume. Tissue preparation and staining of the sections were carried out via Haematoxylin and Eosin method as previously described[18].

Enzyme Linked Immunosorbent Assay (ELISA) of Concentrations of Ki67 and Multidrug Resistance1 (MDR1) Genes in Liver Tissues of Rats

Liver parts were cut from each rat and placed in 10% formalin for histo-pathological examinations, processed for light microscopy using conventional histological procedures and obtained slides were stained with Hematoxyline and Eosin[17]. In addition, separate liver tissues were isolated and then subjected to thorough homogenization using porcelain mortar and pestle in ice-cold 0.25M sucrose, in the proportion of 1g to 4ml of 0.25M sucrose solution. The tissue homogenates were filled up to 5ml with additional sucrose and collected in a 5ml serum bottle. Homogenates were thereafter centrifuged at 3000 revolution per minute for 15 minutes using a centrifuge (Model 90-1). The supernatant was collected with Pasteur pipettes and placed in a freezer at -4^{0}C, and thereafter assayed for concentrations of Ki67 (Sigma-Aldrich AB9260) and MDR1 (Sigma-Aldrich HPA002199-100UL) proteins in the liver tissues of all rats of Control and Experimental Groups using ELISA technique[16].

The ELISA assay technique employs the quantitative sandwich enzyme immunoassay technique. Antibodies specific for Ki67 and MDR1 proteins were pre-coated onto a microplate. Standards and samples were pipetted into the wells, and Ki67 and MDR1 proteins present were bound by the immobilized antibodies. After removing any unbound substances, biotin-conjugated antibodies specific for Ki67 and MDR1 proteins were added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) was added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of Ki67 and MDR1 proteins bound in the initial step. The colour development was stopped and the intensity of the colour was measured.

Statistical Analyses

All data obtained were expressed as arithmetic means ± standard error of mean, and were subjected to statistical analyses using T-test to compare Group 2 with

Groups 1 and 3 – 12. Differences were tested and considered statistically significant when $p \leq 0.05$ using Graph Pad Prism software package (Graph Pad Software Inc., San Diego, CA, USA; version 7 for Windows) and Microsoft Excel 2016.

Rats of Group 2 received only the clastogen (Sodium arsenite) used for the induction of toxicity in this study, and without further treatment with plants' extracts. Hence, Control Group 1 was compared with Group 2 to establish the adverse effects of Sodium arsenite (SA) on Ki67 and MDR1 levels. Similarly, Groups 3 – 12 were compared with Group 2 to confirm the degree of ameliorative potentials of doses of ML and AM on the effects of SA on Ki67 and MDR1 levels.

RESULTS AND DISCUSSION

Gross Morphological and Behavioural Observations

Morphological observations showed normal gross morphology of liver of rats of Groups 1 - 12. In addition, no behavioural anomalies were observed in rats of Groups 1 - 12. This implied that administrations of doses of SA, ML and AM to rats did not result in adverse effects on the gross morphology of rats, the liver and behavioural functions of rats.

Changes in Bodyweights of Rats

Results showed decreases in bodyweight of rats per week, and decreases in the final bodyweight compared to the initial bodyweight of Group 2, which received only SA. This implied that SA-induced toxicity resulted in decreased bodyweights of rats.

Does AM have ameliorative potentials against SA-induced adverse effects on bodyweight of rats? Results showed increases in bodyweight of rats per week, and increases in the final bodyweight compared to the initial bodyweight of Control Group 1 and Experimental Groups 3 - 4 and 6 - 11 (Table 2). This implied that post-treatments of SA-induced toxicity with 7.5 and 15mg/kg bodyweight of ML ameliorated the adverse effects on bodyweight of rats of Groups 3 and 4.

Table 2. Changes in Bodyweight (g) of rats.

Groups of rats	Doses of drug/extract administered	Initial Bodyweight (g)	Final Bodyweight (g)	% Bodyweight change
1	Physiological saline	119.2±0.49	178.6±3.78	49.79±2.66
2	10mg/Kg bodyweight Sodium arsenite (SA)	192.6±8.02	170.0±12.05	13.61±6.29
3	10mg/Kg bodyweight SA (2 weeks) + 7.5mg/Kg bodyweight *Morinda lucida* (3 weeks)	170.8±7.67	199.0±7.12	17.11±5.23
4	10mg/Kg bodyweight SA (2 weeks) + 15mg/Kg Bodyweight *Morinda lucida* (3 weeks)	132.4±8.79	183.8±6.98	41.68±12.20
5	10mg/Kg SA (2 weeks) + 7.5mg/Kg bodyweight *Annona muricata* (3 weeks)	201.0±5.26	185.8±12.35	12.34±5.72
6	10mg/Kg bodyweight SA (2 weeks) + 10mg/Kg bodyweight *Annona muricata* (3 weeks)	138.0±4.37	171.2±3.65	24.64±5.32
7	7.5mg/Kg bodyweight *Morinda lucida*	142.0±9.06	185.8±12.35	33.31±10.85
8	15mg/Kg bodyweight *Morinda lucida*	124.4±0.40	169.0±11.78	33.90±9.66
9	7.5mg/Kg bodyweight *Annona muricata*	110.8±0.49	170.4±4.55	53.79±4.03
10	10mg/Kg bodyweight *Annona muricata*	111.2±0.49	182.0±11.70	63.75±10.74
11	Co-administration of 15mg/Kg bodyweight *Morinda lucida* + 10mg/Kg bodyweight SA (5 weeks)	109.6±0.40	150.6±8.82	37.46±8.26
12	Co-administration of 10mg/Kg bodyweight *Annona muricata* + 10mg/Kg bodyweight SA (5 weeks)	171±5.69	165.4±16.03	11.83±4.73

Does ML have preventive potentials against SA-induced adverse effects on bodyweight of rats? Our findings implied that co-administration of 10mg/kg bodyweight of SA with 15mg/kg bodyweight of ML prevented the adverse effects of SA-induced toxicity on bodyweight of rats of Group 11.

Does AM have preventive and/or ameliorative potentials against SA-induced adverse effects on bodyweight of rats? Results showed decreases in the final bodyweight compared to the initial bodyweight of rats of Groups 5 and 12, which received 10mg/kg bodyweight of SA and were treated or co-administered with doses of AM (Table 2). This implied that post-treatments of SA-induced toxicity with 7.5mg/kg bodyweight of AM did not ameliorate the adverse effects on bodyweight of rats of Group 5. In addition, the co-administration of 10mg/kg bodyweight of SA with 10mg/kg bodyweight of AM did not prevent the adverse effects of SA-induced toxicity on bodyweight of rats of Group 12.

Histo-pathological Evaluations of the Liver

Histo-pathological evaluations showed normal histo-architectures of the liver (Figures 1 - 12) in all rats of Groups 1 - 12. There were normal cellular density and staining characteristics of hepatocytes, hepatic sinusoids and central veins. The nuclei of hepatocytes were well characterized with no apparent large vacuolations around them. This implied that administrations of doses of SA,

ML and AM to rats did not result in evident histopathology of the liver after 5 weeks of exposure. This is due to the fact that the cyto-toxicity of adverse chemical agents is exposure-dependent and drug-induced toxicity is usually first elicited on molecular markers, while further exposure will result in evident histo-pathology at tissue level.

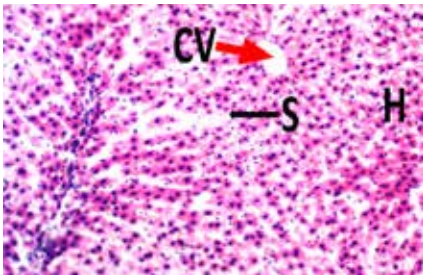

Figure 1. Photomicrograph of liver of rat of Control Group 1, which received Normal Saline. Haematoxylin and Eosin X 400. Scale Bar: 100µm. H = Hepatocytes, S = Blood sinusoids and CV = Central Vein. Histo-pathological evaluations showed normal histoarchitecture of the liver components.

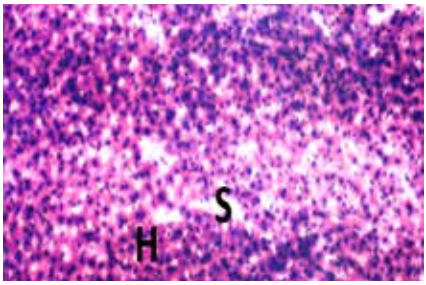

Figure 2. Photomicrograph of liver of rat of Experimental Group 2, which received 10mg/kg bodyweight of Sodium arsenite only. Haematoxylin and Eosin X 400. Scale Bar: 100µm. H = Hepatocytes, S = Blood sinusoids and CV = Central Vein. Histo-pathological evaluations showed normal histoarchitecture of the liver components.

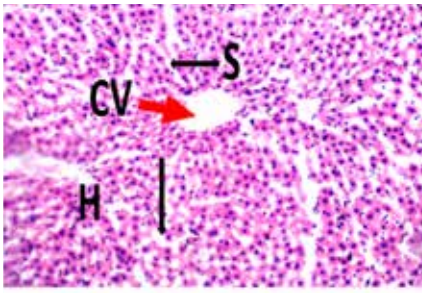

Figure 3

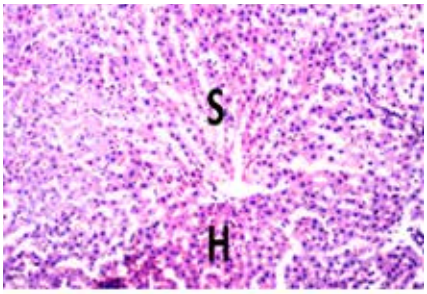

Figure 4

Figures 3 & 4. Photomicrograph of liver of rat of Experimental Groups 3 and 4, which received 10mg/Kg bodyweight Sodium arsenite (2 weeks) + 7.5 and 15mg/Kg bodyweight *Morinda lucida* (3 weeks) respectively. Haematoxylin and Eosin X 400. Scale Bar: 100µm. H = Hepatocytes, S = Blood sinusoids and CV = Central Vein. Histo-pathological evaluations showed normal histoarchitecture of the liver components.

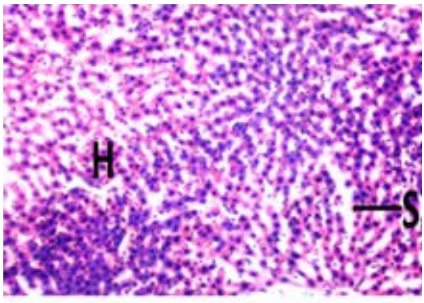

Figure 5

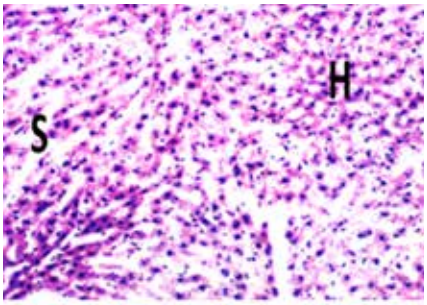

Figure 6

Figures 5 & 6. Photomicrograph of liver of rat of Experimental Groups 5 and 6, which received 10mg/Kg bodyweight Sodium arsenite (2 weeks) + 7.5 and 10mg/Kg bodyweight *Annona muricata* (3 weeks) respectively. Haematoxylin and Eosin X 400. Scale Bar: 100µm. H = Hepatocytes, S = Blood sinusoids and CV = Central Vein. Histo-pathological evaluations showed normal histoarchitecture of the liver components.

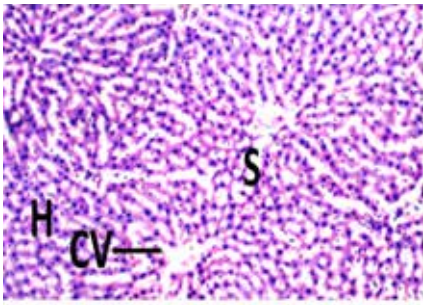

Figure 7

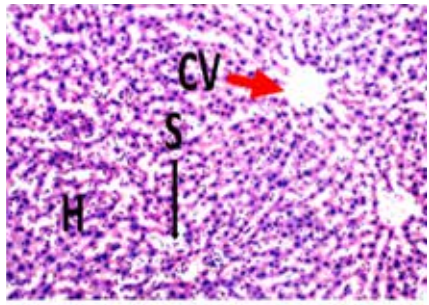

Figure 8

Figures 7 & 8. Photomicrograph of liver of rat of Experimental Groups 7 and 8, which received only 7.5 and 15mg/Kg bodyweight *Morinda lucida* (3 weeks) respectively. Haematoxylin and Eosin X 400. Scale Bar: 100µm. H = Hepatocytes, S = Blood sinusoids and CV = Central Vein. Histo-pathological evaluations showed normal histoarchitecture of the liver components.

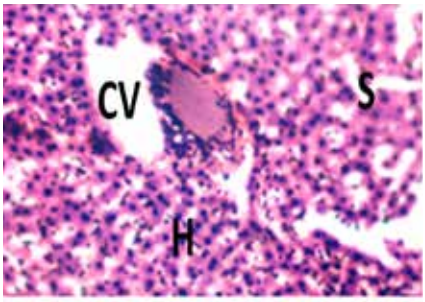

Figure 9

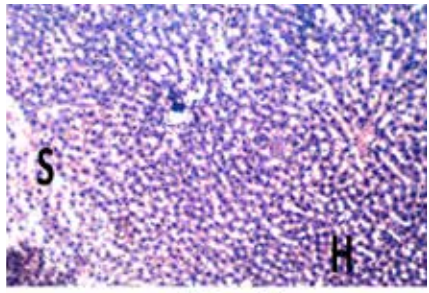

Figure 10

Figures 9 & 10. Photomicrograph of liver of rat of Experimental Groups 9 and 10, which received only 7.5 and 10mg/Kg bodyweight *Annona muricata* (3 weeks) respectively. Haematoxylin and Eosin X 400. Scale Bar: 100µm. H = Hepatocytes, S = Blood sinusoids and CV = Central Vein. Histo-pathological evaluations showed normal histoarchitecture of the liver components.

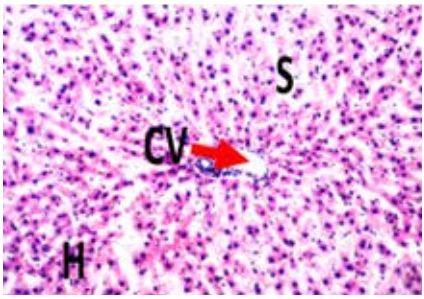

Figure 11. Photomicrograph of liver of rat of Experimental Group 11, which received co-administration of 10mg/Kg bodyweight SA + 15mg/Kg bodyweight *Morinda lucida* (5 weeks). Haematoxylin and Eosin X 400. Scale Bar: 100µm. H = Hepatocytes, S = Blood sinusoids and CV = Central Vein. Histo-pathological evaluations showed normal histoarchitecture of the liver components.

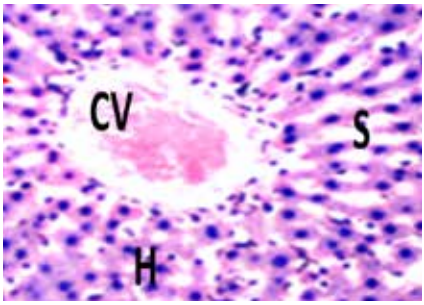

Figure 12. Photomicrograph of liver of rat of Experimental Group 12, which received co-administration of 10mg/Kg bodyweight SA + 10mg/Kg bodyweight *Annona muricata* (5 weeks). Haematoxylin and Eosin X 400. Scale Bar: 100µm. H = Hepatocytes, S = Blood sinusoids and CV = Central Vein. Histo-pathological evaluations showed normal histoarchitecture of the liver components.

ELISA Concentrations of Ki67 in Liver Tissues of Rats

Ki-67 protein is an established biomarker of cellular proliferation[10-12]. Hence, increased Ki67 concentrations is associated with hyperplasia and aggressiveness of malignancies[10-12].

Does SA have any adverse effects on Ki67 levels? Results showed statistically non-significant higher ($p \geq 0.05$) levels of Ki67 in rats of Group 2 when compared with Group 1 (Table 3). This result implied SA-induction of abnormal proliferation (hyperplasia) and upregulation of Ki67 in rats of Group 2. This observation agrees with the views of[12], which opined that all proliferating cells tested expressed Ki67, and that there is no evidence to the contrary that proliferating cells do not express Ki67.

Can ML and AM ameliorate SA-induced upregulations of Ki67 and hyperplasia? Post-treatments of SA-induced hepato-toxicity with 7.5 and 15mg/kg bodyweight of ML, 7.5 and 10mg/kg bodyweight of AM resulted in statistically non-significant higher ($p \geq 0.05$) Ki67 levels in rats of Groups 3 - 6 when compared with Group 2 (Table 3). This implied that the tested doses of ML and AM did not ameliorate SA-induced abnormal proliferations in rats of Groups 3 - 6 respectively.

Table 3. Ki67 concentrations (Mean±SEM) (ng/ml) in Liver tissues of rats.

Groups of rats	Doses of drug/extract administered	Ki67 (Mean±SEM) (ng/ml)	P≤0.05: Group 2 versus Groups 3 - 12
1	Physiological saline	4.37±1.21	0.11
2	10mg/Kg bodyweight Sodium arsenite (SA)	9.99±1.70	
3	10mg/Kg bodyweight SA (2 weeks) + 7.5mg/Kg bodyweight *Morinda lucida* (3 weeks)	11.31±3.81	0.78
4	10mg/Kg bodyweight SA (2 weeks) + 15mg/Kg Bodyweight *Morinda lucida* (3 weeks)	15.47±4.79	0.39
5	10mg/Kg SA (2 weeks) + 7.5mg/Kg bodyweight *Annona muricata* (3 weeks)	12.46±9.66	0.83
6	10mg/Kg bodyweight SA (2 weeks) + 10mg/Kg bodyweight *Annona muricata* (3 weeks)	14.67±4.34	0.42
7	7.5mg/Kg bodyweight *Morinda lucida*	9.10±3.09	0.65
8	15mg/Kg bodyweight *Morinda lucida*	2.84±0.29	0.05*
9	7.5mg/Kg bodyweight *Annona muricata*	3.85±0.89	0.09
10	10mg/Kg bodyweight *Annona muricata*	2.28±0.19	0.05*
11	Co-administration of 15mg/Kg bodyweight *Morinda lucida* + 10mg/Kg bodyweight SA	6.954±0.39	0.22
12	Co-administration of 10mg/Kg bodyweight *Annona muricata* + 10mg/Kg bodyweight SA	2.92±0.84	0.07

*Means with superscript are significantly different ($P<0.05$).

Do ML and AM have cyto-protective potentials against SA-induced upregula-

tions of Ki67 and abnormal proliferations in rats? Results showed statistically non-significant lower ($p \geq 0.05$) Ki67 levels in rats of Groups 11 and 12 when compared with Group 2 (Table 3). Our findings implied that co-administrations of 10mg/kg bodyweight of SA with 15mg/kg bodyweight of ML and 10mg/kg bodyweight of AM resulted in downregulation of Ki67 levels and, therefore, offered cyto-protective potentials against SA-induced abnormal proliferations in rats. These observations are in agreement with previous studies which noted anti-proliferation and tumour inhibitory potentials of ML leaves[19] and AM leaves[20] against HL-60 leukemia cells and breast cancer MCF-7 cells respectively.

Are there any adverse effects on Ki67 levels following exposures of rats to only the evaluated doses of ML and AM? Results showed statistically significant ($p \leq 0.05$) decreased Ki67 levels in rats of Groups 8 and 10, which received only 15mg/kg bodyweight of ML and 10mg/kg bodyweight of AM respectively when compared with Group 2 (Table 3). In addition, it must be noted that the Ki67 concentrations in Groups 8 and 10 were non-significant statistically lower ($p \geq 0.05$) than that of Control Group 1, which received physiological saline (Table 3). These results implied that the administrations of doses of ML and AM only, resulted in the downregulation of Ki67 levels. Therefore, ML and AM possess anti-proliferation potentials.

Furthermore, Ki67 is a biomarker of Cancer Stem Cells (CSCs), hence our findings indicate that ML and AM possibly possess anti-cancer compounds that can specifically target and eliminate CSCs.

ELISA Concentrations of MDR1 in Liver Tissues of Rats

MDR1 or P-glycoprotein is a cell membrane protein, which by its pharmacological function as an active drug efflux transporter protein enhances drug resistance capacity of CSCs[9,13-15]. Hence, significant upregulation of MDR1 is characteristic of drug resistant tumours and has been associated with cancer cells survival[9,13-15].

Does SA have any adverse effects on MDR1 levels? Results showed statistically significant higher ($p \leq 0.05$) levels of MDR1 in rats of Group 2 when compared with Group 1 (Table 4). This result implied SA-induction of drug resistance and significant upregulation of MDR1 and in rats of Group 2.

Table 4. MDR1 concentrations (Mean±SEM) (ng/ml) in Liver tissues of rats.

Groups of rats	Doses of drug/extract administered	MDR1 (Mean±SEM) (ng/ml)	P≤0.05: Group 2 versus Groups 3 - 12
1	Physiological saline	17.44±1.12	0.04*
2	10mg/Kg bodyweight Sodium arsenite (SA)	22.59±0.07	
3	10mg/Kg bodyweight SA (2 weeks) + 7.5mg/Kg bodyweight *Morinda lucida* (3 weeks)	17.92±2.45	0.20
4	10mg/Kg bodyweight SA (2 weeks) + 15mg/Kg Bodyweight *Morinda lucida* (3 weeks)	16.54±2.93	0.12
5	10mg/Kg SA (2 weeks) + 7.5mg/Kg bodyweight *Annona muricata* (3 weeks)	35.30±5.27	0.13
6	10mg/Kg bodyweight SA (2 weeks) + 10mg/Kg bodyweight *Annona muricata* (3 weeks)	36.89±4.93	0.11
7	7.5mg/Kg bodyweight *Morinda lucida*	18.77±3.32	0.26
8	15mg/Kg bodyweight *Morinda lucida*	18.59±3.23	0.23
9	7.5mg/Kg bodyweight *Annona muricata*	21.54±1.30	0.52
10	10mg/Kg bodyweight *Annona muricata*	15.37±0.58	0.02*
11	Co-administration of 15mg/Kg bodyweight *Morinda lucida* + 10mg/Kg bodyweight SA	15.33±0.75	0.01*
12	Co-administration of 10mg/Kg bodyweight *Annona muricata* + 10mg/Kg bodyweight SA	25.69±1.88	0.27

*Means with superscript are significantly different (P<0.05).

Can ML and AM ameliorate SA-induced upregulations of MDR1 and induction of drug resistance? Post-treatments of SA-induced hepato-toxicity with 7.5 and 15mg/kg bodyweight of ML resulted in statistically non-significant lower (p≥0.05) levels of MDR1 in rats of Groups 3 and 4 when compared with Group 2 (Table 4). These results implied that ML ameliorated SA-induced upregulation of MDR1 and drug resistance in rats of Groups 3 and 4.

In contrast, post-treatments of SA-induced hepato-toxicity with 7.5 and 10mg/kg bodyweight of AM resulted in statistically non-significant higher (p≥0.05) levels of MDR1 in rats of Groups 5 and 6 when compared with Group 2 (Table

4). These results implied that AM did not ameliorate SA-induced upregulation of MDR1 and drug resistance in rats of Groups 5 and 6.

Do ML and AM have cyto-protective potentials against SA-induced drug resistance in rats? Results showed statistically significant ($p \leq 0.05$) lower levels of MDR1 in rats of Group 11 when compared with Group 2 (Table 4). Our findings implied that 15mg/kg bodyweight of ML offered cyto-protective potentials against SA-induced upregulation of MDR1 and drug resistance when co-administered with 10mg/kg bodyweight of SA.

In contrast, results showed statistically non-significant higher ($p \geq 0.05$) levels of MDR1 in rats of Group 12 when compared with Group 2 (Table 4). This result implied that 10mg/kg bodyweight of AM did not offer cyto-protective potentials against SA-induced upregulation of MDR1 and drug resistance when co-administered with 10mg/kg bodyweight of SA.

Furthermore, MDR1 is a biomarker of CSCs, hence our findings indicate that ML possibly possesses anti-cancer compounds that can specifically target and eliminate CSCs.

There is paucity of studies, which evaluated the anticancer potentials of ML and AM on MDR1 levels (drug resistance) for comparative analyses, hence the interpretations and implications of results were limited to the observations of this study.

In conclusion, our findings in this study implied that post- and preventive-treatments of SA-induced hepato-toxicity with doses of ML resulted in decreased Ki67 and MDR1 levels. Therefore, ML possibly contains chemical components that may target cancer stem cells, and it possesses hepato-protective, anti-proliferation, anti-drug resistance and anticancer potentials. Hence, the use of ML as nutritional supplements may be further evaluated. In contrast, post-treatments of SA-induced hepato-toxicity with doses of AM resulted only in decreased Ki67 levels. Hence, AM though possibly possesses hepato-protective and anti-proliferation potentials, it does not possess anti-drug resistance potentials.

STATEMENTS OF ETHICS

Ethical approval for this study was sought and received via UERC/ASN/2018/1161 from the University Ethical Review Committee (UERC) of the institution where the study was primarily conducted. This research study was conducted in accordance with the internationally accepted principles for laboratory animal use and care as provided in the European Community guidelines

(EEC Directive of 1986; 86/609/EEC) and the US guidelines (NIH publication #85-23, revised in 1985).

CONFLICT OF INTEREST STATEMENT

The authors wish to confirm that there are no known conflicts of interest associated with this study.

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In vivo inhibitory effect of hydro-ethanol extract of *Xylopia aethiopica* fruits on mediators of acute inflammation

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ABSTRACT

Xylopia aethiopica is traditionally employed, as fruit decoction, in the management of bronchitis, asthma and arthritis in Ghana. This study is to evaluate the involvement of the hydro-ethanol extract of the dried fruit of *X. aethiopica* on some inflammation pathways by employing *in vivo* murine models. *X. aethiopica* (30, 100, 300 mg kg^{-1}) suppressed the mean maximal swelling attained at 60-90 min and also decreased the total paw swelling induced over 2.5-3 h significantly ($p < 0.05$). The extract suppressed mean maximal swelling attained at 60-90 min in the respective histamine, serotonin, bradykinin and prostaglandin E_2-induced oedema models when compared with their respective mean inflamed control responses. Total paw swellings induced over the 2.5-3 h were also significantly suppressed in all mediator-challenged mice. The current study establishes that *X. aethiopica* extract has inhibitory effect on histamine, serotonin, bradykinin and prostaglandin E_2 in acute inflammation.

Key words: Acute inflammation, anti-inflammatory, oedema, *Xylopia aethiopica* extract

INTRODUCTION

The inflammatory process is a protective response involving host immune cells, blood vessels, specialized proteins and lipid mediators, that aims at eliminating the offending agent or noxious stimuli such as tissue necrosis and infection [1]. Although beneficial, the inflammatory response can become destructive if overexaggerated or unable to resolve, such as in septic shock [2,3].

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Upon initiation of the inflammatory response by various inducers such as infection or signals from necrotic tissues, various specialized molecules are activated with subsequent stimulation of the release of inflammatory mediators such as histamine, serotonin, platelet activating factor (PAF), arachidonic acid metabolites, reactive oxygen species (ROS), cytokines and chemokines [4]. These endogenous compounds can activate or inhibit inflammation, stimulate or impair tissue repair, and also activate the effectors, which are the tissues and cells [5].

The inflammatory response is a chain of organized, dynamic responses and can be categorized into acute and chronic responses with each mediated by a different mechanism with different mediator-release profiles [6]. During acute inflammation, responses in the microvasculature occurs rapidly, usually within few minutes following microbial invasion or tissue insult leading to vasodilation and increased capillary permeability [7,8]. Increased capillary permeability facilitates interstitial oedema formation and recruitment of neutrophils to the site of injury [9]. Leukocyte infiltration during acute inflammation is stimulated by chemokines and basophil-derived histamine, PAF and leukotriene B [10,11]. Chronic inflammation on the other hand, is characterized by monocyte and lymphocyte infiltration, fibroblasts proliferation, connective tissue formation, release of reactive oxygen and nitrogen species, and protease secretion [12]. Together, these mediators give rise to diverse pathways in the inflammatory process, offering an array of targets for pharmacological intervention.

Xylopia aethiopica (Dunal) A. Rich. (Annonaceae) is a tropical evergreen plant with green fruits and aromatic seeds [13]. Traditionally, it is used in the form of the dried fruit decoction to treat bronchitis, asthma, arthritis and rheumatism in Ghana, Nigeria and Cameroon [14]. Our earlier findings have established its anti-inflammatory activity in both acute and chronic inflammation in murine subjects [15,16]. Phytochemical analysis and high-performance liquid chromatography (HPLC) fingerprint of *Xylopia aethiopica* fruit extract revealed the presence of kaurenoic acid and xylopic acid which are diterpenes known as kauranes [17]. These kauranes play a major role in the observed biologic effect of the plant [18,19]. The current study therefore aims at expounding some of the mechanisms involved in the anti-inflammatory action of the fruit extract of *Xylopia aethiopica* by investigating the activity of the extract on some prominent mediators of inflammation.

METHODOLOGY

Preparation of Plant Extract

Hydro-ethanol extraction was carried out as described by Obiri et al. [15]. *Xylopia aethiopica* fruits were obtained from the university botanical garden (6°41'7" N 1°33'48" W) in Kumasi between September and November of 2019. Identification of the fruit was made at the Department of Herbal Medicine, KNUST, voucher specimen number assigned (No. FP/09/77) and sample deposited at the herbarium of the department. The *Xylopia aethiopica* fruits were dried in open air and 3 kg of the dried material milled employing a heavy-duty blender. The pulverized material was macerated with 5 L, 70% w/v ethanol for 24 h. The filtrate was concentrated using a rotary evaporator and additionally dried in an oven to produce a 167 g solid extract. The obtained extract from the dried fruit was emulsified employing Tween-80 and normal saline and referred to as XAE.

Experimental Animals

ICR mice (25 – 30 g) were procured from the Noguchi Memorial Institute for Medical Research, University of Ghana, Accra, Ghana. The animals were housed in a temperature-controlled room in the Department of Pharmacology, KNUST animal house facility and had free access to chow and water. Animals were randomly group after a period of acclimatization. Animals were humanely handled throughout the study with ethical approval granted by the Department's ethics committee for the study.

Chemicals and reagents

Granisetron was purchased from Roche, Basel, Switzerland; diclofenac from Novartis Int AG, Basel, Switzerland; chlorpheniramine was procured from DWD Pharmaceuticals Ltd, Mumbai, India; histamine, serotonin hydrochloride, bradykinin acetate salt and prostaglandin E_2 were obtained from Sigma-Aldrich (St Louis, USA).

Histamine-induced paw oedema

Oedema was induced as described by Mazumder and colleagues [20]. Briefly, induction of paw oedema in mice was made by subplantar injection of 0.1 mg histamine after prophylactic or therapeutic administration of normal saline (1 ml kg^{-1} p.o.), chlorpheniramine (10 mg kg^{-1} p.o.) or XAE (30, 100, 300 mg kg^{-1} p.o.). Induced oedema (as paw thickness) was measured every 30 min for 3 h. The maximal oedema and total oedema were then calculated using the equation:

$$\% \text{ change in paw thickness} = 100 \times \frac{T_t - T_i}{T_i}$$

Where, Ti is paw thickness before phlogenic agent injection

Tt is paw thickness at time T.

The recorded paw thicknesses were individually normalized as percentage change from paw thickness before subplantar injection and averages computed. The total oedema was obtained in arbitrary determination as the area under the time course curve (AUC) and percentage oedema inhibition was established using the relation:

$$\% \text{ 100 inhibition of oedema} = \frac{AUC_{(control)} - AUC_{(treatment)}}{AUC_{(control)}} \times 100$$

Serotonin-induced paw oedema

Briefly, subplantar administration of 0.1 mg of serotonin was made as the phlogenic agent. Prophylactic or therapeutic administration of normal saline (1 ml kg^{-1} p.o.), granisetron (100 μg kg^{-1} p.o.) or XAE (30, 100, 300 mg kg^{-1} p.o.) were made to respective groups and paw thickness measured with electronic calipers every 30 min for 3 h. The maximal oedema and total paw oedema responses were determined as explained under histamine-induced paw oedema [20].

Bradykinin-induced paw oedema

Mice in respective groups were pre-treated with 5 mg kg^{-1} captopril subcutaneously. The subsequent process is as portrayed in histamine-induced paw oedema section. Briefly, subplantar administration of 1 μg of bradykinin was made. Prophylactic or therapeutic administration of normal saline (1 ml kg^{-1} p.o.) or XAE (30, 100, 300 mg kg^{-1} p.o.) were made to respective groups and paw thickness measured with calipers every 30 min for 3 h. The maximal oedema and total paw oedema responses were determined as described under histamine-induced paw oedema [20].

Prostaglandin E_2-induced paw oedema

The process is as described in section on histamine-induced paw oedema. Briefly, subplantar injection of 1 nM of prostaglandin E_2 was made. Prophylactic or therapeutic administration of normal saline (1 ml kg^{-1} p.o.), diclofenac (0.93 mg kg^{-1} p.o.) or XAE (30, 100, 300 mg kg^{-1} p.o.) were made to respective groups and paw thickness measured with calipers every 30 min for 2.5 h. The maximal oedema and total paw oedema responses were determined as explained above [20].

Statistical analysis

All obtained data are presented as the mean ± SEM (n = 5). Two-way ANOVA followed by Bonferroni's test were used to analysed the time-course curves while one-way ANOVA followed by Dunnett's post hoc test was employed in analysing the AUCs. Graphs were plotted with GraphPad Prism version 8.00 for MacBook (GraphPad, San Diego, CA)

RESULTS AND DISCUSSION

During infection or tissue damage, the immune system reacts through a sequence of organized events involving molecular, cellular and physiological alterations, in an attempt to eliminate the noxious stimuli and restore homeostasis [4]. This chain of immune responses is coordinated by inflammatory mediators, produced and released by blood, resident inflammatory cells and damaged tissue in response to the noxious stimulus [21,22]. The release of these mediators from mast cells, neutrophils, monocytes/macrophages, platelets, fibroblasts, smooth muscle cells and endothelial cells makes the acute inflammatory response immediate, adaptive and specific [4]. However, the process is sometimes dysregulated resulting in detrimental effects such as observed in septic shock [2]. These mediators can act together and give rise to diverse pathways in the inflammatory process, and can offer a variety of targets for pharmacological intervention.

Histamine–induced paw oedema

Mast cell activation in response to noxious stimuli increases synthesis and release of vasoactive amines such as histamine, which promotes vasodilation and increases vascular permeability, vascular hydrostatic pressure and efflux of intravascular fluid into interstitial space. Thus, resulting in the development of oedema [23,24]. It was observed that XAE (30, 100, 300 mg kg^{-1}) when administered before the induction of the histamine-induced paw oedema caused the mean maximal swelling attained at 60 min to be reduced to 76.80 ± 11.44%, 79.36 ± 10.95% and 49.57 ± 2.29% respectively relative to the control response of 92.14 ± 6.44% (Fig 1A). The total paw swellings induced over the 3 h (measured as the area under the time course curve, AUC) were also significantly suppressed to 352.38 ± 47.72%, 328.42 ± 51.55% and 204.44 ± 6.70% respectively from 490.10 ± 28.75% (Fig 1B).

In the therapeutic protocol, XAE (30 – 300 mg kg^{-1}) suppressed the mean maximal swelling attained at 60 min to 38.37 ± 3.41%, 40.88 ± 4.94% and 28.46 ± 5.56% respectively relative to the inflamed control response of 61.63 ± 4.55% (Fig 1C). The total paw swellings induced over the 3 h were also significantly suppressed to 156.66 ± 14.80%, 155.70 ± 15.33% and 143.94 ± 20.00% respectively from 283.54 ± 17.73% (Fig 1D).

The inhibition of paw oedema by XAE is believed to be mediated via the inhibition of histamine release, and/or interference of histamine's activity on H_1 and H_4 receptors. Antagonism of the former leads to an inhibition of histamine-mediated vasodilation, while H_4 antagonism results in inhibition of propagation of the inflammatory response [24,25].The extract thus inhibits acute inflammatory response mediated by this vasoactive amines, that is mainly characterized by the development of interstitial oedema owing to exudation of fluid and plasma proteins [26].

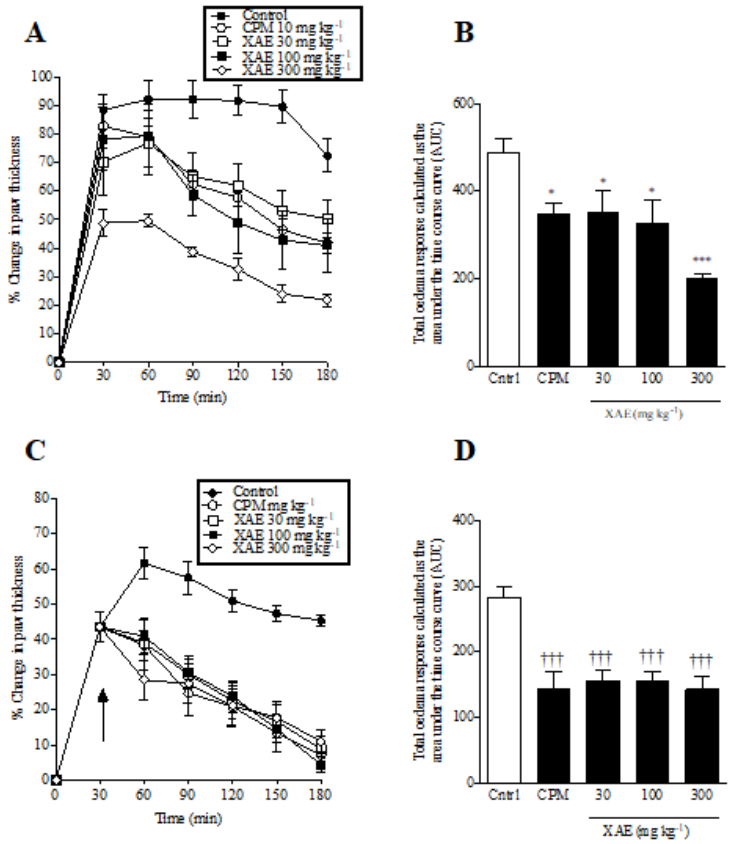

Figure 1. Effect of *Xylopia aethiopica* on histamine-induced paw oedema in mice. Oedema was monitored at 30 min intervals over 3 h as percentage increase in paw thickness (A and C). Total oedema induced during the 3 h was calculated as area under the time course curves, AUC (B and D). *P < 0.04, ***P < 0.0001, †††P< 0.0004 when compared with vehicle-treated control mice. Arrow indicates point of Chlorpheniramine or XAE administration

Serotonin - induced paw oedema

The increased synthesis and release of vasoactive amines such as serotonin upon mast cell activation promotes vasodilation, capillary permeability and plasma exudation with subsequent development of oedema [23,24]. XAE (30, 100, 300 mg kg^{-1}) when administered before the induction of the serotonin-induced paw oedema caused the mean maximal swelling attained at 90 min to be reduced to 50.74 ± 5.30%, 39.81 ± 3.16% and 34.66 ± 3.35% respectively relative to the inflamed control response of 71.38 ± 5.61% (Fig 2A). The total oedema response induced over the 3 h were also significantly suppressed to 230.58 ± 21.08% and 190.92 ± 9.96% in the 100 mg kg^{-1} and 300 mg kg^{-1} XAE-treated groups respectively from 363.62 ± 34.54% (Fig 2B).

XAE (30, 100, 300 mg kg^{-1}) when given therapeutically suppressed the mean maximal swelling attained at 90 min to 44.00 ± 6.60%, 36.34 ± 3.47% and 30.31 ± 8.94% respectively relative to the inflamed control response of 71.38 ± 5.61% (Fig 2C). The total paw swellings induced over the 3 h were also dose-dependently and significantly suppressed to 233.48 ± 35.49%, 195.44 ± 18.58% and 153.22 ± 24.10% respectively from 344.94 ± 28.30% (Fig 2D).

Inhibition of serotonin-induced paw oedema indicates that XAE mitigates the inflammatory process, possibly via inhibition of peripheral 5-hydroxytryptamine 2 ($5\text{-}HT_2$) receptor family-mediated decrease in vascular resistance and increase in intravascular hydrostatic pressure [27]. These observed effects on histamine and serotonin are supported by the findings that, xylopic acid, isolated from XAE inhibits paw oedema induced by these mediators [19].

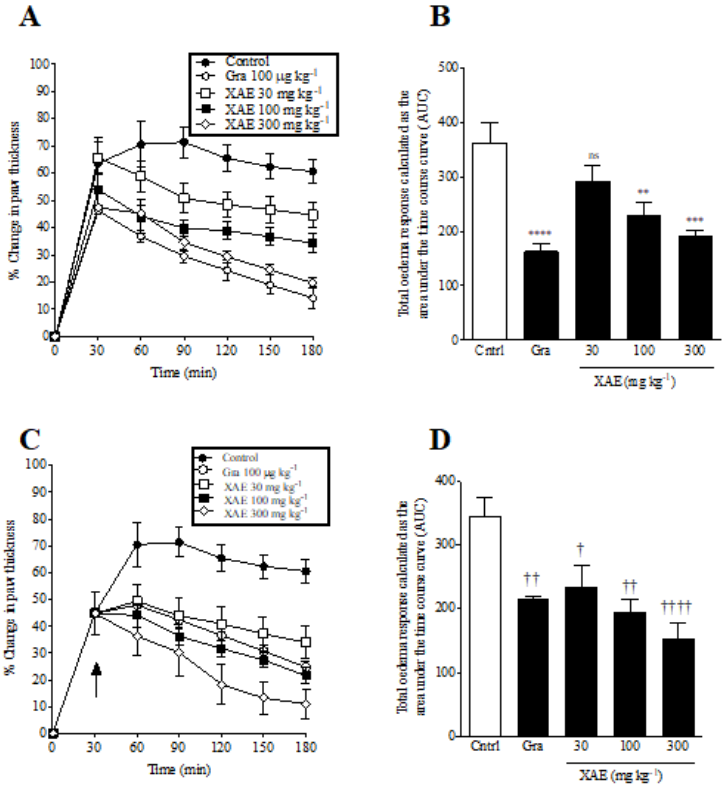

Figure 2. *Effect of Xylopia aethiopica* on serotonin-induced paw oedema in mice. Oedema was monitored at 30 min intervals over 3 h as percentage increase in paw thickness (A and C). Total oedema induced during the 3 h was calculated as area under the time course curves, AUC (B and D). **P = 0.0023, ***P = 0.002, ****P < 0.0001, nsP > 0.05, †P = 0.015, ††P < 0.0045, ††††P < 0.0001 when compared with vehicle-treated control mice. Arrow indicates point of Granisetron or XAE administration.

Bradykinin–induced paw oedema

Plasma mediators such as kinins, following their activation by selective proteolytic enzymes, initiate and amplify the inflammatory response via modulation of specific receptors and signaling pathways. Their activities on these targets control the nature and duration of tissue response to noxious stimuli [5]. One such mediator is the nonapeptide, bradykinin, released during the early stages of tissue damage by action of kallikreins on kininogens, the glycoprotein precursors [28]. In bradykinin-induced paw oedema, bradykinin acts on B_2 receptors which increases vascular permeability via nitric oxide-induced relaxation of perivascular smooth muscles [4]. In addition, kinins upon activation, up-regulate immune activation to enhance the inflammatory response. Kinins

also induce pain either directly or indirectly through the enhancement of the expression of PGE_2 and PGI_2 by endothelial cells and fibroblasts, and the production of tachykinins, ultimately aiding inflammation and subsequent tissue damage [29,30].

Results from the study shows that XAE (30, 100, 300 mg kg^{-1}) when administered before the induction of the bradykinin-induced paw oedema caused the mean maximal swelling attained at 90 min to be reduced to 18.13 ± 5.84%, 24.26 ± 2.88% and 14.31 ± 5.85% respectively relative to the mean inflamed control response of 45.22 ± 5.38% (Fig 3A). The total oedema induced over the 3 h were also significantly suppressed to 96.45 ± 25.66%, 106.11 ± 16.30% and 72.74 ± 20.35% respectively from 186.20 ± 23.58% (Fig 3B). Therapeutically, XAE (30, 100, 300 mg kg^{-1}) suppressed the mean maximal swelling attained at 90 min to 42.53 ± 6.60%, 36.42 ± 3.19% and 30.94 ± 9.03% respectively relative to the mean inflamed control response of 70.40 ± 4.74% (Fig 3C). The total oedema induced over the 2.5 h were also dose-dependently and significantly suppressed to 186.38 ± 24.84%, 170.06 ± 14.07% and 137.82 ± 20.91% from 279.40 ± 24.50% respectively (Fig 3D). Thus, *X. aethiopica* extract inhibits bradykinin-mediated inflammatory response possibly through inhibition of bradykinin B_2 receptor activity.

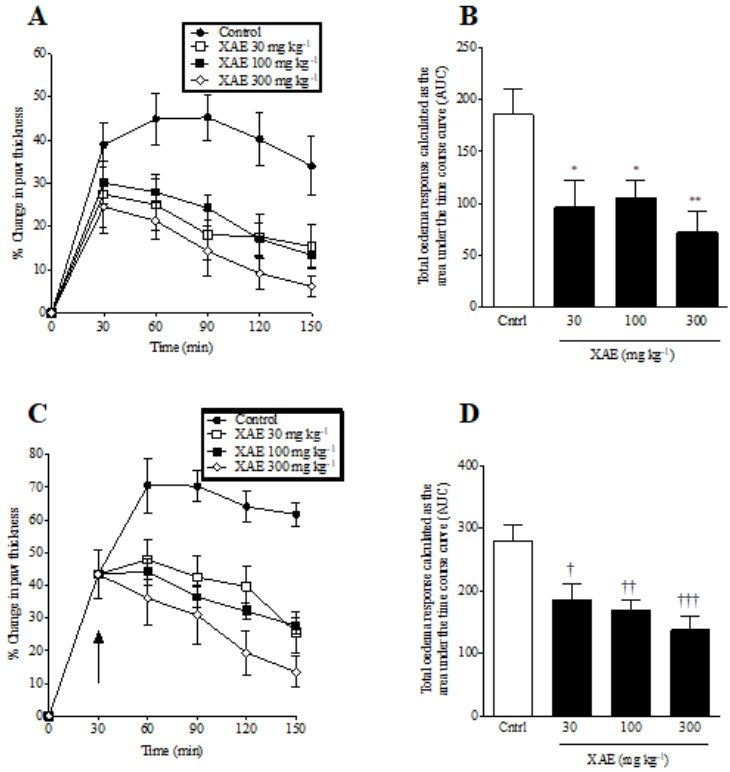

Figure 3. *Effect of Xylopia aethiopica* on bradykinin-induced paw oedema in mice. Oedema was monitored at 30 min intervals over 3 h as percentage increase in paw thickness (A and C). Total oedema induced during the 2.5 h was calculated as area under the time course curves, AUC (B and D). *P < 0.049, **P < 0.0054, †P = 0.02, ††P = 0.007, †††P = 0.007 when compared with vehicle-treated control mice. Arrow indicates point of XAE administration.

Prostaglandin E_2-induced paw oedema

During tissue injury, phospholipids and fatty acids from plasma membranes are metabolized by inflammatory cells and injured tissues into mediators and homeostatic regulators [31]. Unlike majority of inflammatory mediators, eicosanoids are not stored in granules after transcription and mRNA translation, but are produced from arachidonic acid metabolism following the inflammatory stimulus prior to enrollment of leukocytes and the infiltration of immune cells [4]. One such eicosanoid is Prostaglandin E_2 (PGE_2) which is very abundant and also plays critical role in mediating the cascade of events that culminates in classic inflammation signs, notably, arterial dilatation and increased vascular permeability and subsequent oedema [32].

Prophylactically, XAE (30, 100, 300 mg kg^{-1}) caused the mean maximal swelling attained at 60 min to be reduced to 34.53 ± 8.91%, 20.61 ± 7.29% and 18.32 ± 1.00% respectively compared with inflamed control response of 48.60 ± 3.68% (Fig 4A). The total oedema induced over the 2.5 h were also dose-dependently and significantly suppressed by 92.54 ± 35.49%, 77.10 ± 21.52% and 40.57 ± 3.01% respectively from 179.60 ± 21.87% (Fig 4B). In the therapeutic model, XAE (30, 100, 300 mg kg^{-1}) treatment suppressed the mean maximal swelling attained at 60 min to 39.66 ± 5.06%, 39.95 ± 3.51% and 27.93 ± 5.18% respectively compared with the inflamed control response of 61.63 ± 4.55% (Fig 4C) with the total oedema induced over the 2.5 h were also significantly suppressed to 149.92 ± 17.58%, 150.23 ± 15.25% and 131.71 ± 19.92% respectively from 241.82 ± 15.26% (Fig 4D).

The ability of XAE to inhibit paw oedema was partly mediated via interference of PGE_2 receptor activity due to the presence of xylopic acid [33]. As observed in this study, XAE inhibits the action of PGE_2 on E prostanoid receptors 1–4 (EP1–4) which may subsequently inhibit the activation of the rhodopsin-like G protein-coupled receptors [31,34]. In addition to the presence of xylopic acid, these findings may also be attributed to the presence of kaurenoic acid, another kaurane diterpene found in XAE. This kaurene diterpene is known from earlier findings to inhibit the inflammatory response via suppression of PGE_2 synthesis [35].

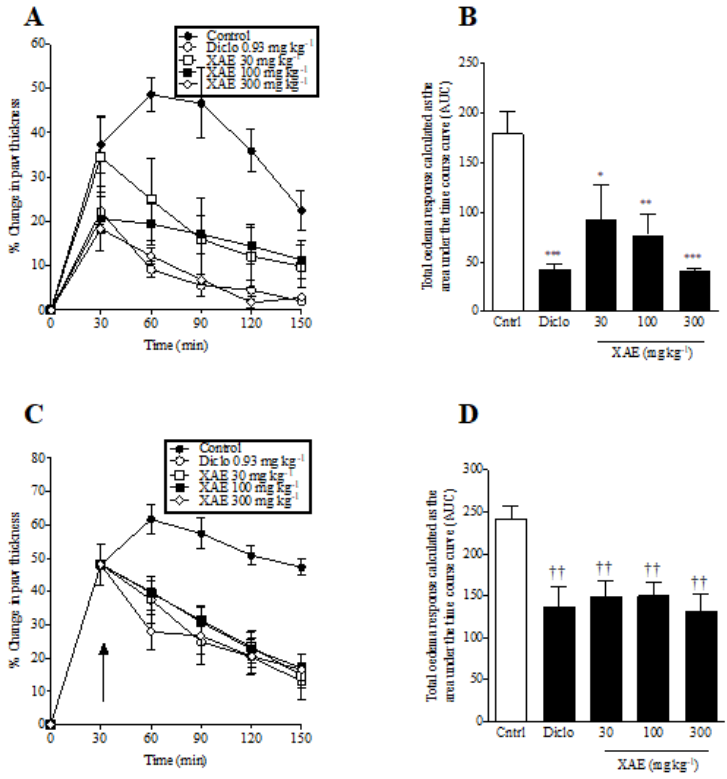

Figure 4. *Effect of Xylopia aethiopica* on prostaglandin E2-induced paw oedema in mice. Oedema was monitored at 30 min intervals over 2.5 h as percentage increase in paw thickness (A and C). Total oedema induced during the 2.5 h was calculated as area under the time course curves, AUC (B and D). *P = 0.03, **P = 0.01, ***P < 0.0007, ††P < 0.008 when compared with vehicle-treated control mice. Arrow indicates point of Diclofenac or XAE administration.

STATEMENT OF ETHICS

In accordance with internationally accepted principles for laboratory animal use and care (EEC Directive of 1986: 86/609 EEC), the animals were considerately handled throughout the experiment. Additionally, all animal experiments were approved by the Department Ethics Committee [Approval No. DPEC/FPPS/18/009. Valid from 1st June 2018 to 31st May 2019].

CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTION

NO, Conceived the idea of the experimental design and analysed the obtained the data; OKY, Performed the experiment and drafted the manuscript.

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Effects of Putrescine, Spermidine and Spermine on Growth and Serum Lipid Levels in *Sprague-Dawley* Rat Offspring

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ABSTRACT

Polyamines are short-chain, basic biogenic amines that are essential for cell growth and reproduction. This study was conducted to examine the effects of maternal polyamine intake on growth and serum lipid levels in first generation rat offspring. Female Sprague-Dawley rats (n=35) of 8 weeks old were used in the study. Rats were divided into five groups according to the polyamine they are fed as putrescine, spermidine, spermine, putrescine-spermidine-spermine, and control group. Before pregnancy, during pregnancy and lactation polyamines were administered to rats by oral gavage. After the offsprings were born, weights were measured every two days. Blood samples were taken when they were one month old and serum lipid analyzes were performed. When the groups were compared with the control group, it was shown that spermidine and spermine significantly increased the total cholesterol level, spermidine and putrescine-spermidine-spermine significantly decreased the triglyceride level and significantly increased the HDL level of the spermine. When birth weight averages and final weight averages were compared, it was seen that the group given putrescine-spermidine-spermine had the highest value. In conclusion, this study shows the effects of maternal polyamine intake on growth, total cholesterol, HDL, LDL and triglyceride levels of rat offsprings.

Key words: Polyamine, putrescine, spermidine, spermine, lipid profile

INTRODUCTION

Polyamines are short-chain, basic biogenic amines that are found in almost all living organisms and are necessary for cell growth and reproduction. Putresci-

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ne, spermidine and spermine consisting of arginine, ornithine, and methionine amino acids, are common polyamines found in eukaryotes[1-4].

Putresin, spermidine, and spermine are synthesized in mammalian cells. In addition, it can be taken into the body through diet and intestinal bacteria. Polyamine synthesis in tissues and organs decrease with aging[5]. Polyamine biosynthesis occurs in the G1 phase of the cell cycle and is required for process that initiate cell differentiation[6]. The first synthesis step of polyamines is the synthesis of ornithine, the precursor of polyamines, from the amino acid arginine. Synthesis of three important polyamines found in the cells of all mammals takes place starting from ornithine. Putrescine is synthesized from ornithine by means of ornithine decarboxylase enzyme. Spermidine is synthesized from putrescine by spermidine synthase and spermine is synthesized from spermidine by spermine synthase[4,7-11]. Polyamine levels in cells are altered by synthesis, catabolism, and transport[12,13]. Dietary polyamine, which is one of the important factors determining the amount of polyamine in the body, is absorbed in the small intestine before entering the systemic circulation, passed through the duodenum and proximal jejunum lumen into the blood by passive diffusion to the whole body and used for cell growth[9,14]. High levels of polyamine are found in a variety of foods and beverages such as broccoli, mushrooms, green peppers, rice bran, green tea, mushrooms, soybeans and oranges[14]. Polyamines are also synthesized microbially in the intestine. This synthesis varies according to the type of microorganisms[12].

In fast-growing tissues, metabolic polyamine requirement is quite high during normal growth and development[15]. Polyamine biosynthesis is therefore high in conditions requiring rapid cell growth such as neonatal period and newborn period[11,16]. When polyamine is absent or insufficient in the cell, cell proliferation is inhibited and sometimes results in cell death. In case of inhibition of enzymes involved in polyamine biosynthesis, cell growth slows down[17]. Polyamine deficiency may have negative effects on reproduction. Therefore, it is recommended that diets rich in polyamine be used in reproductive studies. It is recognized that polyamines are essential in reproductive processes and have an important role in embryo and placental development[18]. ODC activity in the placenta is much higher than in the fetus. However, the increase in the amount of polyamine in the fetus is significantly higher than in the placenta. With this increase, the growth rate of fetal tissue increases[13]. Polyamines are important for the uterus during pregnancy during myometrial cell proliferation and development of hypertrophy. Spermine has a relaxing effect on the contraction of the uterus. It also exerts a similar effect by reducing intracellular calcium concentration in vascular smooth muscle[19].

Breast milk, which is the main source of newborns, contains putrescine, spermidine and spermine. While spermidine and spermine are found in similar concentrations in human milk, both decrease during lactation period. In rat milk, spermidine is found more than spermine and increases in lactation period.

Polyamines have an important role in the growth and development of the digestive system of newborn mammals. It is also important for normal growth and maintenance of the general characteristics of the adult digestive system. Spermidine and spermine have effects such as structural and functional development of the small intestine, enlargement of villi, elongation of crypts, cell proliferation, increase in maltose and sucrose activity, and decrease in lactase activity in rats[20].

Because polyamines are cationic, they tend to interact with anionic structures such as nucleic acid, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), adenosine triphosphate (ATP), some proteins, and phospholipids. These interactions are essential to the biological functions of polyamines. It stabilizes the negative charges of phosphate groups on DNA. It acts on protein synthesis by affecting the secondary structure of RNA and by binding to ribosomes and bringing its subunits together[3,14,21-23]. It also plays an important role in cell growth, proliferation, differentiation, development, immunity, migration, gene transcription, gene expression, and DNA stability as well as nucleic acid and protein synthesis[3,9,13,14,24,25].

Polyamines (3-6%) bind to the membrane with anionic phospholipids and stabilize the membrane. In addition, the polyamines in the serum interact by binding with anionic phospholipids on the surface of proteins and lipoproteins[1]. Thus, lipid peroxidation is inhibited by polyamines. Spermine, spermidine and putrescine, respectively, have a strong antioxidant effect on LDL oxidation. This is due to the ability of polyamines to form complexes with Cu^{2+} and is positively correlated with the number of amine groups of these molecules[26].

According to all this information, polyamines have effects on both growth and development and serum lipid levels. Therefore, in this study, it was aimed to investigate the effects of maternal polyamine intake on growth and serum lipid levels in first generation offspring rats.

METHODOLOGY

The study was approved by Istanbul Medipol University Animal Experiments Local Ethics Committee, number 38828770-604.01.01-E.706, decision number 10 and dated 14.01.2016, ethical rules were followed during the study.

Experimental Animals

This study was carried out in Istanbul Medipol University Medical Research Center (MEDITAM). *Sprague-Dawley* rats used in the study were obtained from the same center.

Female *Sprague-Dawley* rats (n=35), 8 weeks old, weighing an average of 156 grams (between 128-182 grams) were used in the study. The number of rats was kept to a minimum in accordance with the literature[27]. All rats were kept at standard environmental conditions (20-22°C temperature, 55-65% humidity and 12 hours night - 12 hours day), and standard pellet feed and fed *ad libitum* with tap water. Marking was made on rats by tail staining method so that each rat could be followed.

Study Design

After adaptation period, the rats were randomly divided into five groups. Tap water for control was given to the first group, putrescine to the second group, spermidine to the third group, spermine to the fourth group, putrescine, spermidine and spermine to the fifth group through oral gavage. The offspring of the rats given different substances were divided into five groups as follows:

- Group 1 (G1): offspring rats born and fed to mothers given water by oral gavage (control)
- Group 2 (G2): offspring rats born and fed to mothers given putrescine by oral gavage
- Group 3 (G3): offspring rats born and fed to mothers given spermidine by oral gavage
- Group 4 (G4): offspring rats born and fed to mothers receiving spermine by oral gavage
- Group 5 (G5): offspring rats born and fed from mothers given putrescine, spermidine and spermine by oral gavage

Dietary intervention

The rats in the control group were fed only with pellet feed during the experiment. The rats in the other groups were fed dietary polyamine as well as pelleted feed.

Putrescine [Putrescine dihydrochloride, 98.0% (TLC), 25g, Sigma], spermidine [Spermidine trihydrochloride, ≥99.5% (AT), 5g, Sigma] and spermine [Spermine tetrahydrochloride, ≥ 99.5% (AT), 1g, Sigma] was dissolved in distilled water. The amounts given to rats were calculated as 90 μg/g putrescine, 41.5 μg/g spermidine, 9.5 μg/g spermine. The amount of putrescine, spermidine and spermine given to rats is similar to previous studies and does not exceed the no observed adverse effect level (NOAEL) in experimental animals[27].

Before oral gavage, the amount of polyamine to be given was calculated by measuring the weights of all rats with precision weighing. Oral gavage was performed every three days for four weeks before pregnancy. At the end of one month, all rats were mated for a week and oral gavage was not performed during this period. Oral gavage was performed every three days during pregnancy (three weeks) and every two days during lactation (four weeks). The reason for not being able to perform oral gavage every day is the possibility that rats may harm their health by irritating the esophagus. The rats in the control group were exposed to the same stress as the rats in the other groups by oral gavage with water. Offspring rats were fed with breast milk for four weeks. Thus, polyamines given to mother rats by gavage were passed on to the newborn through breast milk.

Analyzing blood samples

One month after birth, the offspring rats were anesthetized by intraperitoneal administration of Rompun (Xylazine) (2%) and Ketasol (Ketamine) (10%), and 1 mL of blood samples were taken from the jugular vein. The blood samples taken were centrifuged at 3000 rpm for 10 minutes at +4°C within 30 minutes, the serum was separated and transferred into eppendorf tubes and kept at -20°C until analyzed.

Determination of serum lipid levels

Serum samples were analyzed in the Biochemistry Laboratory of Medipolitan Sağlık ve Eğitim Hizmetleri A.Ş. by applying standard methods, and total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol and triglyceride (TG) levels were determined. Cholesterol value was analyzed by enzymatic method, HDL and triglyceride value by colorimetric method, LDL value calculation method [LDL=Cholesterol-(TG/5+HDL)][28] Cobas 6000 (Roche, Tokyo, Japan) by working in biochemistry autoanalyzer.

Termination of the study

The adult and offspring rats were given anesthesia by intraperitoneal injection of Rompun and Ketasol. The study was terminated by performing cervical dislocation as a result of general anesthesia. Adult rats were euthanized after 4 weeks of lactation, and offspring rats 4 weeks after birth.

Calculation of specific growth rates

The first weight of newborn rats was measured and recorded one day after birth. Weight measurement was continued every two days for a month. Since the offspring in the groups could not be marked individually, weight tracking could not be made individually, so the total weight averages were recorded as group average.

The individual growth rate was calculated as follows[29,30].

Specific growth rate (%) = [(ln w2–ln w1)/days]x100

w1: initial body weight (gram); w2: final body weight (gram); days: number of days between recordings of w1 and w2.

Statistical analysis

All analysis was performed using IBM SPSS Statistics, v.18.0. The normality of the distribution of the variables was evaluated with the Kolmogorov-Smirnov test. When comparing more than two groups, we used analysis of variance (ANOVA) with one factor or the nonparametric Kruskal Wallis test. Differences between groups were analyzed using the Tukey test when the variances of the groups were homogeneous and the Tamhane's T2 test when they were not homogeneous. P values below 0.05 were considered statistically significant. The data are presented as the mean ± standard deviation (SD).

RESULTS AND DISCUSSION

The average of the first and last weights and specific growth rates of the offspring rats according to the groups are shown in Table 1. It was determined that the group with the highest first and last birth weight averages was G5, and the group with the highest specific growth rate was G4.

Table 1. Weight averages and specific growth rates of offspring rats

	*Initial weight, gram	*Final weight, gram	Specific growth rate, %
G1 (n=34)	5.77	63.61	8.00
G2 (n=36)	5.65	71.44	8.46
G3 (n=50)	5.70	63.63	8.04
G4 (n=16)	7.02	90.30	8.52
G5 (n=9)	7.44	93.22	8.43

G1, Group 1; G2, Group 2; G3, Group 3; G4, Group 4; G5, Group 5

*Results are expressed as the mean

Offspring body weight change

Body weight changes of offspring rats are shown in Figure 1. Growth and development of offspring rats differ according to the polyamines they receive from their mothers via the placenta. When the data are examined, it is seen that the offspring in the G1 group reach the lowest weight and the ones in the G5 group reach the highest weight.

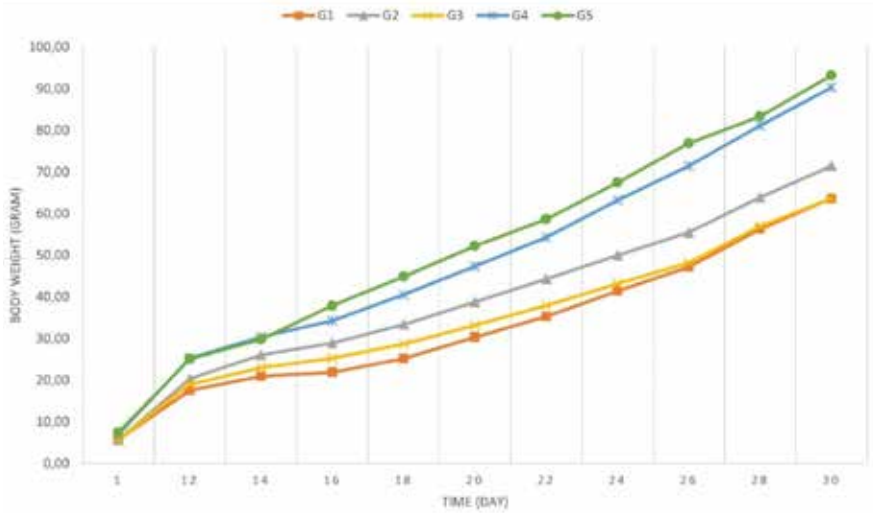

Figure 1. Body weight change of offspring during growth

G1, Group 1; G2, Group 2; G3, Group 3; G4, Group 4; G5, Group 5

Body weights are expressed as the mean

Changes in serum lipid levels

Total cholesterol, triglyceride, HDL, and LDL cholesterol levels of offspring rats according to the type of polyamine taken from both placenta and breast milk are shown in Table 2. When the data obtained were examined, it was found that there were significant differences between groups are shown in Table 3 in terms of total cholesterol, HDL cholesterol and triglycerides.

Table 2. Results of serum lipid levels of offspring

	Total cholesterol mg/dL	Triglyceride mg/dL	HDL cholesterol mg/dL	LDL cholesterol mg/dL
G1	51.77±6.23^{ab}	64.21±17.12^{fg}	45.69±5.27^{j}	0.07±0.26
G2	48.28±6.43^{cd}	55.54±13.27^{h}	41.73±5.80^{klm}	0.34±1.07
G3	56.90±8.02^{ac}	44.02±13.31^{fhi}	48.46±6.28^{kn}	1.55±2.77
G4	62.16±6.51^{bde}	60.20±12.64^{i}	57.57±5.72^{ilno}	0.50±1.37
G5	50.86±5.23^{e}	49.12±10.09^{g}	48.16±5.78^{mo}	0.00±0.00
p	<0.001*	<0.001*	<0.001*	0.055$^{£}$

G1, Group 1; G2, Group 2; G3, Group 3; G4, Group 4; G5, Group 5; HDL, high-density lipoprotein; LDL, low-density lipoprotein

Results are expressed as the mean ± SD

$^{a, b, c, d, e, f, g, h, i, j, k, l, m, n, o}$: Means that have superscript in common are significantly different from each other.

******One-Way-Anova Test*

£*Kruskal Wallis Test*

Table 3. Statistical results (p values) of total cholesterol, triglyceride, and HDL levels between groups

		G1	G2	G3	G4	G5
Total Cholesterol	**G1**	-	0.294	**0.018***	**<0.001*****	0.997
	G2	0.294	-	**<0.001*****	**<0.001*****	0.863
	G3	**0.018***	**<0.001*****	-	0.074	0.125
	G4	**<0.001*****	**<0.001*****	0.074	-	**0.001****
	G5	0.997	0.863	0.125	**0.001****	-
Triglyceride	**G1**	-	0.117	**<0.001*****	0.888	0.043*
	G2	0.117	-	**0.004****	0.812	0.742
	G3	**<0.001*****	**0.004****	-	**0.001****	0.853
	G4	0.888	0.812	**0.001****	-	0.322
	G5	**0.043***	0.742	0.853	0.322	-
HDL	G1	-	0.069	0.271	**<0.001*****	0.805
	G2	0.069	-	**<0.001*****	**<0.001*****	**0.034***
	G3	0.271	**<0.001*****	-	**<0.001*****	1.000
	G4	**<0.001*****	**<0.001*****	**<0.001*****	-	**0.002****
	G5	0.805	**0.034***	1.000	**0.002****	-

G1, Group 1; G2, Group 2; G3, Group 3; G4, Group 4; G5, Group 5; HDL, high-density lipoprotein

p *<0.05 **<0.01 ***<0.001

Polyamines are essential for various reproductive processes such as early embryogenesis, implantation, placental growth, and angiogenesis[9,10,18]. Polyamines found in mammalian placentas are key regulators of DNA synthesis, protein synthesis, cell proliferation and cell differentiation. Beneficial changes in the metabolic profiles of polyamines during pregnancy can have a long-term effect on postnatal growth and metabolism. Maternal protein intake during pregnancy and lactation is an important factor affecting fetal and neonatal development, child health and diseases in all life[11,18,25].

Putrescine functions as a growth factor in the rat intestine and can directly induce DNA, RNA, and protein synthesis. Spermine regulates growth responses[14]. Spermidine is the unique hypusine (N^{δ}-4-amino-2-hydroxybutyl(lysine)) of eukaryotic translation initiation factor 5A (eIF5A), which is necessary for protein synthesis and growth is included as a substrate for the modification[9,14].

During pregnancy, polyamine levels are high in plasma and urine as well as in amniotic fluid. Especially the 11-14th week of pregnancy. Putrescine, spermidine and spermine levels are at the highest level in amniotic fluid[31]. Plasma putrescine, spermidine and spermine levels gradually increase by the third trimester of pregnancy and reach the highest concentration at the end of pregnancy[32].

Ornithine decarboxylase (ODC) is the rate limiting enzyme in polyamine biosynthesis. ODC activity is also increased to increase polyamine concentration, especially during embryogenesis and cell growth. This enzyme activity changes rapidly against hormonal stimulus. Progesterone an important hormone in pregnancy, controls the increase in ODC activity associated with cell proliferation activity[9,33]. ODC level is found more in the placenta than the fetus[13]. The role of putrescine synthesis in the fetus is important because putrescine is required for spermidine and spermine conversion. ODC and polyamine synthesis are very important for embryo development as it increases the growth rate in tissues.

In a study with mice[13], when the polyamines found in mouse placenta, yolk sac and fetus in the second half of gestation were examined, it was determined that spermidine was synthesized the most. In maternal deficiency of arginine, which is the precursor of polyamines, supplementation with dietary arginine is effective in positive change in embryonic and fetal survival and growth[9,11,34]. In our study, when we look at the first weights measured after birth (Table 1), it was determined that the offspring born from rats that received three polyamines together with the diet were the largest. This suggests that this may be different from polyamine synthesis under normal conditions since dietary polyamine is taken during pregnancy.

Putrescine, spermidine and spermine are found in breast milk, which is the main food source of newborn. While spermidine and spermine are found in similar concentrations in human milk, both decrease during the lactation period. While there is more spermidine than spermine in rat milk, its amount increases during the lactation period. Polyamines play an important role in the growth of newborn mammals. It is also important for normal growth and maintenance of the general characteristics of the adult digestive system[20,36-37].

Analysis of growth data shows that arginine plays a very important role in postnatal development and that arginine in the diet is responsible for growth in infancy. Arginine deficiency in the diet can restrict the growth and development of newborn animals. Because the amount of arginine obtained from breast milk and its own synthesis often cannot meet nutritional requirements[34,38,39]. In a study in which neonatal rats were given oral spermidine and spermine[20], no significant difference was observed between the control group and the body weight of rats given polyamine It is thought that this result may be caused by the lack of polyamine synthesis as a result of feeding a diet containing insufficient spermidine and spermine. When the last weight measurements of the offspring in our study are examined **(**Table 1**)**, it was observed that the offspring born from rats that received three type of polyamine in the diet during lactation had the highest weight, and the offspring born from rats that did not take polyamine had the lowest weight. When the specific growth rate was examined, it was determined that the offspring born from rats that did not take polyamine was the lowest. This shows how important dietary polyamine intake is in neonatal growth. However, since the group averages were taken for each weight measurement in our study, it could not be determined whether the result we obtained was significant.

Polyamines that are polycationic are bound with phospholipids that are polyanionic at physiological pH. Polyamines stabilize the membrane by binding to the membrane with anionic phospholipids. The amount of spermine (2.3 mol/100 mol) bound to phospholipids is greater than the amount of spermidine (0.23 mol/100 mol)[1,23]. Cationic polyamines in serum interact by binding with anionic phospholipids on the surface of lipoproteins called HDL and LDL[40]. In recent years, polyamines have been shown to be powerful antioxidant agents as they protect cellular components such as polyunsaturated fatty acids in membranes from oxidative damage[14,41]. Of the polyamines, especially spermine has a strong antioxidant effect on human LDL oxidation compared to α-tocopherol, followed by spermidine and putrescine[26]. In our study, it was determined that the HDL level of the group given spermine was significantly higher than the other groups, and it is in accordance with the literature. However, it was shown that there was no statistically significant difference in LDL levels between the groups.

Methionine and arginine are required for polyamine biosynthesis. In a study examining the serum lipid profiles of broiler chickens fed with different levels of arginine[42], it was found that the triglyceride level was significantly lower compared to the control group. In another study with broiler chickens[43], serum

triglyceride levels were found to be high in 40-day-old broiler chickens fed a diet containing insufficient methionine. Similarly, Ebrahimi et al.[44] observed that the expression of the gene encoding FAS was decreased when L-arginine was added to the diet of broiler chickens. In addition, in the study by Filho et al. [45], it is estimated that the effects of dietary L-arginine on lowering serum triglyceride and total cholesterol levels are associated with decreased gene expression of FAS and HMG-CoA. These results are in line with our study. In other words, the triglyceride level of the G1 group is significantly higher than that of the G3 and G5 groups.

In a study with treated diabetic rats, a significant increase in triglyceride, HDL and LDL levels was found as a result of feeding spermine for five months[46]. In our study, when the groups that were given spermine and control were examined, a significant increase in total cholesterol and HDL levels (respectively $p < 0.001$, $p < 0.001$), a decrease in triglyceride level ($p = 0.888$) (Table 3) and an increase in LDL ($p = 0.927$) were observed. This result suggests that rats with diabetes may have been using spermine for a much longer time. Also, maternal spermine intake and direct dietary spermine intake are likely to have different results on serum lipid levels.

In conclusion, this study demonstrated the efficacy of maternal polyamine intake on growth and development and serum lipid levels in first generation offspring rats. Positive effects on growth and development of rat offspring were observed as a result of using polyamines both separately and together. In addition, when the serum lipid levels of the offspring were examined, significant differences were observed between groups and the effectiveness of polyamines on total cholesterol, triglyceride, HDL, and LDL was demonstrated. These results are proof that the dietary polyamines are passed on to offspring through the placenta and breast milk.

STATEMENT OF ETHICS

The study was approved by Istanbul Medipol University Animal Experiments Local Ethics Committee.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Fatma Mert-Biberoğlu and Nihal Büyükuslu. The first draft of the manuscript was written by Fatma

Mert-Biberoğlu and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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