ACTA PHARMACEUTICA SCIENCIA

International Journal in Pharmaceutical Sciences, Published Quarterly

ISSN: 2636-8552
e-ISSN: 1307-2080,
Volume: 59, No: 4, 2021
Formerly: Eczacılık Bülteni / Acta Pharmaceutica Turcica
Founded in 1953 by Kasım Cemal GÜVEN

ACTA PHARMACEUTICA SCIENCIA

International Journal in Pharmaceutical Sciences
is Published Quarterly
ISSN: 2636-8552
e-ISSN: 1307-2080,
Volume: 59, No: 4, 2021
Formerly: Eczacılık Bülteni/Acta Pharmaceutica Turcica
Founded in 1953 by Kasım Cemal Güven

Editor
Şeref Demirayak
Associate Editors
Gülden Zehra Omurtag
Barkın Berk
Zafer Şahin
Publication Coordinator
Sevde Nur Biltekin Kaleli
Ayşegül Çaşkurlu
Büşra Işıl Tok
Language Editor
Recep Murat Nurlu
Mert Sarsağ
Neda Taner
Biostatistics Editor
Pakize Yiğit
Editorial Board
Complete list of editors and reviewers can be found on http://www.actapharmsci.com/static.php?id=2
Art Director
Levent Karabağlı - Medicomia
Graphic-Design
Sertan Vural - Medicomia

Address
İstanbul Medipol Üniversitesi
Kavacık Güney Kampüsü
Göztepe Mah. Atatürk Cad.
No: 40 34810 Beykoz/İSTANBUL
Tel: 0216 681 51 00
E-mail
editor@actapharmsci.com
secretary@actapharmsci.com
Web site
http://www.actapharmsci.com
Printing Office
Has Kopyalama Baskı ve Kırtasiye A.Ş
Kavacık Mah. Ekinciler Cad. No:19
Medipol Üniversitesi Kuzey Yerleşkesi
Tel: (0216) 681 53 72

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. Years of the references should be written boldly. More than one reference from the same author(s) in the same year must be identified by the letters "a", "b", "c", etc., placed after the year of publication. References should conform to APA style and be numbered consecutively in the order in which they are cited in the text.

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

1. Scope and Editorial Policy

1.1 Scope of the Journal

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

The original studies accepted for publication must be unpublished work and should contain data that have not been published elsewhere as a whole or a part.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

All manuscripts has to be written in clear and concise English. Starting from 2016, the journal will be issued quarterly both in paper and 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 statement that study samples were obtained through the informed consent of the donors, or in lieu of that evidence, by the authority of the institutional board that licensed the use of such material. Authors are requested to declare the identification or case number of institution approval as well as the name of the licensing committee in a statement placed in the section describing the studies' Material and Methods.

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

1.6 Issue Frequency

The Journal publishes 4 issues per year.

2. Preparing the Manuscript

2.1 General Considerations

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

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

It is best to use the font "Times". 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 experimental descriptions. General reaction, isolation, preparation conditions should be given only once. The title of an experiment should include the chemical name and a bold Arabic identifier number; subsequently, only the bold Arabic number should be used. Experiments should be listed in numerical order. Molar equivalents of all reactants and percentage yields of products should be included. A general introductory section should include general procedures, standard techniques, and instruments employed (e.g., determination of purity, chromatography, NMR spectra, mass spectra, names of equipment) in the synthesis and characterization of compounds, isolates and preparations described subsequently in this section. Special attention should be called to hazardous reactions or toxic compounds. Provide analysis for known classes of assay interference compounds.

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

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

2.2.6 Ancillary Information. Include pertinent information in the order listed immediately before the references.

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

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

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

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

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

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

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

2.2.7 References and Notes. 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. APA 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.

APA Style examples:

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

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

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

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

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

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

below for TIFF file production resolutions.

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

- Black & White line art: 1200 dpi

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

- Color art (RGB color mode): 300 dpi

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

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

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

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

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

2.3 Specialized Data

2.3.1 Biological Data. Quantitative biological data are required for all tested compounds. Biological test methods must be referenced or described in sufficient

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

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

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

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

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

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

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

2.3.2 Purity of Tested Compounds.

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

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

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

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

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

3. Submitting the Manuscript

3.1 Communication and log in to Author's Module All submissions to Acta Pharmaceutica Sciencia should be made by using e-Collittera (Online Article Acceptance and Evaluation) system on the journal main page (www. actapharmsci. com)

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

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

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

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

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

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

3.2.2 Institutions Authors should give their institutional information during submission.

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

3.2.4 Title should be English, explaining the significance of the study. If the title includes some special characters such as alpha, beta, pi or gamma, they can easily be added by using the **Title** window. You may add the character by clicking the relevant button and the system will automatically add the required character to the text.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.3.4.1 Articles Not Completed to be Sent After the sending transaction was started, if article is not able to continue until the ninth step or could not be sent due to technical problems shown at this part. Here you can find the information such as the article's number which is assigned by system, title and formation date. You may delete the articles by using **Delete** button on the right column, if the article is not considered to send into the system.

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

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

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

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

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

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

Article review process

Articles uploaded to the Manuscript submission system are checked by the journal administration for format consistency and similarity rate which is required to be less than 20%. Then sent to the chief editor if found appropriate.

Articles that are not suitable are sent back to the author for correction and re-submit (sent back to the author). Studies that have not been prepared using the draft for submitting to Acta Pharmaceutica Sciencia "acta_msc_tmp" and that have not been adapted in terms of format, will be directed to the editor-in-chief, after the 3rd time, by giving the information that "the consistency requirements have not been met".

The manuscripts sent to the chief editor will be evaluated and sent to the "language and statistics editor" if deemed appropriate.

Studies found appropriate after language-statistics editor will be sent to field editors. If the field editor does not deem it appropriate after evaluating the article scientifically, he/she will inform the editor-in-chief of its negative comments, otherwise, at least two independent referee comments will be asked.

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

Our review system is double-blind. The editor, who evaluates according to the comments of the referees, submits his/her comment and suggestion to the editor-in-chief. In this way, the article takes one of the acceptance, rejection, or revision decisions. In the case of revision, after the author revises, the editor submits his/her final opinion to the editor in chief. Editor-in-Chief conveys his final decision to the author. After the accepted articles are subjected to the final control by the journal and the corresponding author, the article starts to be included in the "accepted papers" section by giving the inactive DOI number. When the article is placed in one of the following issues, the DOI number will be activated and displayed in the "current issue" section on the journal homepage.

ORIGINAL ARTICLES

Acta Pharm. Sci. Vol 59:(4), 2021
DOI: 10.23893/1307-2080.APS.05931

Correlation Between Oxidant and Antioxidant Status (MDA and SOD Expression) and the Improvement of Outer Hair Cells Function Due to Curcumin Administration in Noise-Exposed Rattus Norvegicus Cochlea Assessed by DPOAE Examination

Tengku Siti Hajar HARYUNA[1*], Reastuty REASTUTY[1], Tengku Siti Harilza ZUBAIDAH[2], Delfitri MUNIR[1], Wibi RIAWAN[3], Juliandi HARAHAP[4]

1 Department of Otorhinolaryngology, Faculty of medicine, Universitas Sumatera Utara, Medan, Sumatera Utara, 20155, Indonesia

2 Department of Ophtalmology, Faculty of medicine, Universitas Sumatera Utara, Medan, Sumatera Utara, 20155, Indonesia

3 Department of Biochemistry and Molecular Biology, Faculty of medicine, Brawijaya University, Malang, East Java, 65145, Indonesia

4 Department of Community Medicine, Faculty of medicine, Universitas Sumatera Utara, Medan, Sumatera Utara, 20155, Indonesia

ABSTRACT

NIHL caused by excessive noise exposed to cochlea. Curcumin is a medicine that has antioxidant effects. The purpose of this study was to look at the effect of curcumin on NIHL which was assessed with DPOAE, value and the expression of SOD and MDA. This research was conducted on 36 rats which were divided into 6 groups, group 1 was a control group, group 2 was a group receiving only noise treatment, groups 3 and 4 were the treatment group receiving curcumin after exposed to noise, groups 5 and 6 were preventive groups receiving curcumin starting from 14

* Corresponding author:
Address: Department of Otorhinolaryngology, Faculty of medicine, Universitas Sumatera Utara, 5 dr. Mansur street, Medan, Sumatera Utara, Indonesia. 20155
email: tengkusitihajarharyuna@gmail.com / tengkusitihajarharyuna@usu.ac.id
Mobile : +62812606194
ORCID Number :
Tengku Siti Hajar Haryuna : 0000-0003-2779-3285
Reastuty : 0000-0003-4608-8629
Tengku Siti Harilza Zubaidah : 0000-0002-9433-0873
Delfitri Munir : 0000-0002-8526-6398
Wibi Riawan : 0000-0001-5027-5051
Juliandi Harahap : 0000-0002-1090-2003
(Received 07 Oct 2020, Accepted 29 Mar 2021)

days before their get noise. The study result showed the correlation between SOD and MDA expression and DPOAE value due to curcumi*n* Administration in Noise-Exposed rats Cochlea. The conclussion of the study showed that curcumin was able to change the value of DPOAE, SOD and MDA expressions in rats exposed to noise.

Keywords : Curcumin, noise, cochlea, antioxidants, superoxide dismutase

INTRODUCTION

Nowadays there is a lot of noise at work that causes workers to suffer from hearing loss due to work. Besides environmental noise also increased, this resulted in around 1-1.6 million people in western Europe or 1.1 million world population suffer from Noise Induced Hearing Loss (NIHL) every year. The UN World Health Organization estimates that 1.1 billion people in the world are at risk of suffering from NIHL because of the fallacy in listening behavior. In high and middle income countries, adolescents and young adults aged 12-35 years are exposed to excessive sound through personal audio devices and around 40% of them occur in clubs, discos and bars[1].

Noise will cause a shearing force that results in changes in the stereocilia of hair cells outside the cochlear basilar membrane and cell death will occur if the process occurs excessively[2]. Studies in animals exposed to noise showed anatomic changes occur ranging from stereocilia distortion of outer and inner hair cells to loss of cortical organs and rupture of the Reissner membrane. In general, changes in blood vessels, spirals or limb ligaments are not found. Stria vascular edema occurs a few minutes after exposure to noise and persists for several days[3]. Noise can cause Temporary Treshold Shift (TTS) or Permanent Threshold Shift (PTS). Generally, in TTS, the hearing will return to normal within 24-48 hours[4]. The cause of cortical organ damage can occur in two mechanisms, namely mechanical damage and continuous metabolic activity[1].

Many studies carried out over nearly 40 years have shown that otoaccoustic emission (OAE) is useful for determining the differential diagnosis of sensorineural hearing loss; cochlear screening of infants, toddlers and other patients that are difficult for hearing tests; assess outer hair cells in patients exposed to excessive noise, ototoxic or other progressive disease sufferers[5]. One type of OAE examination is Distortion Product Otoacoustic Emission (DPOAE), this examination is very suitable and sensitive to assess ear responses to dangerous stimuli such as toxic, trauma and cochlear degenerative processes[6].

Oxidative stress due to noise exposure damages the antioxidant defense mechanism in the cochlea so that Reactive Oxygen species (ROS), Reactive Nitrogen Species (RNS) and other free radicals are formed excessively, to overcome this

detoxification process occurs. This defense mechanism consists of enzymes and antioxidants. Enzymes such as Superoxide Dismutase (SOD) catalyze the removal of O2- to H_2O_2, Glutathione peroxidase (GPx), Glutathione reductase (GR) and Catalase (CAT)[7]. Oxygen free radicals can attack proteins, nucleic acids and lipid membranes that disrupt the normal function and integrity of cells. An increase in neurotransmitters in the brain area after exposure to noise and can persist for up to 15 days after exposure. In addition there is also an increase in Malondialdehyde (MDA) as the end result of lipid peroxidation[8] and is a marker for damage caused by free radicals[9].

In the future prevention and treatment efforts for NIHL can be increased by the use of chemoprotective agents such as antioxidants and identification of risk factors[2]. Turmeric (*Curcuma longa Linn*) is one of the most well-known herbal medicines and has many pharmacological activities such as antioxidants, anti-inflammation, anti-tumor, anti-proliferation, anti-angiogenic, anti-aging, antiprotozoa, anti-microbial, and anti-malaria[10]. Turmeric has the main active components namely *curcuminoids*, *monoterpenoids* and *sesquiterpenoids*[11]. Whereas *curcuminoids*, consisting of *curcumin*, *demethoxycurcumin* and *bisdemethoxycurcumin*[10,11].

Previous studies have shown that curcumin has the effect of preventing and treating the damage of supporting fibroblast cells and the lateral wall of the cochlea due to noise exposure with increased SOD and CAT expression[12] and decreased MDA and H_2O_2 expression[13].

This study is different from the previous studies, in this study we want to prove that curcumin is able to cause changes in oxidant and antioxidant status in hair cells / cochlear cortical organs so that the outer hair cell function improves as evidenced by DPOAE examination in rats exposed to noise.

METHODOLOGY

This study used a randomized post test only control group laboratory experimental design. This study founded by Research Institute of the Universitas Sumatera Utara under the TALENTA Research Implementation Contract of the Universitas Sumatera Utara 2018 fiscal year No: 2590 / UN.5.1.R / PPM / 2018 on March 16, 2018.

Ethical Approval

Before the study was conducted, this study approved ethical clearance from the ethics research institute of the Faculty of Medicine, Universitas Sumatera Utara with number 509 / TGL / KEPK FK USU-RSUP HAM / 2018.

The Groups of The Study

The study was conducted on 36 male *Rattus norvegicus* pure strain rats, weighing 200-300 grams and declared healthy by veterinarians. All rats were treated in a polycarbonil cage to ensure the temperature of the cage remained at 20°C-26°C with humidity around 30-70%. It is ensured that all rat receive sufficient light sources and get adequate food and drink[14].

This study was divided into 6 groups, each consisting of 6 rats. The first group is a control group without administration of curcumin and noise exposure. Group 2 is a group of rats with only noise exposure without curcumin. Group 3 and group 4 are curative groups. Group 3 is a group with noise exposure and curcumin 100 mg / day for 2 days after exposure to noise. Group 4 is a group with noise exposure and curcumin 200 mg / day for 2 days after giving noise exposure. Group 5 is a preventive group that starts with 100 mg of curcumin / day for 2 weeks followed by noise exposure and 100 mg / day of curcumin for 2 days. Group 6 is a preventive group that starts with 200 mg / day of curcumin for 2 weeks followed by noise recording given at a frequency of 1-10,000Hz with an intensity of 110 dB for 2 hours / day, for 2 days. Noise treatment is carried out by placing rats on a box (with a size of 64.5 x 45 x 40) cm made of cork coated by foam, speakers are placed attached to the roof of the box cover and a hole is made at the base of the box to measure the intensity, measurements are made at eight points where the cage will be placed with a noise difference not exceeding 1 dB using a sound level meter.

Procedures of DPOAE examination.

Examination of DPOAE using Distortion Product Otoacoustic Emissions (DPOAE) of the brand Elios Elito Otodia (Echodia Ltd., London, UK) was carried out 2 times; before the noise treatment was carried out and after 2 days of noise treatment. Before examination, rats were first anesthetized using Ketamine at a dose of 90 mg / kgBW and Xylazine at a dose of 10mg / kgBW which was injected intraperitoneally[15,16]

Procedures for Curcumin Administration

In this study, curcumin given was extracted from *Curcuma longa L* (turmeric, with certificate number 0532 / SA / V / 2016, certified by Dr. Rer. Nat. M. Yuwono, MS, Department of Pharmacy, Airlangga University) with a concentration of 16.62% ± 0.14% w / w calculated by the TLC-densitometry method. Curcumin given at a dose of 100 mg and 200 mg, which was diluted in CMC / Carboxy methyl cellulose 0.5% was given using Nasogastric Tube (NGT). After all treatments were completed, the rats were terminated by inhalation of ether and necropsing of the rat's temporal bone.

Immunohistochemistry Staining

Temporal bone taken was fixed with 10% formalin buffer solution, then by using EDTA for 4 weeks it was expected for decalcification to occur. Next, each tissue sample was prepared in a paraffin block and sliced into 4 μm thick sections and placed in a slide and then stained with hematoxylin-eosin and immunohistochemical staining. SOD staining with primary antibodies (SOD-2 (A-2) (Santa Cruz Biotechnology, Inc. cat # sc-133134)) and MDA with anti-malondialdehyde antibodies (abcam cat # ab6463) to assess SOD and MDA expression in cortical organs cochlea. The XC 10 Olympus microscope using 40x magnification was used to calculate SOD and MDA expressions using a double-blind method performed by two examiners separately (researcher and anatomist pathologist). SOD and MDA expressions were assessed by a broad score (P) and Intensity score (I) of the brown color on the cytoplasm. Intensity score: 1-3, broad score 0: 0%; 1: <10%; 2: 11% -50%; 3: 51-80%; 4:> 80% to obtain an immune-reactive score which is the product of P and I multiplication, with a value of 0-12[17].

Statistical Analyze

All data collected was analyzed statistically using the ANNOVA test to assess differences in each treatment and bivariate analysis was also performed using the Pearson test to assess the correlation between DPOAE examination with SOD and MDA expressions.

RESULT AND DISCUSSION

To get the appropriate cut of the cochlea, hematoxylin-eosin staining is done before immunohistochemical staining. A picture of cochlea with hematoxylin eosin staining can be seen in Figure 1 below.

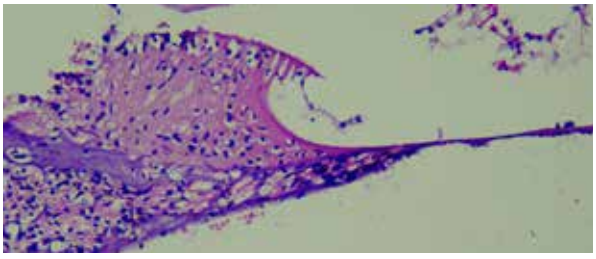


Figure 1: Figure of *Rattus norvegicus* (red arrow) cochlear cortical organ with hematoxylin-eosin staining under 400 magnification.

The SNR value of group 2, the group that received only the lowest noise of 110 dB when compared with the control group (group 1) and the group receiving curcumin (groups 3,4,5, and 6) as shown in table 1. ANOVA test found significant differences between groups (p = 0,000) as seen in table 2. Table 3 shows the significant differences between the control group (group 1) with the group that only gets noise treatment (group 2) and the group given curcumin (group 4,5 and 6) with a value of p <0.05. In this study no significant differences were found in changes in SNR values between the preventive group and between the curative groups regarding the difference in curcumin doses of 100 and 200 mg.

Table 1. Average SNR scores between groups

	N	Mean	Standard Deviation
Group 1	6	9,833	2,2923
Group 2	6	4,167	2,4147
Group 3	6	7,767	1,1759
Group 4	6	6,800	,6197
Group 5	6	6,833	,9245
Group 6	6	6,000	,4382
Total	36	6,900	2,2386

Table 2. ANOVA Test of SNR Value in Each Group

Group	Mean Difference ± Standard Deviation	p Value
Group 1	9,833 ± 2,2923	,000*
Group 2	4,167 ± 2,4147	
Group 3	7,767 ± 1,1759	
Group 4	6,800 ± 0,6197	
Group 5	6,833± 0,9245	
Group 6	6,000± 0,4382	

** Differences are statistically significant*

Table 3. Post Hoc Test Value SNR Value

		Mean difference	p Value
Group 1	Group 2	5,6667	0,000*
	Group 3	2,0667	0,206
	Group 4	3,0333	0,019*
	Group 5	3,0000	0,021*
	Group 6	3,8333	0,002*
Group 2	Group 3	-3,6000	0,004*
	Group 4	-2,6333	0,055
	Group 5	-2,6667	0,051
	Group 6	-1,8333	0,321
Group 3	Group 4	0,9667	0,877
	Group 5	0,9333	0,892
	Group 6	1,7667	0,360
Group 4	Group 5	-0,0333	1,000
	Group 6	0,8000	0,941
Group 5	Group 6	0,8333	0,930

** Differences are statistically significant*

SOD expression decreased in the group that only received noise treatment (group 2) when compared to the control group (group 1) and increased in the group with noise treatment and curcumin administration (groups 3,4,5, and 6) seen in table 4. In the figure 2 the brown color shows SOD expression where there is a decrease in the intensity of brown color in the cytoplasm of cochlear cortical organs of group 2 compared to the control group and seen an increase in the intensity of brown color in group 3,4,5, and 6 when compared to group 2. As seen in Table 5 in this study was found significant differences between groups. Table 6 shows that in this study there were significant differences between the groups that only received noise treatment, namely group 2 and all groups. In the group that given curcumin, the difference in curcumin dose in the curative group (groups 3 and 4) and the preventive group (groups 5 and 6) did not have a significant difference with a p value of <0.05.

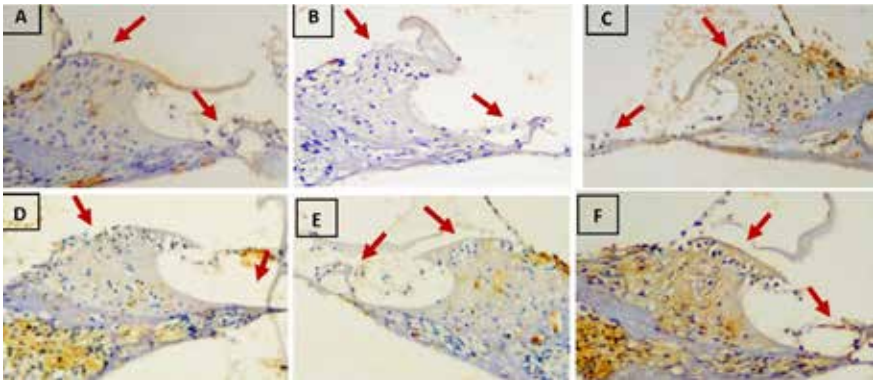


Figure 2: SOD expression in each group under 400 magnification, namely A; Group 1, B; Groups 2 and C; Group 3, D; group 4, E; group 5 and F; group 6, arrows show SOD expression in cochlear cortical organs which are marked in brown.

Table 4. Average SOD Expressions for each group

	N	Mean	Standard Deviation
Group 1	6	9,00	1,897
Group 2	6	4,00	1,897
Group 3	6	8,17	2,563
Group 4	6	9,83	2,563
Group 5	6	8,50	1,975
Group 6	6	10,00	2,449
Total	36	8,25	2,912

Table 5. ANOVA Test on SOD Expression in Each Group

Group	Mean Difference ± Standard Deviasi	p Value
Group 1	9,00 ± 1,897	,001*
Group 2	4,00 ± 1,897	
Group 3	8,17 ± 2,563	
Group 4	9,83 ± 2,563	
Group 5	8,50 ± 1,975	
Group 6	10,00 ± 2,449	

** Differences are statistically significant*

Table 6. Post Hoc Test for SOD Expression

		Mean Differnce	p Value
Group 1	Group 2	5,000	0,007*
	Group 3	0,833	0,987
	Group 4	-0,833	0,987
	Group 5	0,500	0,999
	Group 6	-1,000	0,970
Group 2	Group 3	-4,167*	0,033*
	Group 4	-5,833*	0,001*
	Group 5	-4,500*	0,018*
	Group 6	-6,000*	0,001*
Group 3	Group 4	-1,667	0,790
	Group 5	-0,333	1,000
	Group 6	-1,833	0,718
Group 4	Group 5	1,333	0,904
	Group 6	-0,167	1,000
Group 5	Group 6	-1,500	0,853

** Differences are statistically significant.*

Figure 3 shows an increase in the intensity of the brown color in the cytoplasm of the cochlear cortical organ which shows increased expression of MDA in group 2 compared to the control group. In groups 3,4,5 and 6 there was a decrease in the intensity of the brown color in the cytoplasm of the cochlear cortical organ when compared to group 2. As shown in table 7, this study found an increase in MDA expression in the group that received only noise treatment (group 2) when compared with the control group (group 1), and found a decrease in MDA expression in the group with the administration of noise and curcumin (groups 3, 4, 5 and 6) but not lower or equal to the value of MDA expression in group 1. With the ANOVA test was found a significant difference between groups as seen in table 8. In this study was found significant differences in the expression of MDA in the group that only received noise treatment (group 2) with all groups both the control group (group 1) as well as the noise and curcumin administration group (group 3,4 , 5, and 6) as shown in table 9. Differences in dose did not show significant differences either in the curative group and preventive groups.

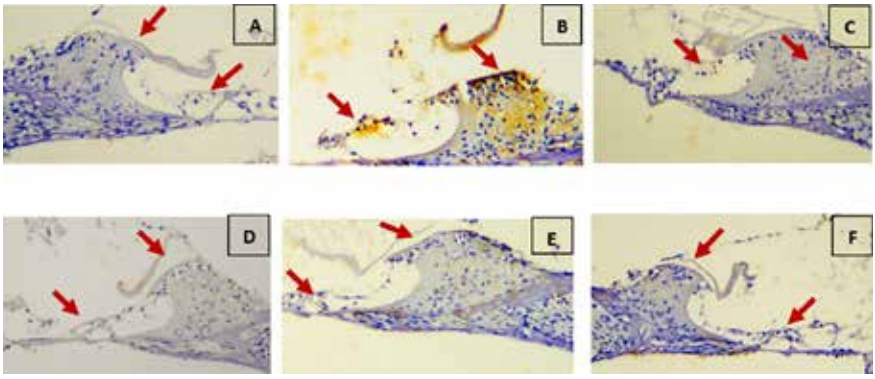


Figure 3: MDA Expressions in each group under 400 magnification, namely A; Group 1, B; Groups 2 and C; Group 3, D; group 4, E; group 5 and F; group 6, arrows show MDA expression in cochlear cortical organs which are marked in brown.

Table 7. The mean values of MDA expressions for each group

	N	Mean	Standard Deviation
Group 1	6	2,00	1,095
Group 2	6	7,33	3,670
Group 3	6	3,33	1,751
Group 4	6	3,17	1,941
Group 5	6	3,33	1,966
Group 6	6	3,17	2,317
Total	36	3,72	2,700

Table 8. ANOVA Test of MDA Expressions in Each Group

Group	Mean Difference ± Standard Deviation	p Value
Group 1	2,00 ± 1,095	,007*
Group 2	7,33 ± 3,670	
Group 3	3,33 ± 1,751	
Group 4	3,17 ± 1,941	
Group 5	3,33 ± 1,966	
Group 6	3,17 ± 2,317	

** Differences are statistically significant*

Table 9. Post Hoc Test MDA expression values

		Mean Difference	p Value
Group 1	Group 2	-5,333	0,004*
	Group 3	-1,333	0,907
	Group 4	-1,167	0,945
	Group 5	-1,333	0,907
	Group 6	-1,167	0,945
Group 2	Group 3	4,000	0,048*
	Group 4	4,167	0,036*
	Group 5	4,000	0,048*
	Group 6	4,167	0,036*
Group 3	Group 4	0,167	1,000
	Group 5	0,000	1,000
	Group 6	0,167	1,000
Group 4	Group 5	-0,167	1,000
	Group 6	0,000	1,000
Group 5	Group 6	-0,167	1,000

* *Differences are statistically significant*

All SNR values of SOD and MDA expression in each group are normally distributed so that the Pearson test can be performed on all groups. Pearson test results show there is a siginificant positive correlation between the value of SNR with SOD expression and siginificant negative correlation between the value of SNR with MDA expression in groups 2, 3, 4, 5, and 6 as shown in table 10.

Table 10. Person Correlation betweem SNR values with SOD expressions and MDA expressions of the Groups

Group		Mean ± Standard Deviation	R	p Value
Group 2	SOD	4,00 ± 1,897	0,891	0,17*
	MDA	7,33 ± 3,670	-0,816	0,48*
	SNR	4,167± 2,4147		
Group 3	SOD	8,17 ± 2,563	0,905	0,13*
	MDA	3,33 ± 1,751	-0,907	0,13*
	SNR	7,767 ± 1,1759		
Group 4	SOD	9,83 ± 2,563	0,831	0,040*
	MDA	3,17 ± 1,941	-0,865	0,026*
	SNR	6,800 ± 0,6197		
Group 5	SOD	8,50 ± 1,975	0,822	0,045*
	MDA	3,33 ± 1,966	-0,887	0,018*
	SNR	6,833± 0,9245		
Group 6	SOD	10,00 ± 2,449	0,894	0,016*
	MDA	3,17 ± 2,317	-0,867	0,025*
	SNR	6,000± 0,4382		

* *Statistically significant*

Noise exposure causes a variety of damage to the cochlea both during noise exposure and after the noise exposure is stopped so that it will ultimately affect hearing sensitivity[18]. Regular or prolonged exposure to noise can cause damage to the sensory cells and other structures gradually, which cannot be recovered, leading to PTS (Permanent Threshold Shift)[1]. Noise can cause damage to cochlear function through 7 mechanisms, namely free radical formation, mechanical damage, release of glutamate into hair cells in the cochlea, excessive stimulation of N methyl-D-aspartame receptors that cause nitric oxide release, decrease in magnesium which causes changes in intracellular activity, increased intracellular calcium activity and protein damage[19].

The formation of free radicals will cause oxidative stress. Antioxidants in general function as free radical scavenger which can repair damage caused by free radicals. Curcumin is known as an antioxidant and is reported to have free radical scavenger activity[20]. Other studies have concluded that administration of curcumin before and during paclitaxel can significantly protect the morphology and function of cochlea in pacoxitaxel-induced ototoxic rats assessed by using a light microscope and DPOAE examination to evaluate histopathology, immunohistochemistry, and hearing functional changes[21].

In this study group 2 that received noise treatment for 2 days with an intensity

of 110 dB for 2 hours the SNR value decreased compared to the control group, this shows that in group 2 there was NIHL. In groups 3, 4, 5, and 6 which are groups with the same noise treatment group as group 2 followed by administration of curcumin there was an increase in SNR value ≥ 6. In this study also found a significant difference in SNR values between group 1 and group 2, 4, 5 and 6 ($p < 0.05$) which can be interpreted that the administration of curcumin in the group with the noise treatment can increase the SNR value, this is in accordance with previous studies conducted by Yamaguchi et al who found that the administration of curcumin orally for 3 days before and each days during noise exposure significantly reduce hearing loss caused by repeated noise exposure. In that study the auditory hearing used was BERA and was found that dose of 100 mg / kgBW of curcumin was capable of partially but significantly attenuating the noise-induced elevation of the auditory threshold at 12 and 20 KHz frequencies[22].

SOD expression decreased in the noise treatment group only (group 2) when compared to the control group (group 1) and increased in the group with noise treatment and curcumin administration (groups 3,4,5, and 6). Significant differences were found between the groups that were only exposed to noise (Group 2) with the control group and the group that received noise exposure followed by the administration of curcumin (groups 3,4,5, and 6) ($p < 0.05$). This study is in accordance with research conducted by Kavakli et al who found that administration of curcumin protected spinal cord tissue from oxidative damage through a mechanism of increasing SOD enzyme activity and a decrease in MDA levels in rat animals which were subjected to spinal cord trauma and laminectomy[23]. Curcumin can increase the antioxidant status and expression of caspase-9 gene and inhibit the process of oxidative stress and lipid peroxidation in rat colon cancer tissue induced by 1,2-dimethylhydrazine. Curcumin has been shown to increase SOD and GST activity and levels of GSH, caspase-9 and DNA fragmentation and reduce the increase in MDA and NO concentrations that occur in the colon tissue[24]. This study is also in accordance with the research conducted by Meshkibav et al who found that curcumin increases the level of antioxidant enzymes such as SOD, CAT and GPx in rats with arthritis models. The study also found that the increase in MRSA levels was due to the antioxidant effect of curcumin[25].

This study found an increase in MDA expression in the group that received only noise treatment (group 2) when compared to the control group (group 1). This is in accordance with the research by Demirel et al which showed an increase in MDA levels, indicators of lipid peroxidation, as well as NO levels

and GSH-Px activity by noise exposure indicating oxidative stress which can cause various levels of damage in cells, especially through lipid peroxidation pathways[8]. In the group with noise treatment followed by administration of curcumin of group 3, 4, 5, and 6 was found a decrease in the value of MDA and found significant differences between the group that only received treatment (group 2 compared) to all groups that received noise treatment followed by administration of curcumin (groups 3,4,5, and 6). Research conducted by Zheng et al (2009) found that administration of curcumin was able to reduce MDA levels and expression of c-fos protein in the brains of rats that were damaged due to ischemic hypoxia. In this study an improvement was seen in changes in the structure and morphology of neuron cells in the cortex of the rat brain after administration of curcumin[26].

In the previous study, curcumin was found to be able to significantly increase SOD expression[11] and decrease MDA expression[12] in noise-exposed cochlear fibroblasts ($p < 0.05$). However, this study proves that curcumin can increase SOD expression and decrease MDA expression in outer cochlear hair cells in rats that are exposed to noise, causing improvement in outer hair cell function as measured by the increase of SNR values on DPOAE examination. Was also found a positive correlation between SNR values with SOD expression and negative correlations between SNR values with MDA expression in all treatment groups. Increased SOD expression and decreased MDA values led to an increase in the SNR value after administration of curcumin in groups 3,4,5, and 6. In the end this study, the results could perform as the basis of benchmarks for further studies in humans that prove the benefits of curcumin in repairing cochlear cortical organ damage which is assessed by DPOAE examination. Research by Soyalıç et al found that curcumin can protect cochlear tissue from acoustic trauma in rat. Intra-peritonial curcumin injection before and after acoustic trauma reduces cochlear hair cell damage and protects from hearing damage assessed by DPOAE, histopathological and immunohistochemical examinations[27].

This research proves the existence of Correlation between Oxidant (MDA) and Antioxidant (SOD) Status and the improvement of outer hair cell function which is assessed by DPOAE examination in the cochlea of *Rattus norvegicus* which is exposed to noise due to curcumin administration, where positive correlation was found between SOD expression and SNR value and negative correlation between MDA expression and SNR value.

ACKNOWLEDGEMENTS

The authors would thank the Research Institute of the Universitas Sumatera Utara for funding support under the TALENTA Research Implementation Contract of the University of North Sumatra 2018 fiscal year No: 2590 / UN.5.1.R / PPM / 2018 on March 16, 2018.

FUNDING SOURCES

This work was supported by Research Institute of the Universitas Sumatera Utara under the TALENTA Research Implementation Contract of the Universitas Sumatera Utara 2018 fiscal year No: 2590 / UN.5.1.R / PPM / 2018 on March 16, 2018.

CONFLICT OF INTEREST

Nothing to declare

AUTHOR CONTRIBUTION

Design: Tengku Siti Hajar Haryuna

Acquisition of data: Reastuty

Analysis of data: Tengku Siti Hajar Haryuna and Reastuty (these authors contributed equally)

Drafting of the manuscript: Tengku Siti Hajar Haryuna and Reastuty (these authors contributed equally)

Critical revision of the manuscript: Tengku Siti Hajar Haryuna and Delfitri Munir (these authors contributed equally)

Statistically analisis: Tengku Siti Hajar Haryuna, Reastuty and Juliandi Harahap (these authors contributed equally)

Technical and Financial Support: Tengku Siti Hajar Haryuna, Tengku Siti Harilza Zubaidah and Delfitri Munir (these authors contributed equally)

Supervision: Tengku Siti Hajar Haryuna (this author contibuted fully)

Other (Specify): Histopatological and molecular biology expertise: Wibi Riawan

REFERENCES

1. World Health Organization. Hearing loss due to recreational exposure to loud sounds: a review. World Health Organization. 2015 https://apps.who.int/iris/handle/10665/154589

2. Rabinowitz ME, Noise-induced hearing loss. Am Fam Physician, **2000;** 61(9):2749-56

3. Mathur NN, Noise induced hearing loss. emedicine **2020** July 13 https://emedicine.medscape.com/article/857813-overview

4. Le NT, Straatman VL, Lea J, Westerberg B. Current insights in noise-induced hearing loss: a literature review of the underlying mechanism, pathophysiology, asymmetry, and management options. J Otolaryngol Head Neck Surg, **2017;**46(1):1-15. https://doi.org/10.1186/s40463-017-0219-x.

5. Martin LLB, Martin GK, Otoacoustic emissions disorders of peripheral and central auditory processing. Handbook of Clinical Neurophysiology. **2013;** 10(6):115-34

6. Saurini P, Nola G, Lendvai D, Otoacoustic emissions: a new method for newborn hearing screening. Eur Rev Med Pharmaco Sci. **2004;**8(3):129-133

7. Choi CH, Mechanisms of noise-induced hearing loss and treatment. Audiol. **2011;**7:124-32. http://doi.org/10.21848/audiol.2011.7.2.124

8. Demirel R, Mollaoğlu H, Yeşilyurt H, Üçok K, Ayçiçek A, Akkaya M,et al. Noise induces oxidative stress in rat. Eur J Gen Med. **2004;**6(1):20-24

9. Özgüner MF, Delibaş N, Tahan V, Koyu A, Köylü H, Effects of industrial noise on the blood levels of superoxide dismutase, glutathione peroxidase and malondialdehyde. Eastern Jour Med. **1999;**4(1):13-15

10. Amalraj A, Pius A, Gopi S, Gopi S, Biological activities of curcuminoids, other biomolecules from turmeric and their derivatives A review. J Tradit Complement Med. **2016;**7(2):205-33. https://doi.org/10.1016/j.jtcme.2016.05.005

11. Hadi S, Artanti AN, Rinanto Y, Wahyuni DSC, Curcuminoid content of Curcuma longa L. and curcuma xanthorrhiza rhizome based on drying method with NMR and HPLC-UVD. Mater. Sci. Eng. **2018;**349. https://doi.org/10.1088/1757-899X/349/1/012058

12. Haryuna TSH, Riawan W, Reza M, Saragih AR, Modulation of antioxidant status by curcumin prevents cochlear damage after noise exposure. J. Chem. Pharm. Res. **2015;**7(11)593-7

13. Haryuna, TSH, Riawan W, Reza M, Purnami N, Adnan A, Curcumin prevents cochlear oxidative damage after noise exposure. Int J Pharm Pharm Sci. **2016;**8(1):175-8

14. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Guide for the Care and Use of Laboratory Animals. 8th ed. Washington (DC): National Academies Press (US). **2011:**1-220

15. Albert Einstein College of Medicine. Recommended Methods of Anesthesia, Analgesia, and Euthanasia for Laboratory Animal Species. Institute for Animal Studies Veterinarians. 2014. https://www.spandidos-publications.com/var/RecommendedMethodsAnesthesiaAnalgesiaEuthanasia.pdf

16. Toydemir T, Kanter M, Erboga M, Oguz S, Erenoglu C, Antioxidative, antiapoptotic and proliferative effect of curcumin on liver regeneration after partial hepatectomy in rats. Toxicol Ind Health. **2015;**31(2):162-72. https://doi.org/10.1177/0748233712469658

17. Czogalla B, Kahaly M, Mayr D, Schmoekel E, Niesler B, Kolben T, et al. Interaction of ERα and NRF2 impacts Survival in Ovarian Cancer Patients. Inj J Mol Sci. **2018;**20(1):112. https://doi.org/10.3390/ijms20010112

18. Hu BH, Henderson D, Nicotera MT, Involvement of apoptosis in progression of cochlear lesion following exposure to intense noise. Hear Res. **2002;**16(1-2):62-71. https://doi.org/10.1016/s0378-5955(02)00286-1

19. Alcarás PAS, Lüders D, França DMVR, Klas RM, lacerda ABML, Gonçalves CGO, Evoked otoacoustic emissions in workers exposed to noise: a review. Int Arch Otorhinolaryngol. **2012;** 16(4):515-22. https://doi.org/10.7162/S1809-97772012000400014

20. Choudhury AK, Raja S, Mahapatra S, Nagabhushanam K, Majeed M, Synthesis and Evaluation of the Anti-Oxidant Capacity of Curcumin Glucuronides, the Major Curcumin Metabolites. Antioxidants. **2015;** 4(4):750–767. https://doi.org/10.3390/antiox4040750

21. Bucak AK, Ozdemir C, Ulu S, Gonul Y, Aycicek A, Uysal M, et al. Investigation of Protective Role of Curcumin Against Paclitaxel-Induced Inner Ear Damage in Rats. Laryngoscope. **2015;**125(5):1175-82. https://doi.org/10.1002/lary.25031

22. Yamaguchi T, Yoneyama M, Onaka Y, Imaizumi A, Ogita K, Preventive effect of curcumin and its highly bioavailable preparation on hearing loss induced by single or repeated exposure to noise: A comparative and mechanistic study. J Pharmacol Sci. **2017;**134(4):225-33. https://doi.org/10.1016/j.jphs.2017.07.003

23. Kavakli, HS, Koca C, Alici O, Antioxidant effects of curcumin in spinal cord injury in rats. Ulus Travma Acil Cerrahi Derg. **2011;**17(1):14-8.

24. Aziza, SAH, Abdel-Aal SA, Mady HA, Chemopreventive effect of curcumin on oxidative stress, antioxidant status, DNA fragmentation and caspase-9 gene expression in 1,2-dimethylhydrazine-induced colon cancer in rats. Am J Biochem Mol Biol. **2014;**4(1):22-34.

25. Meshkibaf MH, Maleknia M, Noroozi S, Effect of curcumin on gene expression and protein level of methionine sulfoxide reductase A (MSRA), SOD, CAT and GPx in Freund's adjuvant inflammation-induced male rats. J Inflamm Res. **2019;**12:241-9. https://doi.org/10.2147/JIR.S212577

26. Zheng YY, Yu LS, Zhang YG, Ye GH, Yi JP, Effects of curcumin on malondialdehyde and c-fos protein in hypoxia ischemia brain tissue in rats. Fa Yi Xue Za Zhi. **2009;**25(1):6-8.

27. Soyalıç H, Gevrek F, Karaman S, Curcumin protects against acoustic trauma in the rat cochlea. Int J Pediatr Otorhinolaryngol. **2017;**99:100-16. https://doi.org/10.1016/j.ijporl.2017.05.029

Acta Pharm. Sci. Vol 59:(4), 2021
DOI: 10.23893/1307-2080.APS.05932

Point Prevalence Survey of Antibiotic Prescribing in the Hospital Sector in Albania

Silvi BOZO[1*], Carlo De Giuli MORGHEN[1], Elena CAPPARELLI[1], Eftiola POJANI[2]

1 Department of Chemical-Pharmaceutical and Biomolecular Technologies, Faculty of Pharmacy, Catholic University "Our Lady of Good Counsel", Tirana, Albania.

2 Department of Pharmaceutical Sciences, Faculty of Pharmacy, Catholic University Our Lady of Good Counsel, Tirana, Albania.

ABSTRACT

Antibiotic resistance represents a serious threat worldwide. The onset and spread of resistance is mostly related to irrational and increased use of antibiotics. The aim of this study was to evaluate antibiotic prescribing patterns and identify areas for quality improvement in the hospital sector in Albania. A Point Prevalence Survey study was conducted, at a University Hospital Centre in Tirana, Albania. On the day of the survey, 65.7% of the inpatients were on antibiotic treatment, mostly for medical prophylaxis (67.1%). The higher prevalence was found in Intensive Care Units (81.0%). The most commonly used antibiotics were the cephalosporins (53.1%) followed by metronidazole (16.2%) and fluoroquinolones (14.7%). High rates of antibiotic use were found in all wards, while some indicators related to prescribing patterns were critical. A close and continuous surveillance of the antibiotic use, along with measures at national level could contribute in improving a proper use of antibiotics.

Keywords: prevalence survey, antibiotic use, Albania, antibiotic surveillance.

INTRODUCTION

Antibiotics are among the most frequently prescribed drugs worldwide. According to the studies carried out in the European countries and the United States, 23-38% of inpatients are on systemic antibiotic treatment[1]. Moreover, from the data collected worldwide it appears that the consumption of antibiotics is constantly increasing and in the years 2000-2010 there was an increase of 36% in their global consumption[2]. One of the main causes for the onset and

*Corresponding Author: s.bozo@unizkm.al
ORCIDs:
Silvi Bozo : https://orcid.org/0000-0002-6444-0799
Carlo De Giuli Morghen : https://orcid.org/0000-0002-4127-3596
Elena Capparelli : https://orcid.org/0000-0002-7031-4422
Eftiola Pojani : https://orcid.org/0000-0001-5402-4065
(Received 24 Jan 2020, Accepted 29 March 2021)

diffusion of antibiotic resistance is their increased and irrational consumption and administration. This correlation is well documented in the medical literature[3,4,5,6,7]. Antibiotic resistance has a significant impact on patients morbidity, mortality, occurrence of side effects, toxicity and prolonged hospitalization. Another important consequence is the increased costs incurred by government, patients, insurance companies, or other third-parties when antibiotics are misused. A large portion of antibiotic use appears to be for viral or spontaneously resolving bacterial infections. The Centers for Disease Control and Prevention (CDC) estimates that about 100 million courses of antibiotics are prescribed by office-based physicians each year, and that approximately one-half of those prescriptions are unnecessary[8,9,10,11]. Studies evaluating physicians' prescribing patterns have found that many of these antibiotic courses are unnecessary, with a projected 40%-60% of patients receiving antibiotic treatment for viral upper respiratory tract infections. This prescribing pattern persists despite the fact that antibacterial agents have no significant benefit for the resolution of viral diseases, such as the common cold[12,13,14,15,16].

The hospital sector, with an increased antibiotic consumption and a facilitated diffusion of resistance, is a major driver of the spread of multidrug-resistant bacteria. In fact, patients who are hospitalized for prolonged periods are more at risk of acquiring antibiotic-resistant nosocomial infections, which are also very difficult to treat and lead to increased treatment costs[17]. This may also require the use of innovative and more expensive antibiotics which may not be readily available. Some of these second-line antibiotics are not necessarily more effective than first line agents but are the last line of antibacterial defense. The courses of treating resistant microorganisms are usually longer than the courses of treating susceptible microorganisms[18,19].

Antibiotic prescribing patterns differ not only between different countries, but also between hospitals of a same country. These differences may be related to hospital and patient features, antibiotic policies at a hospital level, prescribing physician preferences and differences in public education and health systems[2].

Antibiotic resistance is a global problem, affecting both developing and developed countries. Especially in developing countries there is lack of centralized systems for monitoring resistance patterns and antibiotic consumption[20,21,22]. In fact, in Albania, the data on trends of resistance and antibiotic consumption are scarce and fragmentary and there is a lack of consistent representative data at national level. The aim of the present work was to evaluate antibiotic consumption, investigate antibiotic prescribing patterns and identify areas for quality improvement in the hospital sector in Albania towards a more rational use of antibiotics.

METHODOLOGY

Study design

The study was carried out at the University Hospital Center "Mother Teresa" (QSUT) located in the capital of Albania, Tirana. QSUT is the only university and tertiary hospital in Albania. Moreover, this hospital represents a reference center for all the regional State hospitals and therefore can be considered a representative institution for the use and consumption of antibiotics nationwide.

For the evaluation of antibiotic consumption, a Point Prevalence Survey (PPS) was conducted in May 2017, including 11 wards (2 medical wards, 5 surgery wards and 4 Intensive Care Units, ICU) with a total 356 bed capacity. Only adult patients were enrolled in the study.

Data collection

Data were collected mainly by consulting patient prescribing charts, supplemented by information acquired by the Hospital Information System, laboratory data and interviews with physicians, nurses and pharmacists within the hospital, using an *ad-hoc* method according to the Global PPS methodology[23]. A patient form was completed only for those patients on antibiotic treatment at 8 o'clock on the day of the survey. Only data related to antibiotics for systemic use included in the Anatomical Therapeutic Classification, ATC class J01, were collected[24]. Antibiotics for topical use were excluded from the study.

However, all patients whether or not on antibiotic treatment were counted in the denominator data. In our study were enrolled a total of 315 patients, corresponding to the number of patients admitted to each of the wards included on the specific study day.

Overall, bed occupancy rate was 88.5% (315/356). The collected data included age, gender, type of antibiotic, dosage, reasons and indications for treatment, as well as compliance to guidelines. All patients were de-identified and to every patient record was given a unique not identifiable survey number.

Statistical analysis

Data were analyzed using the SPSS version 19.0 software. Chi-square tests were used for comparing the percentages between variables. P-value ≤0.05 was considered as significant.

RESULTS AND DISCUSSION

On the day of the survey, 207 (65.7%) out of 315 total inpatients were on antibiotic treatment for any reason. The overall bed occupancy rate was 88.5% (315/356 beds). Distribution rates of antibiotic use and bed occupancy rate according to the ward type are presented in Table 1.

Table 1. Distribution rates of antibiotic use and bed occupancy rate according to the ward type

Ward type	No. of beds	No. of inpatients	Bed occupancy rate (%)	Prevalence*
Medical				
Infectious diseases	75	68	90.7	57 / 83.8
Hematology	34	34	100.0	17 / 50
Total	109	102	93.6	**74 / 72.5**
Surgery				
General Surgery, Clinic 1	35	35	100.0	16 / 45.7
Urology	45	40	88.9	38 / 95.0
General Surgery, Clinic 3	51	51	100.0	35 / 68.6
Neurosurgery	32	24	75.0	1 / 4.2
Burn and plastic surgery	33	21	63.6	9 / 42.8
Total	196	171	87.2	**99 / 57.9**
Intensive Care Unit (ICU)				
Infectious diseases	5	2	40.0	2 / 100.0
Central ICU	15	14	93.3	14 / 100.0
Cardiology	21	21	100.0	13 / 61.9
Neurosurgery	10	5	50.0	5 / 100.0
Total	**51**	**42**	**82.3**	**34 / 81.0**

**Prevalence is expressed as the ratio of No. of antibiotic-treated patients/ the percentage of patients under antibiotic treatment*

** chi – square test; the highest rate of antibiotic use was observed in the ICU wards ($\chi 2=2.3$ $p=0.5$)*

In our study, the prevalence of antibiotic use is relatively high, if compared to the other European countries. According to the Global PPS report of 2015, the prevalence of antibiotic use in Europe is of 31.1%[26]. The prevalence rate in our study is higher compared even with countries reported with the highest prevalence of antibiotic use, respectively West & Central Asia (42.1%), followed by

South America (39.5%), Australia and New Zealand (38.5%), North America (35.2%), East & South Asia (33.3%)[23]. Different rates can also be seen depending on the country and the hospital in which other studies are conducted. For instance, prevalence rates similar to our study were reported in a study performed in four tertiary hospitals in Nigeria (69.7%), in 11 hospitals from the Democratic Republic of Congo (68%) and some other developing countries[26,27]. However it's important to note that QSUT is a reference center for all regional hospitals in Albania, where are hospitalized patients suffering from serious infectious diseases that cannot effectively be treated in the hospital of origin. Therefore, the high levels of prevalence of antibiotic consumption deriving from the data of our study could be partly explained from a presumed high prevalence of patients affected by bacterial infections in this hospital.

The prevalence levels of antibiotic consumptions found in our study were higher if compared also with previous data from Albania. The data reported from an Albanian study performed as part of the Global PPS project of 2015, where were involved 3 Albanian hospitals (including the QSUT), show a lower level of prevalence (47.5%) compared to our study[23]. Higher values also result in comparison with the prevalence levels reported in another PPS study conducted in Albania in the QSUT in 2003, where the prevalence was 46.9% [28].

The differences observed between different studies carried out in Albania may be due to the number of wards of the various specialties included in the study, considering that in different specialties, the rate of administration of antibiotics presents considerable differences, as well as the period and the temporal distance between the studies. In our case, sampling was of convenience, and therefore were included in the study those wards were we expected to find the highest rate of antibiotic administration. In other studies previously conducted in Albania, the goal was to include as many wards as possible, but without paying particular attention to the quality and types of wards in terms of antibiotic consumption levels.

The highest rate of antibiotic use in our study was observed in the ICU wards ($\chi 2=2.3$ $p=0.5$), ranging from 61.9% in the cardiology ICU to 100 % in the infectious diseases, central and neurosurgery ICU. This could be partially related to the debilitated physical condition and deficiencies of the immune system in the patients in these units which require intense antibiotic therapy for longer periods and for severe infectious complications, including nosocomial infections. In fact, a study conducted in the Danish hospitals estimated that the total antibiotic consumption in the ICUs is approximately tenfold greater than in general hospital wards[29]. However, high levels of prevalence in our study were also found in the medical (72.5%) and surgery wards (57.9%), although not comparable with the ICU wards.

The treated patients (207) received a total of 327 antibiotics (1.6 antibiotics/patient). In 53.1% of the patients receiving antibiotic therapy, was administered only one antibiotic, while in 46.9% of patients the therapy consisted of 2 or 3 antibiotics simultaneously. None of the patients were given more than 3 antibiotics at the same time.

The types of indication were defined and classified according to the GLOBAL PPS protocol, respectively as Community-acquired infection (CAI), Hospital-acquired infection (HAI), Medical Prophylaxis (MP), Surgical Prophylaxis (SP) or Unknown indication[23]. The most common indication for antibiotic use (67.1% of the patients), was medical prophylaxis ($\chi 2=197$ $p<0.01$), followed by community-acquired infection (CAI) (26.6%), hospital-acquired infection (HAI) (4.8%) and surgical prophylaxis (2.9%) (Table 2).

Table 2. Distribution rates of antibiotic use according to the type of indication

Type of indication	No./% of patients			
	Medical wards†	Surgical wards§	ICU‡	Total*
Community-acquired infection (CAI)	46 / 62.2	5 / 5.1	4 / 11.8	55 / 26.6
Hospital-acquired infection (HAI)	4 / 5.4	3 / 3.0	3 / 8.8	10 /4.8
Medical Prophylaxis (MP)	25 / 33.8	89 / 89.9	25 / 73.5	139 / 67.1
Surgical Prophylaxis (SP)	0 / 0	6 / 6.1	0 / 0	6 / 2.9
Unknown indication	2 / 2.7	0 / 0.0	0 / 0	2 / 1.0

†§‡ chi – square test;*

†In medical wards, the most common indication for antibiotic use was CAI ($\chi 2=197$ $p<0.01$).

§In surgical wards, the most common indication for antibiotic use was MP ($\chi 2=197$ $p<0.01$).

‡In ICU wards, the most common indication for antibiotic use was MP ($\chi 2=197$ $p<0.01$).

**In the total sample, the most common indication for antibiotic use was MP ($\chi 2=197$ $p<0.01$).*

Medical Prophylaxis is classified as the indication when the antibiotic therapy is used as general prophylaxis, without targeting a specific site, to prevent HAIs and/or surgical site infections, but in any case without the presence of

any identified or confirmed infection. HAIs included post-operative surgical site infection (30.0%), intervention related infections (30.0%) such as Ventilator Associated Pneumonia (VAP) or Catheter related-Urinary Tract Infection (C-UTI) or other hospital acquired infections such as Hospital Acquired Pneumonia (HAP). On the other hand, prolonged surgical prophylaxis for more than one day, was very common. These results are similar to other studies conducted in Albania with a relatively high percentage of patients (67.1%) who received antibiotic therapy as medical prophylaxis[23]. If compared to European data, this percentage is almost 10 times the relative value reported by the ESAC 2006 study conducted in 20 European hospitals (6.6%)[14]. In our study, the surgical departments are those with the highest proportion of patients under antibiotic therapy as medical prophylaxis (89.9%), followed by ICU departments (73.5%). This result could partially be justified by the critical conditions of the patients admitted to these wards, with a high sensitivity to nosocomial infections. However, in many cases, the administration of the antibiotic therapy as a medical prophylaxis in the patients of our study did not have a precise and/or controlled well-based clinical justification. From the information received from the hospital's healthcare staff, the administration of antibiotics throughout the patient's hospitalization turns out to be a common practice for physicians. This suggests that initiatives needs to be taken in order to strengthen infection preventive practices and reduce associated complications, This would improve physicians confidence in infection prevention and control programs, which would lead to substantial reductions and more rational use of antibiotics in the hospital setting as well. Type, frequency of antibiotic use and the ATC code for each antibiotic and class is shown in Table 3.

Table 3. Type and frequency of antibiotics

ATC code	Antibiotic	No. of patients	Frequency of use (%)*
J01A	**Tetracyclines**	4	1.2
	Tigecycline	2	0.6
	Doxycycline	2	0.6
J01B	**Amphenicols**	0	0
J01C	**Beta-lactam antibacterials, penicillins**	3	0.9
	Piperacillin	1	0.3
	Piperacillin and Tazobactam	2	0.6
J01D	**Other beta-lactam antibacterials**	174	53.2
	Cefazolin	91	27.8
	Ceftriaxone	41	12.5
	Cefuroxime	24	7.3
	Cefepime	12	3.7
	Ceftazidime	2	0.6
	Cefotaxime	2	0.6
	Cefalexine	2	0.6
J01E	Sulfonamides and trimethoprim	5	1.5
	Sulfamethoxazole and trimethoprim	5	1.5
J01F	Macrolides, lincosamides and streptogramins	0	0
J01G	Aminoglycoside antibacterials	36	11.0
	Gentamicin	23	7.1
	Amikacin	13	4.0
J01M	Quinolone antibacterials	48	14.7
	Ciprofloxacin	25	7.7
	Levofloxacin	23	7.1
J01R	Combinations of antibacterials	0	0
J01X	Other antibacterials	57	17.5
	Metronidazole	53	16.2
	Vancomycin	4	1.2

** chi – square test;*

The class of antibiotics used most frequently is the J01D group (Other beta-lactams) ($\chi 2 = 468$ $p < 0.001$).

Among all antibiotics, the most frequently used antibiotic is cefazolin ($\chi 2 = 987$ $p < 0.001$).

The results of our study show that among the antibiotics of the ATC class J01, the class of antibiotics used most frequently is the J01D group (Other beta-lactams) (53.2%) ($\chi 2 = 468$ $p < 0.001$), followed by the J01X group (Other antibacterials) (17.5%), the J01M group (Quinolones) (14.7%) and finally the J01G group (Aminoglycosides) (11.0%).

Cefazolin was found to be the most frequently used among all antibiotics (27.8%) ($p<0.001$) followed by metronidazole (16.2%) and ceftriaxone (12.5%). These results are consistent with those reported for Albania in Global PPS, except for metronidazole where, the frequency of use in our study is higher[23]. Similar results are found also in a PPS study involving 13 Chinese hospitals[30]. On the other hand, different prescription trends are reported in other studies[13,31,32]. In a large-scale PPS study at European level, the combination of penicillins with a beta-lactamase inhibitor (24%) was the most widely used class of antibiotics, followed by macrolides (15%), fluoroquinolones (11%) and third-generation cephalosporins (10%)[14]. In another study conducted in 130 U.S. hospitals, it appeared that the classes of antibiotics most frequently administered to adults were fluoroquinolones, cephalosporins, metronidazole and vancomycin[33]. Globally, it appears that high levels of cephalosporin use, similar to our study, are found in Russia (65.8%) and Serbia (52.9%)[34].

The prescription practices observed in our study, with a large use of broad-spectrum antibiotics could be attributed to the fear for inadequate antibiotic coverage by the physicians. Changes in antibiotic use in hospitals undergoing these studies can also be attributed to differences in microbial resistance trends between countries, the presence and implementation of guidelines, the duration of empirical therapy, the clinical conditions of the patients and the common habits in prescribing this class of drugs[30]. Furthermore, we have observed that the antibiotics choice was also conditioned by their availability and cost in the hospital setting rather than by the clinical needs of the patients. As a matter of fact, since Albania is a still developing country and the healthcare system has limited financial resources, the availability of a wide class of antibiotics is very limited. In fact, antibiotics such as carbapenems do not appear to be administered in any of the patients in our study and antibiotics such as glycopeptides (vancomycin) have been used in a very small number of patients due to their prohibitive cost for the hospital and the patient.

Different trends in antibiotic use were observed across the different wards in our study. For instance, in the medical wards, the most widely used antibiotic is cefuroxime (19.1%). The antibiotics used in surgical wards, in decreasing order of frequency are cefazolin (37.6%), metronidazole (24.4%) and ceftriaxone (8.8%).

Unlike the data on the total sample, in these wards there is also a high percentage of gentamicin prescriptions, while in ICUs, the most widely used antibiotic is ceftriaxone (34.7%), followed by cefazolin (24.5%) and ciprofloxacin (12.3%). Compared to the other specialties, the variety of antibiotics used in the ICU is generally lower.

Several hospital quality indicators regarding antibiotic prescription were also evaluated and the results according to the ward type and the total sample are shown in Table 4[13]. The results are represented as the number and percentage of antibiotics on the total number of antibiotics administered on the day of PPS.

Table 4. Hospital quality indicators for antibiotic use

Parameter description	No./% of antibiotics			
	Medical wards*	Surgical wards*	ICU*	Total*
Targeted treatment	8/7.3	2/1.2	0/0.0	10/3.1
Guideline compliance	83/75.4	2/1.2	6/12.2	91/27.8
Treatment based on biomarker data	55/50.0	30/17.9	6/12.2	91/27.8
Reason written in notes	41/37.3	75/44.6	10/20.4	126/38.5

** chi – square test; p<0. 001*

Data indicate a remarkably high proportion of empirically administered antibiotics (96.9%), compared to those administered according to microbiological findings (3.1%) (p<0.001). Results are similar to other studies performed in Albania as well as in other low-income countries such as Congo (neither of the hospitalized patients received targeted therapy) and Mongolia (targeted therapy was prescribed in 9.2% of the cases)[27, 35]. Conversely, the European countries show a significantly higher prevalence of targeted prescriptions. For instance, in the ESAC 2009 study, the average percentage is 43%, while in many Swedish hospitals it reaches levels of 70%[14, 36].

In our study, the specialties with the highest rate of empirical therapy were the ICUs. Especially in patients with a severe clinical condition, it is well known that initiation of empiric therapy is associated with an increased survival rate of critically ill patients, and therefore some authors suggest empiric therapy with antibiotics (alone or in combination) in the case of serious bacterial infections[37]. Nevertheless, the revision of therapy in the ICU should be carried out on the third day of therapy to review the therapy used after the outcome of the microbiological examination (de-escalation)[38]. Therefore, the analysis of the patient's microbiological cultures remains a fundamental requirement to ensure patient safety, especially when it comes to hospital acquired infections that are often caused by multi-drug resistant bacteria pathogens.

In Albania there are no prescription guidelines for the administration of antibiotics, neither at a national nor a local level. As a result, at the QSUT no protocols and guidelines were found that would serve as a reference for comparing the concordance of antibiotic therapy found for each individual patient on the day of the study. Thus, since in our study the treatment was considered appropriate if prescribed by an infection specialist, the results showed that guideline compliance occurred only in 27.8% of the prescriptions ($p<0.001$). Clinical guidelines should therefore be considered crucial to improve the quality of antibiotic prescriptions, both to promote the effectiveness of empirical therapy and to reduce the inappropriate use of antibiotics in the ongoing fight against resistant pathogens. The absence of clinical guidelines in Albania, highlights the need for national and local guideline design and implementation that can assist the physician in taking appropriate decisions in specific clinical settings and pathologies.

On the other hand, treatment was based on biomarker data in 27.8% of the prescriptions and the mainly biomarker was the C-reactive protein (PCR) (15.3%) ($p<0.001$). Other biomarkers used were the erythrocyte sedimentation index, the HIV-related immune dysfunction, the brucellosis and the cerebrospinal fluid biomarkers.

The reason for which antibiotics were prescribed was recorded in 38.5% of the patients medical records. Moreover, in many of these cases, the physician's notes were unclear and it was often necessary to refer to the entire patient's medical history.

In conclusion, this study reported high rates of antibiotic use in Albanian hospitals. In addition, some other indicators related to the patterns of prescribing were critical, such as lack of microbiology data, low documentation of the reasons for treatment, its duration, and the lack of hospital guidelines. This situation is problematic also in comparison with other industrialized countries or developing countries and the data reported at a regional and international level.

The present study has some limitations such as the data bias due to the selection bias and the type of wards included in the study which could provide unrepresentative data at a hospital level. The partial lack of information in the patient charts encountered in some cases, may have also influenced the data represented for some indicators related to the antibiotic prescriptions. However, this research, was performed in accordance with the guidelines of the Global PPS methodology, contributing to provide useful information regarding the use of antibiotics in the hospital setting and highlighted some differences with other studies previously conducted in Albania.

The results of our study, as well as those from previous ones conducted in Albania, suggest that more guidance on the use of antibiotics for the treatment of infections or prophylaxis is needed as well as policies of antibiotic use in the hospitals in terms of more rational prescriptions. A close and continuous surveillance of the antibiotic use, as part of the structuring of policies, along with measures at national level such as development of local guidelines and continuous education, could contribute to improving the appropriate and proper use of antibiotic therapy. These results need further investigation through qualitative or quantitative studies.

STATEMENT OF ETHICS

Approval was obtained from the Republic of Albania Medical University of Tirana Ethical Council prot. nr. 2104/1 on 07/09/2021.

ACKNOWLEDGEMENT

We would like to express our gratitude to A. Radaelli, Department of Medical Biotechnologies and Translational Medicine, State University of Milano, Italy for lexically revising the manuscript.

REFERENCES

1. The European Centre for Disease Prevention and Control (ECDC). Available from: http://ecdc.europa.eu/en/eaad/Documents/antibiotics-consumption-EU-data-**2014.**pdf.

2. Van Boeckel T.P, Gandra S, Ashok A, Caudron Q. Grenfell B.T, Levin S.A, et al. Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. Lancet Infect Dis, **2014;** 14(8): 742-750.

3. Pouwels K, Freeman R, Muller-Pebody B, Rooney G, Henderson K, Robotham L, et al. Association between use of different antibiotics and trimethoprim resistance: going beyond the obvious crude association. J Antimicrob Chemother, **2018;** 73: 1700–1707.

4. Klein E.Y, Van Boeckel T.P, Martinez E.M, Pant S, Gandra S, Levin S.A, et al. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. Proc Natl Acad Sci U S A, **2018;** 115(15): 3463–3470.

5. Cusini A, Herren D, Bütikofer L, Plüss-Suard C, Kronenberg A, Marschall J. Intra-hospital differences in antibiotic use correlate with antimicrobial resistance rate in Escherichia coli and Klebsiella pneumoniae: a retrospective observational study. Antimicrob Resist Infect Control, **2018;** 7: 89.

6. Laxminarayan, R, Sridhar, D, Blaser, M, Wang, M, Woolhouse, M. Achieving global targets for antimicrobial resistance. Science, **2016;** 353: 874–875.

7. Almagor J, Temkin E, Benenson I, Fallach N, Carmeli Y, DRIVE-AB consortium. The impact of antibiotic use on transmission of resistant bacteria in hospitals: Insights from an agent-based model. PLoS One, **2018;** 13(5): e0197111.

8. Bengtsson-Palme J, Kristiansson E, Joakim Larsson D. Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiol Rev, **2018;** 42(1): fux053.

9. The Centers for Disease Control and Prevention. Available from: https://www.cdc.gov/antibiotic-use/healthcare/implementation/core-elements.html.

10. Klein E, Van Boeckel T, Martinez E, Pant S, Gandra S, Levin S, et al. Laxminarayana R. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. Proc Natl Acad Sci U S A, **2018;** 115(15): 3463-3470.

11. Imanpour S, Nwaiwu O, McMaughan D, De Salvo B, Bashir A. Factors associated with antibiotic prescriptions for the viral origin diseases in office-based practices, 2006–2012. JRSM Open, **2017;** 8: 1–8.

12. Havers FP, Hicks LA, Chung JR, Gaglani M, Murthy K, Zimmerman R.K, et al. Outpatient Antibiotic Prescribing for Acute Respiratory Infections During Influenza Seasons. JAMA Netw. Open, **2018;**1(2): e180243.

13. Zarb P, Amadeo B, Muller A, Drapier N, Vankerckhoven V, Davey P, et al. Identification of targets for quality improvement in antimicrobial prescribing: the web-based ESAC Point Prevalence Survey 2009. J Antimicrob Chemother, **2011;** 66: 443–449.

14. Ansari F, Erntell M, Goossens H, Davey P. The European Surveillance of Antimicrobial Consumption (ESAC) point-prevalence survey of antibacterial use in 20 European hospitals in 2006. Clin Infect Dis, **2009;** 49: 1496-504.

15. Schroeck J.L, Ruh C.A, Sellick J.Aa Jr, Ott M.C, Mattappallil A, Mergenhagen K.A. Factors Associated with Antibiotic Misuse in Outpatient Treatment for Upper Respiratory Tract Infections. Antimicrob Agents Chemother, **2015;** 59: 3848-52.

16. McDonagh M, Peterson K, Winthrop K, Cantor A, Holzhammer B, Buckley D.I. Improving Antibiotic Prescribing for Uncomplicated Acute Respiratory Tract Infections. Rockville (MD): Agency for Healthcare Research and Quality (US); **2016.** Report No.: 15(16)-EHC033-EF.

17. Zarb P, Coignard B, Griskeviciene J, Muller A, Vankerckhoven V, Weist K, et al. The European Centre for Disease Prevention and Control (ECDC) pilot point prevalence survey of healthcare-associated infections and antimicrobial use. Euro surveill, **2012;** 17(46): 20316.

18. Pristaš I, Baršić B, Butić I, Zarb P, Goossens H, Andrašević A.T. PPS on antibiotic use in a Croatian Infectious Disease Hospital. J Chemother, **2013;** 25(4): 222-8.

19. Li B, Webster T.J. Bacteria antibiotic resistance: New challenges and opportunities for implant-associated orthopedic infections. J Orthop Res, **2018;** 36(1): 22–32.

20. Ayukekbong JA, Ntemgwa M, Atabe AN. The threat of antimicrobial resistance in developing countries: causes and control strategies. Antimicrob Resist Infect Control, **2017;** 6: 47.

21. Bhatia R, Katoch VM, Inoue H. Creating political commitment for antimicrobial resistance in developing countries. Indian J Med Res, **2019;** 149(2): 83–86.

22. Klein E, Gandra S, Pant S, Mojica N, Martinez E, Laxminarayan R. Global Antibiotic Use and Resistance. Open Forum Infect. Dis, **2016;** 3/1: 1487.

23. Global point prevalence survey of antimicrobial consumption. Available from: http://www.global-pps.com.

24. WHO Collaborating Centre for Drug Statistics Methodology, Guidelines for ATC classification and DDD assignment, **2017.** Available from: https://www.whocc.no/atc_ddd_index/?code=J01 .

25. Versporten A, Zarb P, Caniaux I, Gros M.F, Drapier N, Miller M, et al. Global-PPS network. Lancet Glob. Health, **2018;** 6(6): 619 - 629.

26. Oduyebo O.O, Olayinka A.T, Iregbu K.C, Versporten A, Goossens H, Nwajiobi-Princewill P.I, et al. A point prevalence survey of antimicrobial prescribing in four Nigerian Tertiary Hospitals. Ann Trop Pathol, **2017;** 8: 42-6.

27. Mulwahali Wambale J, Liesse Iyamba JM, Masika Mathe D, Kabuyaya Kavuo S, Takaisi-Kikuni. Point prevalence study of antibiotic use in hospitals in Butembo. Int. J. Med. Med. Sci, **2016;** 8: 133-139.

28. Faria S, Sodano L, Dauri M, Sabato AF, Gjata A, Kito I, et al. First point prevalence survey of nosocomial infections in the intensive care units of a tertiary care hospital in Albania. J Hosp Infect, **2007;** 65: 244-250.

29. Røder B.L, Nielsen S.L, Magnussen P, Engquist A, Frimodt-Møller N. Antibiotic usage in an intensive care unit in a Danish university hospital. J Antimicrob Chemother, **1993;** 32: 633-42.

30. Duo-shuang X, Li-li X, Rui L, Qiao H, Qing-qin L, Wei X. A multicenter point-prevalence survey of antibiotic use in 13 Chinese hospitals. J Infect Public Health, **2015;** 8: 55-61.

31. Aghdassi S.J.S, Gastmeier P, Piening B.C, Behnke M, Peña Diaz L.A, Gropmann A, et al. Antimicrobial usage in German acute care hospitals: results of the third national point prevalence survey and comparison with previous national point prevalence surveys. J Antimicrob Chemother, **2018;** 73(4): 1077-1083.

32. Morioka H, Nagao M, Yoshihara S, Ohge H, Kasahara K, Shigemoto N, et al. The first multi-centre point-prevalence survey in four Japanese university hospitals. J Hosp Infect, **2018;** 99(3): 325-331.

33. Polk R.E, Fox C, Mahoney A, Letcavage J, MacDougall C. Measurement of adult antibacterial drug use in 130 US hospitals: comparison of defined daily dose and days of therapy. Clin Infect Dis, **2007;** 44(5): 664-70.

34. Versporten A, Drapier N, Zarb P, Caniaux I, Gros M.F, Miller M, et al. The Global Point Prevalence Survey of Antimicrobial Consumption and Resistance (Global-PPS): A Worldwide Antimicrobial Web-Based Point Prevalence Survey. Open Forum Infect. Dis, **2015;** 2(1): 147.

35. Ider B.E, Clements A, Adams J, Whitby M, Muugolog T. Prevalence of hospital-acquired infections and antibiotic use in two tertiary Mongolian hospitals. J Hosp Infect, **2010;** 75(3): 214-9.

36. Skoog G, Struwe J, Cars O, Hanberger H, Odenholt I, Prag M, et al. Skärlund K.; Ulleryd P. Repeated nationwide point-prevalence surveys of antimicrobial use in Swedish hospitals: data for actions 2003-2010. Euro Surveill, **2016;** 2: 30264.

37. Liang S.Y, Kumar A. Empiric antimicrobial therapy in severe sepsis and septic shock: optimizing pathogen clearance. Curr Infect Dis Rep, **2015;** 17(7): 493.

38. Viasus D, Simonetti A, Garcia-Vidal C, Niubó J, Dorca J, Carratalà J. Impact of antibiotic de-escalation on clinical outcomes in community-acquired pneumococcal pneumonia. J Antimicrob Chemother, **2017;** 72(2): 547–553.

Acta Pharm. Sci. Vol 59:(4), 2021
DOI: 10.23893/1307-2080.APS.05933

The Study of Phenolic Compounds and Antioxidant Activity of Raw Materials of *Reynoutria Sachalinensis* (F. Schmidt) Nakai

Abdulrazzaq Yasir ALRIKABI, Viktoriia PROTSKA, Nadiia BURDA*, Iryna ZHURAVEL, Viktoriia KUZNETSOVA

Department of Chemistry of Natural Compounds and Nutritiology, National University of Pharmacy, Kharkiv, Ukraine

ABSTRACT

Phenolic composition and quantitative evaluation of herbal part and root extracts for *Reynoutria sachalinensis* studied by HPLC method. Using the same method, we also established the antioxidant activity of *R. sachalinensis* raw materials. Six phenolic compounds were idenified for herbal part of *R. sachalinensis* as, gallic acid, chlorogenic acid, *trans*-cinnamic acid, rutin, hyperoside and isoquercitrin at total amount of 885.37±21.25 mg/kg. Neochlorogenic acid and rutin were found as main compounds for herbal part of *R. sachalinensis,* and gallic acid and 6,7-dihydroisoflavone were determined for root of *R. sachalinensis*. In *R. sachalinensis* roots we found gallic acid and 6,7-dihydroisoflavone.

The HPLC study of antioxidant activity showed almost identical antioxidant potential of bioactive substances (BASs) in *R. sachalinensis* herbal parts and roots that is 3.85±0.09 and 3.59±0.09 mg/g in Trolox equivalent respectively.

The obtained data proved the feasibility of new antioxidant drugs development on the basis of *R. sachalinensis* raw materials.

Keywords: *Reynoutria sachalinensis*, phenolic compounds, HPLC

INTRODUCTION

Reynoutria sachalinensis (F. Schmidt) Nakai (synonyms: *Polygonum sachalinensis* F. Schmidt, *Fallopia sachalinensis* (F. Schmidt) Ronse Decr., *Pleuropterus sachalinensis* (F. Schmidt) H. Gross, *Tiniaria sachalinensis* (F. Schmidt)

*Corresponding Author: Nadiia Burda, e-mail: nadegdaburda@ukr.net
ORCIDs:
Abdulrazzaq Yasir Alrikabi : 0000-0002-2791-6073
Viktoriia Protska : 0000-0002-2439-138X
Nadiia Burda : 0000-0002-7435-5731
Iryna Zhuravel : 0000-0001-8092-733X
Viktoriia Kuznetsova : 0000-0002-4146-689X
(Received 07 August 2020, Accepted 16 Apr 2021)

Janch.) is a perennial herbaceous plant of *Polygonaceae* family. The plant originates from East Asia, growing in Korea, Japan, the Kurile Islands and Sakhalin. This species first appeared in Europe in 1855 and has been grown at botanical gardens as an ornamental plant. *R. sachalinensis* is met as a weed in many European countries, including Ukraine[1, 2].

Anthraquinones (physcion, I-O-methylemodin, emodin) and stilbene (*trans*-resveratrol) possessing cytotoxic activity were separated from methanol extract of herbal part of *R. sachalinensis*[3].

Another species of *Reynoutria* genus which is also under study, *R. japonica,* contains phenolic compounds, including flavonoids, anthraquinones, condensed tanning agents and stilbenes, polysaccharides. Extracts from *R. japonica* demonstrate antipyretic, analgesic and anti-inflammatory activities[4].

HPLC/UV/ESI-MS studies of the rhizomes of *R. japonica*, *R. sachalinensis and Reynoutria* x *bohemica* revealed 171 compounds, comprising stilbenes, carbohydrates, procyanidins, flavan-3-ols, anthraquinones, phenylpropanoids, lignin oligomers, hydroxycinnamic acids, naphthalenes and their derivatives[5].

Antimicrobial activity of acetone extract from rhizome of *R. japonica* and *R. sachalinensis*, as well as of their hybrid *Reynoutria* x *bohemica* was studied as regards the caries-inducing pathogens, against *Streptococcus mutans*. The most active extract was found for *R. japonica rhizome*[6].

In Ukraine R. sachalinensis is not a pharmacopoeia-registered plant, still, issuing from the experience of folk medicine application of this plant in East Asian countries as well as from the results of scientific research, we may foresee its feasibility for medical drugs development.

As the oxidative stress in human organism may provoke the diseases of different severity, including cancers, atherosclerosis, neurodegenerative diseases (Parkinson's, Alzheimer's, etc.), hypertension, diabetes mellitus, cardiovascular diseases, reproductive system dysfunctions, etc, search of promising antioxidants of herbal origin has become an important aspect in pharmacy[7-12].

Antioxidant activity were studied of phenolic compounds as, anthraquinones (emodin, emodin-8-O-beta-D-glucopyranoside and physcion-8-O-beta-D-glucopyranoside) and flavonoids (quercetin-3-O-alpha-L-arabinofuranoside, quercetin-3-O-beta-D-galactopyranoside and quercetin-3-O-beta-D-glucuronopyranoside) for flower extracts of *R. sachalinensis in* our previous study[13].

Therefore, for deeper understanding of *R. sachalinensis* application prospects, it turned out feasible to study phenolic substances in herbal and roots extracts

of this plant as well as its antioxidant activity. We conducted a comparative study of *R. sachalinensis* herbal parts and roots.

METHODOLOGY

Plant materials

In experiments we used air-dried milled roots and herbal parts of *R. sachalinensis*. Herbal parts were collected within blossoming period in June, roots – in September in Kharkiv Region, Ukraine during 2018-2019.

The plant material sample was identified by Prof. *Tatyana* Gontova, Department of Botany, National University of Pharmacy, Ukraine and voucher specimens were deposited at National University of Pharmacy, Ukraine.

Extraction

Extracts for analysis were prepared by extracting 0.3 g milled raw material with 10 mL methanol within 20 min on ultrasonic bath at 20±2°C. The obtained extracts were filtered through a membrane filter (0.45 μm)[14].

General experimental procedures

For study of phenolic compounds in *R. sachalinensis*, as well as determination of antioxidant activity by HPLC method (Waters Corporation, Milford, USA) with Waters 996 PDA photodiode matrix detector, (Waters Corporation, USA), Wise Clean WUC-A06H ultrasonic cleaning set (**Daihan**, Korea), ANG 100 analytical balance (AXIC, Poland), standard samples of substances and solvents for chromatographic analysis from Merck KGaA (Darmstadt, Germany).

Chromatographic analysis by HPLC

Chromatographic separation of phenolic compounds was performed using ACE 5 C18 column 250 mm × 4.6 mm (Pennsylvania, USA). Elution flow rate was 1 ml/min. Mobile phase binary solvent system consisted of solvent A (0.1% acetic acid aqueous solution) and solvent B (acetonitrile). All solvents passed ultrasonic degassing and 0.23 μm pore size membrane filter. Linear gradient program looked as follows:

Time, min	Solvent A, %	Solvent B, %
0–8	5–15	95-85
8–30	15–20	85-80
30–48	20–40	80-60
48–58	40–50	60-50
58–65	50	50
65–66	50–95	50-5

The column had constant temperature of 25°C. 10 μl samples were injected[13].

For determination of antioxidant activity after application of HPLC-PDA detector system the mobile phase containing tested samples was fed with Gilson 305 pump (Middleton, WI, USA) to the column via mixing tee with ABTS reagent in split relation 1:1. A Teflon column (Waters PCR module, Milford, CT, USA) 3 m long and 0.25 mm in diameter was used, its granularity being 1.58 μm. ABTS solution system control parameters: column temperature circa 50°C, mobile phase flow rate 0.5 ml/min[15,16].

Sample color change in mixture with ABTS reagent after reaction ending was recorded using Waters 2487 UV/VIS detector (Waters Corporation) at wavelength of 650 nm.

In selection of analysis terms we were guided by the signal value expressed in negative peak height as a sensitivity indicator. The antioxidant potential of tested samples was determined by the comparison with that of Trolox standard solution in eight different concentrations within the range of 0.625–80 mg/ml. The constructed calibration plot was expressed with the following quadratic equation:

$Y= -1.54 \cdot 10^2X^2 + 4.16 \cdot 4.16 \cdot 10^4x - 2.08 \cdot 10^4$; R^2 (ABTS) = 0.9991.

Antioxidant potential of extracts (X, mg/g) was calculated by formula:

$$X = \frac{m_0 \cdot 20000}{m_1 \cdot (100 - w)},$$

where m_0 – mass of Trolox standard sample, g; m_1 – mass of tested sample, g; w – drying loss, wt %[14, 15].

RESULTS AND DISCUSSION

The results of our study enabled the identification of six phenolic compounds in *R. sachalinensis* herbal parts: phenolcarbonic gallic acid, two hydroxycinnamic acids (neochlorogenic, *trans*-cinnamic) and three flavonoids (rutin, hyperoside and isoquercitrin). In roots of this plant we found only gallic acid and 6,7-dihydroisoflavone. HPLC chromatograms of phenolic compounds in *R. sachalinensis* herbal parts and roots are shown in Fig. 1-2.

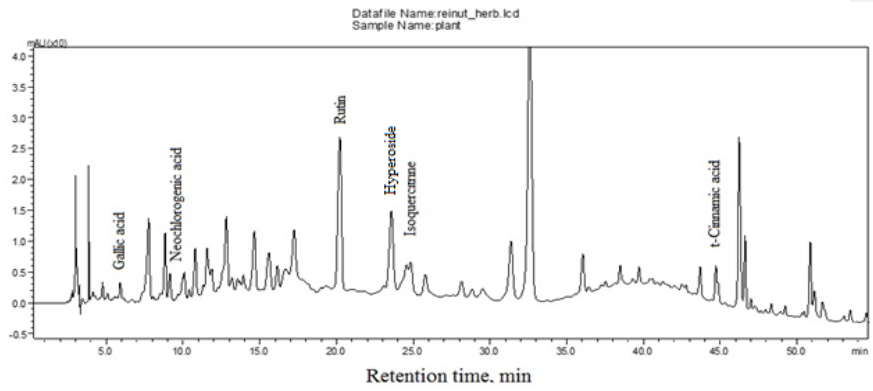


Figure 1. HPLC chromatogram of phenolic compounds in *R. sachalinensis* herbal parts

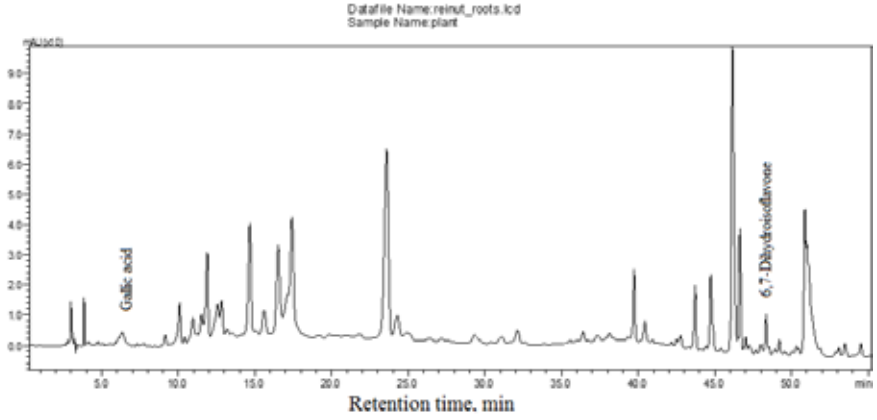


Figure 2: HPLC chromatogram of phenolic compounds in *R. sachalinensis* roots

The qualitative composition and quantitative content of phenolic compounds in *R. sachalinensis* herbal parts and roots are presented in Table 1.

Table 1. Qualitative composition and quantitative content of phenolic compounds of *R. sachalinensis* raw materials

Compound	Herbal parts		Roots	
	Retention time, min	Quantitative content, mg/kg	Retention time, min	Quantitative content, mg/kg
Phenolic acids				
Gallic acid	5.91	21.99 ± 0.55	5.91	6.98 ± 0.17
Total content of phenolic acids	—	21.99 ± 0.55	—	6.98 ± 0.17
Cinnamic acids				
Neochlorogenic acid	8.84	407.17 ± 8.56	—	—
trans -Cinnamic acid	44.72	23.39 ± 0.47	—	—
Total content of cinnamic acids	—	430.56 ± 9.90	—	—
Flavonoids				
Rutin	20.52	189.72 ± 4.55	—	—
Hyperoside	24.55	115.61 ± 2.54	—	—
Isoquercitrin	24.83	127.49 ± 2.93	—	—
6,7-Dihydroisoflavone	—	—	47.99	10.32 ± 0.24
Total content of flavonoids	—	432.82 ± 10.82	—	10.32 ± 0.24
Total content of identified compounds	—	885.37 ± 21.25	—	17.30 ± 0.43

Results are expressed as means ± SD of three measurements; $p<0.05$; «—» - not identified

The total content of identified compounds in *R. sachalinensis* herbal parts was 885.37±21.25 mg/kg. The amounts of hydroxycinnamic acids and flavonoids in this plant: 430.56±9.90 and 432.82±10.82 mg/kg respectively. Gallic acid (21.99±0.55 mg/kg) accounted for circa 2.5 % of the total content of identified compounds.

Neochlorogenic acid and rutin dominated in *R. sachalinensis* herbal parts. This raw material contained 407.17±8.56 mg/kg chlorogenic acid, 189.72±4.55 mg/kg rutin, 115.61±2.54 mg/kg hyperoside and 127.49±2.93 mg/kg isoquercitrin.

The total content of identified phenolic compounds in *R. sachalinensis* roots was 17.30±0.43 mg/kg. Gallic acid content in this part of plant was 3.3 times less than in its herbal parts. 6,7-dihydroisoflavone (10.32±0.24 mg/kg) accounted for almost 60% of the sum of identified compounds.

Antioxidant activity of bioactive substances in *R. sachalinensis* herbal parts and roots were studied *in vitro* by HPLC method in Trolox equivalent. Antioxidant activity chromatograms of *R. sachalinensis* herbal parts are shown in Fig. 3, that of the roots in Fig. 4.

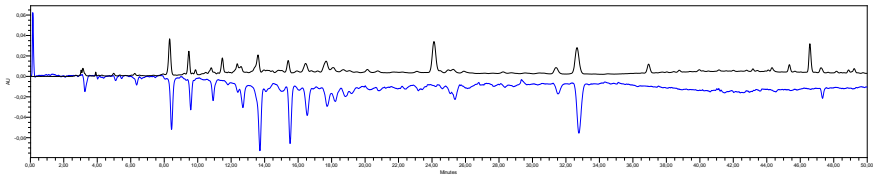


Figure 3: HPLC chromatogram of antioxidant activity determoination of *R. sachalinensis* herbal parts

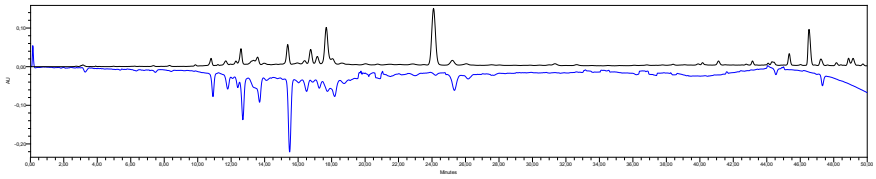


Figure 4: HPLC chromatogram of antioxidant activity determibation in *R. sachalinensis* roots

The experimental results are presented in Table 2.

Table 2. Antioxidant activity of BASs in *R. sachalinensis* raw materials

Extracts	Antioxidant capacity in Trolox equivalent, mg/g
Herbal part of extract	3.85±0.09
Roots	3.59±0.09

Results are expressed as means ± SD of three measurements; $p<0.05$.

The obtained data showed that antioxidant activities of bioactive substances in *R. sachalinensis* herbal parts and roots were in close proximity and made 3.85±0.09 and 3.59±0.09 mg/kg respectively.

Our research proved much higher versatility of phenolic compounds composition in *R. sachalinensis* herbal parts as compared to its roots. The quantitative content of those bioactive substances was much higher in *R. sachalinensis* herbal parts. The study of antioxidant activity confirmed that the antioxidant potential of both parts of tested plant was almost at an identical level. The obtained data enable our deeper knowledge of *R. sachalinensis* chemical composition and pharmacological activity and confirm the feasibility of drugs devel-

opment on the basis of *R. sachalinensis* raw materials, including drugs with antioxidant activity. Besides, this information will be useful in the development of quality control methods for *R. sachalinensis* raw materials.

ACKNOWLEDGMENTS

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

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Širka VH, Lakušić D, Šinžar-Sekulić J, Nikolić T, Jovanović S. Reynoutria sachalinensis: a new invasive species to the flora of Serbia and its distribution in SE Europe. Bot Serb, **2013;** 37(2): 105-112.

2. Alrikabi AYa, Tartynska HS, Zhuravel IO. A study in elementary composition of Sakhalin knotweed (Reynoutria sachalinensis (F. Schmidt) Nakai) herb. Fitoterapiia. A Journal, **2019;** 1: 70-72. – in Ukrainian. https://doi.org/10.33617/2522-9680-2019-1-70

3. Jin W, Na MK, Song GY, Lee YM, Bae KH. Cytotoxic anthraquinones and stilbenes from Reynoutria sachalinensis (Fr. Schm.) Nakai. Korean Journal of Medicinal Crop Science, **2005;** 13(2): 80-84.

4. Patocka J, Navratilova Z, Ovando M. Biologically active compounds of Knotweed (Reynoutria spp.). Mil Med Sci Lett (Voj. Zdrav. Listy), **2017;** 86(1): 17-31.

5. Nawrot-Hadzik I; Ślusarczyk S; Granica S; Hadzik J; Matkowski A. Phytochemical Diversity in Rhizomes of Three Reynoutria Species and their Antioxidant Activity Correlations Elucidated by LC-ESI-MS/MS Analysis. Molecules, **2019;** 24(6): 1136. https://doi.org/10.3390/molecules24061136

6. Nawrot-Hadzik I, Hadzik J, Fleischer M, Choromańska A, Sterczała B, Kubasiewicz-Ross P, et al. Chemical composition of East Asian invasive Knotweeds, their cytotoxicity and antimicrobial efficacy against cariogenic pathogens: an in-vitro study. Med Sci Monit, **2019;** 25: 3279-3287. https://doi.org/10.12659/MSM.913855

7. Uttara B, Singh AV, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. Curr Neuropharmacol, **2009;** 7(1): 65-74. https://doi.org/10.2174/157015909787602823

8. Gupta RK, Patel AK, Shah N, Choudhary AK, Jha UK, Yadav UC, et al. Oxidative stress and antioxidants in disease and cancer: a review. Asian Pac J Cancer Prev, **2014;** 15: 4405-4409. https://doi.org/10.7314/apjcp.2014.15.11.4405

9. Vaziri ND, Rodríguez-Iturbe B. Mechanisms of disease: oxidative stress and inflammation in the pathogenesis of hypertension. Nat Clin Pract Nephrol, **2006;** 2: 582-593. https://doi.org/10.1038/ncpneph0283

10. Pohanka M. Alzheimer´s disease and oxidative stress: a review. Curr Med Chem, **2014;** 21(3): 356-364. https://doi.org/10.2174/09298673113206660258

11. Kruk J, Aboul-Enein HY, Kładna A, Bowser JE. Oxidative stress in biological systems and its relation with pathophysiological functions: the effect of physical activity on cellular redox homeostasis. Free Radic Res, **2019;** 53(5): 497-521. https://doi.org/10.1080/10715762.2019.1612059

12. Pohanka M. Role of oxidative stress in infectious diseases. A review. Folia Microbiol, **2013;** 58: 503-513. https://doi.org/10.1007/s12223-013-0239-5

13. Zhang X, Thuong PT, Jin WY, Su N.D, Sok DE, Bae KH, et al. Antioxidant activity of anthraquinones and flavonoids from flower of Reynoutria sachalinensis. Arch Pharm Res, **2005;** 28(1): 22-27. https://doi.org/10.1007/BF02975130

14. Glushchenko A, Bezruk I, Ivanauskas L, Georgiyants V. Development of the method of simultaneous quantitative determination of loratadine and auxuilary substances in the combined syrup «Loratadin+». ScienceRise: Pharmaceutical Science, **2019;** 2(18): 39-47. https://doi.org/10.15587/2519-4852.2019.169511

15. Raudonis R., Raudone L, Jakstas V, Janulis V. Comparative evaluation of post-column free radical scavenging and ferric reducing antioxidant power assays for screening of antioxidants in strawberries. J Chromatogr *A,* **2012;** 1233: 8-15. https://doi.org/10.1016/j.chroma.2012.02.019

16. Raudonis R, Jakštas V, Burdulis D, Benetis R., Janulis V. Investigation of contribution of individual constituents to antioxidant activity in herbal drugs using postcolumn HPLC method. Medicina, **2009;** 45(5): 382-394.

Acta Pharm. Sci. Vol 59:(4), 2021
DOI: 10.23893/1307-2080.APS.05934

Relationship Between Interleukin 33 and Tissue Factor in Non-Diabetic and Diabetic Obese Patients

Cagri CAKICI[1*], Turkan YIGITBASI[1], Maide Hacer ALAGOZ[2], Pakize YIGIT[3], Nesrin EMEKLI[1]

1 Department of Biochemistry, Faculty of Medicine, Istanbul Medipol University, Istanbul, Turkey

2 Department of Clinical Biochemistry, Cekirge Devlet Hastanesi, Bursa, Turkey

3 Departmant of Department of Biostatistics and Medical Informatics, Faculty of Medicine, Istanbul Medipol University, Istanbul, Turkey

ABSTRACT

The aim of the study is to investigate the relationship between tissue factor (TF) and Interleukin-33 (IL-33) and biochemical parameters in diabetic obese and non-diabetic obese patients. 21 healthy controls, 25 non-diabetic and 36 diabetic-obese patients were included in the study. While there was no difference between the groups in terms of IL-33 levels ($p > 0.05$), TF levels of diabetic obese group were statistically significantly higher than control group ($p < 0.05$). HDL levels of the obese and diabetic obese groups were significantly lower than control group, and triglyceride, glucose, insulin, C-reactive protein (CRP), hemoglobin A1c (HbA1c), and HOMA-IR levels were significantly higher ($p < 0.05$). A positive correlation was found between TF activity, and HbA1c and glucose levels ($p < 0.05$). This suggests that TF may be predictive for diabetes which develop in the background of obesity, and that TF can be used as a prognostic value for diabetes.

Keywords: Diabetes Mellitus, interleukin 33, obesity, tissue factor

INTRODUCTION

Obesity is a chronic disease which is caused by disruption of energy balance,and characterized by increased body fat mass[1]. Adipose tissue with chronic low-grade inflammation can contribute to the metabolic consequences of obesity.

*Corresponding Author: Cagri Cakici, e-mail: ccakici@medipol.edu.tr
ORCIDs:
Cagri Cakici : https://orcid.org/0000-0002-8662-5284
Turkan Yigitbasi : https://orcid.org/0000-0002-0675-1839
Maide Hacer Alagoz : https://orcid.org/0000-0003-2032-6684
Pakize Yigit: https : //orcid.org/0000-0002-5919-1986
Nesrin Emekli : https://orcid.org/0000-0002-0109-5086
(Received 06 February 2021, Accepted 16 April 2021)

Therefore, obesity thought to play a role in both atherosclerosis and diabetes development[2].

Diabetes Mellitus (DM) is a metabolic disease of multiple etiologies. It is characterized by chronic hyperglycemia with impaired carbohydrate metabolism, due to insulin secretion and / or defects in its progression[3].

Despite various studies, the increased rate of obesity and health problems due to obesity comorbidities such as cardiovascular diseases and health expenditures are gradually increased[4].

It is observed that patients, whose body mass index (BMI) remain over 30 kg/m^2 more for than ten years, are twice as much susceptible to the risk of diabetes compared to the those with the same BMI for five years[2]. Type 2 Diabetes Mellitus (T2DM), which is typically caused by obesity, is the most common disease among carbohydrate metabolism disorders[4]. Because of this close relationship between diabetes and obesity, the concept of "diabesity" was coined. "Diabesity" is a term which refers to diabetes occurring in the context of obesity[2].

Besides requiring lifelong treatment, DM also negatively affects quality of life because of acute and chronic complications[4]. Uncontrolled DM is the cause of multiple organ damage as a result of macrovascular and microvascular complications. It also increases the risk of cardiovascular disease by twice[5].

TF, also known as factor III or thromboplastin, is the primary initiator of the extrinsic blood coagulation system[6]. TF is expressed by epithelial cells around the blood vessels, such as adventitial fibroblasts, and plays a critical role in hemostasis. TF also contributes to various forms of thrombosis. Besides these well-known features, TF is highly expressed in many types of cancer, especially adenocarcinomas[7]. It is known that TF levels, which are also associated with increased appetite, decrease as a result of weight loss in obesity. It reduces circulating prothrombotic marker levels, including TF and plasminogen activator inhibitor-1 (PAI-1)[8,9].

Interleukin 33 (IL-33), which is a nuclear associated cytokine and the ligand of the ST2 receptor, belongs to the interleukin 1 (IL-1) family. It is abundantly expressed in endothelial cells, epithelial cells and fibroblast-like cells, during homeostasis and inflammation[10]. Studies have shown that IL-33 modulates inflammatory diseases such as arthritis and atherosclerosis, as well as other inflammatory diseases of the gastrointestinal, and the respiratory systems. At the same time, both IL-33 and ST2 are abundantly expressed in human atherosclerotic plaques[11]. Besides, it was found that both IL-33 and ST2 are expressed in adipocytes and adipose tissues. IL-33 was found to have a protective effect in

adipose tissue inflammation during obesity, in that it induces the production of Th2 cytokines (IL-5 and IL-13), reduces lipid storage, inhibits adipogenesis and promotes lipolysis. In addition, the treatment of obese diabetic (ob/ob) mice with IL-33 has led to protective metabolic effects such as significantly lower adiposity, lower fasting glucose, and increased glucose and insulin sensitivity[12].

It is shown that IL-33 induces TF expression depending on the ST2 receptor and the NF-κ B pathway human umbilical vein endothelial cells (HUVECs) and coronary artery endothelial cells (HCAECs)[11].

However, the relationship between IL-33 and TF in non-diabetic and diabetic obese patients, and their association with other metabolic parameters have not yet been revealed. Therefore, the aim of this study is to investigate the relationship between TF and IL-33 in diabetic and non-diabetic obese patients.

METHODOLOGY

Ethics committee approval was obtained from the Istanbul Medipol University Non-Interventional Clinical Research Ethics Committee (Decision No: 335).

21 healthy controls, 25 non-diabetic obese and 36 diabetic-obese patients who applied to Istanbul Medipol Mega Hospital Biochemistry Laboratory were included in the study. BMI is a person's weight in kilograms divided by the square of body height in meters (kg/m^2). Patients with BMI values ranging between 18.9 and 24.9 kg / m^2 were considered normal, while patients with BMI values greater than 24.9 kg / m^2 were considered obese[13]. Diagnosis of diabetes was identified based on clinical and laboratory findings according to the American Diabetes Association (ADA) criteria, HbA1c levels above 6.5% were included in the diabetic obese group, and the others were included in the non-diabetic obese group[14].

Exclusion criteria were; being under 18 years old, being over 75 years old, smoking, having hypertension, heart disease, polycystic ovarian disease, inflammation, and infection.

Blood collection and storage

After obtaining consent forms from selected reference individuals, blood samples were collected between 08:00-12:00 in the morning, at the end of 8-12 hours of fasting. Blood samples were taken from the antecubital vein in a sitting position into 8-milliliter vacuum gel red-capped tubes. The blood samples were centrifuged at 3000 rpm for 10 minutes with the NUVE (NF-800R) brand centrifuge in the clinical biochemistry laboratory, and serum samples were separated. The separated serum samples were taken into Eppendorf tubes and stored at -80°C until study.

Parameters examined in serum

IL-33 and TF serum concentrations were measured using the ELISA method (Cusabio Elisa kit, catalog no: CSB-E13000h and catalog no: CSB-E07913h, respectively). The *R*-square (R^2) values were calculated as 0.97 and 0.99 respectively on the standard graph created to reflect the results of the measurements. The measurements were taken using BioTek Synergy HTX multimode reader. Glucose, insulin, TC, HDL, LDL, and TG serum concentrations were measured using Roche / Hitachi C501 autoanalyzer, with a commercial kit by a photometric method. Serum concentrations of HbA1c and CRP were measured by Roche / Hitachi Cobas autoanalyzer using a commercial kit by an immune chemiluminescence method. Insulin resistance was calculated using the formula; (HOMA-IR) = [fasting insulin X glucose] / 22.5 [15].

Statistical Analysis

SPSS 22 (IBM, Chicago) software was used for statistical analysis. The results are presented as mean values ± standard deviations ($\bar{x}$ ± SD). T-test was used to compare parameters of obese and control groups which exhibit a normal distribution, and the Mann-Whitney U test was used to compare two groups which do not exhibit a normal distribution. One-way analysis of variance (ANOVA) was used to compare the differences of variables in subgroup analysis. Kruskal Wallis and Post-hoc Dunn tests were also used for parameters which did not exhibit normal distribution. The correlation graphs were created using the SPSS software and the bar graphs were created using the Graphpad Prism 8 software. The significance level was accepted as $p < 0.05$ for all tests.

RESULTS AND DISCUSSION

IL-33 is a proinflammatory cytokine from the IL-1 family, which is located in the homeostatic system and is a ST2 receptor ligand. In the animal study, it was revealed that the IL-33 / ST2 effect played a role in the modulation of obesity, insulin resistance, and inflammatory pathologies of T2DM[16,17].

Elevated levels of TF are found in atherosclerotic plaques, and TF triggers thrombosis after plaque rupture. It is known that IL-33 is expressed in atherosclerotic plaques and endothelial cells. Also, endothelial cells and atherosclerotic plaques are involved TF secretion[11]. Because of this connection, the relationship between IL-33 and TF was the subject of two research studies by Stojkovic et al.[11,18].

In the first study, Stojkovic et al. used HUVECs and HCAECs cell lines to demonstrate that IL-33 induces TF expression of the ST2 receptor and the NF-κ B pathway, and promotes TF activity in microparticles produced from endothelial cells. Moreover, the researchers found a positive correlation between the

expression of TF mRNA and IL-33 mRNA in human carotid atherosclerotic plaques[11]. In the second study, Stojkovic et al. demonstrated that the effects of IL-33 on TF expression are related to the amounts of ST2 receptors on the monocyte surface[18].

This is the first study to investigate the relationship between serum IL-33 and TF in non-diabetic obese and diabetic obese study groups.

As shown in Table 1, the obese group had significantly higher levels of serum TF compared to the control group ($p < 0.05$), whereas IL-33 values showed no difference between the two groups ($p > 0.05$). Comparing the obese group to the control group; HDL levels are significantly lower, while TG, Glucose, insulin, CRP, Hb1Ac and HOMA-IR values were higher ($p < 0.05$). There was no difference between total cholesterol and LDL levels between the two groups ($p > 0.05$) **(Table 1) (Figure 1)**.

Table 1. Biochemical parameters of obese and control groups

	Control Group (n=21)	Obese Group (n=61)	P*
	$\bar{x} \pm SD$	$\bar{x} \pm SD$	
BMI (kg/m²)	23,44 ± 0,84	33,75 ± 6,15	**<0,05**
TF (pg/ml)	53,14 ± 24,75	71,90 ± 34,51	**<0,05**
IL-33 (pg/ml)	5,22 ± 1,58	5,54 ± 2,02	**>0,05**
Glucose (mg/dl)	102,69 ± 17,22	151,96 ± 69,05	**<0,05**
HDL (mg/dl)	53,86 ± 10,00	47,21 ± 9,08	**<0,05**
LDL (mg/dl)	121,52 ± 18,36	121,98 ± 27,41	**>0,05**
TG (mg/dl)	119,73 ± 28,68	158,10 ± 51,44	**<0,05**
TC (mg/dl)	192,57 ± 24,17	198,35 ± 31,96	**>0,05**
Insulin (µIU/ml)	9,70 ± 3,01	17,21 ± 8,53	<0,05
CRP (mg/l)	3,55 ± 2,71	7,73 ± 6,98	<0,05
HbA1c (%)	5,42 ± 0,52	6,86 ± 1,68	<0,05
HOMA-IR	2,43 ± 0,76	6,40 ± 7,46	<0,05

Abbreviations: BMI: Body mass index; TF: Tissue factor; IL-33: Interleukin 33; HDL: High density lipoprotein; LDL: Low density lipoprotein; TG: Triglyceride; TC: Total Cholesterol; CRP: C reactive protein; HbA1c: Hemoglobin A1c; HOMA-IR: Insulin resistance. ***p<0,05 was considered statistically significant.**

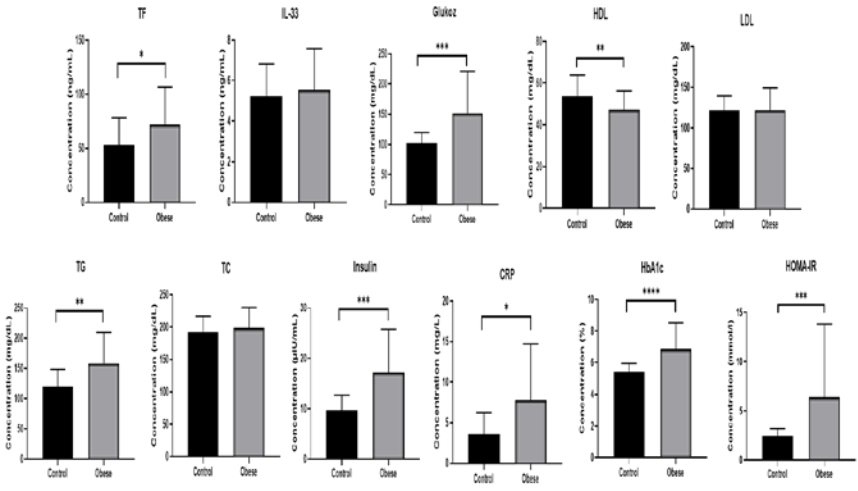


Figure 1: The bar graphs of parameters that show statistical significance in obese and control groups.

Abbreviations: TF: Tissue Factor; IL-33: Interleukin 33; HDL: High density lipoprotein; LDL: Low density lipoprotein; TG: Triglyceride; TC: Total Cholesterol; CRP: C reactive protein; HbA1c: Hemoglobin A1c; HOMA-IR: Insulin resistance. Statistical significances are indicated by asterisks. ***p<0,05; ****p<0,01; *****p<0,001; ******p<0,0001**.

Obesity contributes to the development of diabetes and being obese generally aggravates the prognosis of diabetes[19]. In obesity, inflammation and metabolic changes lead to an increase in TF expression in adipose tissue and macrophages[9]. Confirming our findings, Ayer et al. observed that TF increased in obese patients[20]. In other studies, it was revealed that thrombin formation and TF levels decreased significantly when obese individuals with loose weight[21,22]. Singh et al. showed that TF levels increased in the circulation system during childhood obesity and high levels of TF increased risk of cardiovascular diseases[23]. Studies with obese mice report that there is increased expression of TF mRNA in adipocytes as well as in adipose tissue stromal vascular cells. In addition, there was also increased TF activity in the circulation system and adipose tissue[9,24].

The protective role played by IL-33 against obesity-related inflammation, insulin resistance, and T2DM was also demonstrated in animal studies. However, a limited number of human studies on IL 33 levels in obesity are controversial[25,26]. Similar to our results, a study done by Zeyda et al., showed no significant changes in IL-33 levels, but a significant increase in sST2 levels in

morbidly obese subjects[25]. Tang et al. study's showed that serum IL-33 levels were increased in overweight/obese Chinese population. Increased IL-33 levels have positive correlation with metabolic syndrome[27]. In the other study, IL-33 levels were found to be lower compared to the overweight and obese groups[26]. It was inferred that the high IL-33 levels were due to the protective effect of IL-33 against obesity[28,29].

CRP levels were found to be higher than those of the control group, which underlies inflammation in obesity. In obese individuals, there has been a relationship between CRP levels and basal peripheral blood mononuclear cell TF procoagulant activity which suggests a link between inflammation brought by obesity and thrombosis[20].

When the control, non-diabetic and diabetic obese groups are compared; TF values were examined, and a statistically significant increase was observed in the diabetic obese group compared to the control group ($p < 0.05$). Although the TF levels were numerically higher in the non-diabetic obese group compared to the control group, there was no statistical significance ($p > 0.05$). In IL-33 levels; there was no significant difference between the control, non-diabetic obese, and diabetic obese groups ($p > 0.05$). In the non-diabetic obese group, Insulin and CRP levels were found to be significantly higher than control group ($p < 0.05$). In addition, in the diabetic obese group, glucose, TG, insulin, CRP, Hb1Ac, and HOMA-IR levels were significantly higher, and HDL was significantly lower than in the control group ($p < 0.05$). There was no difference in TC and LDL levels ($p > 0.05$). In the diabetic obese group, Hb1Ac and glucose levels were significantly higher than in the non-diabetic obese group ($p < 0.05$) **(Table 2) (Figure 2).**

Table 2. Biochemical parameters of control, non-diabetic and diabetic obese groups

	Control (1) n=21	Non-Diabetic Obese (2) n=25	Diabetic Obese (3) n=36	P*	*Intergroup Significance
	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD		
BMI (kg/m²)	23,44 ± 0,84	33,91 ± 4,64	33,65 ± 7,07	**<0,05**	1-3;1-2
TF (pg/ml)	53,14 ± 24,75	64,01 ± 32,95	77,38 ± 34,95	**<0,05**	1-3
IL-33 (pg/ml)	5,22 ± 1,58	5,70 ± 2,02	5,43 ± 2,04	>0,05	-
Glucose (mg/dl)	102,69 ± 17,22	100,40 ± 7,84	187,76 ± 70,10	**<0,05**	1-3;2-3
HDL (mg/dl)	53,86 ± 10,00	49,60 ± 9,86	45,54 ± 8,22	**<0,05**	1-3
LDL (mg/dl)	121,52 ± 18,36	123,86 ± 30,08	120,67 ± 25,75	>0,05	-
TG (mg/dl)	119,73 ± 28,68	151,01 ± 51,84	163,03 ± 51,31	**<0,05**	1-3
TC (mg/dl)	192,57 ± 24,17	196,12 ± 33,22	199,90 ± 31,43	>0,05	-
Insulin (µIU/ml)	9,70 ± 3,01	16,85 ± 8,54	17,46 ± 8,64	**<0,05**	1-2;1-3
CRP (mg/l)	3,55 ± 2,71	7,21 ± 6,60	8,47 ± 7,57	**<0,05**	1-2;1-3
HbA1c (%)	5,42 ± 0,52	5,37 ± 0,42	7,89 ± 1,43	**<0,05**	1-3;2-3
HOMA-IR	2,43 ± 0,76	4,00 ± 2,32	8,06 ± 9,20	**<0,05**	1-3

Abbreviations: BMI: Body mass index; TF: Tissue factor; IL-33: Interleukin 33; HDL: High density lipoprotein; LDL: Low density lipoprotein; TG: Triglyceride; TC: Total Cholesterol; CRP: C reactive protein; HbA1c: Hemoglobin A1c; HOMA-IR: Insulin resistance. ***p<0,05 was considered statistically significant.**

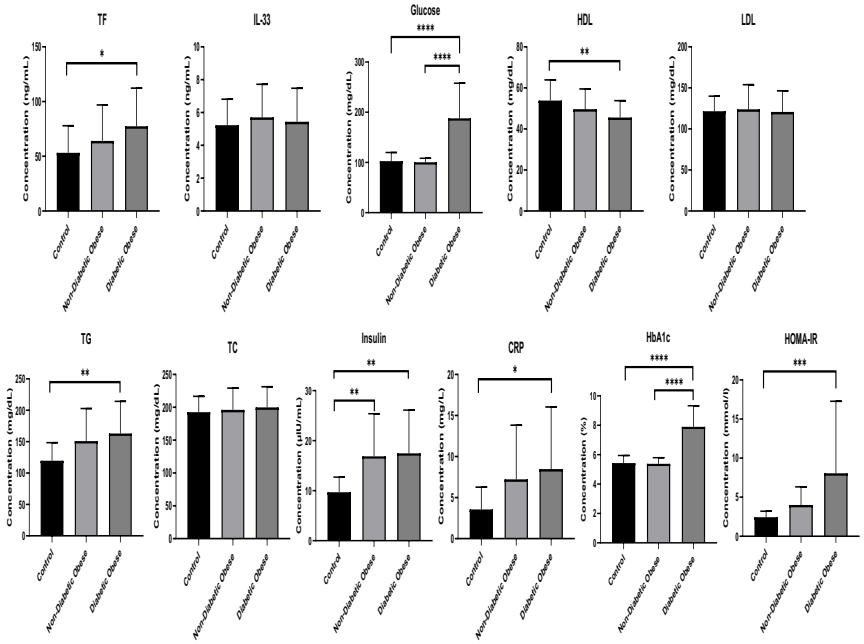


Figure 2: The bar graphs of statistically significant parameters in control, non-diabetic obese and diabetic obese groups.

Abbreviations: TF: Tissue Factor; IL-33: Interleukin 33; HDL: High density lipoprotein; LDL: Low density lipoprotein; TG: Triglyceride; TC: Total Cholesterol; CRP: C reactive protein; HbA1c: Hemoglobin A1c; HOMA-IR: Insulin resistance. Statistical significances are indicated by asterisks. ***p<0,05; **p<0,01; ***p<0,001; ****p<0,0001.**

In the study comparing diabetic obese and non-diabetic obese groups, plasma TF antigen, activity, and adipose tissue TF mRNA were found to be higher in the diabetic obese group in comparison to the non-diabetic obese group[30]. A study on patients with T2DM found that in cases where hyperglycemia and hyperinsulinemia exist together, there is increased expression of monocyte TF as well as increased platelet interaction with monocytes. Hyperglycemia and hyperinsulinemia alone also stimulate platelet activation, and monocyte TF expression is increased by selective hyperinsulinemia[31]. As reported by previous studies, the prevalence of thrombosis is high among diabetes patients and these patients also exhibit high TF activity[32,33,34,35,36]. In diabetic obese group, high TF levels were the result of inflammation, hyperlipidemia, hyperglycemia, and hyperinsulinemia[37].

The effect of IL-33 on TF protein production and TF activity was also concentration-dependent, whereby substantial effects were observed at concentrations > 0.1 ng / mL in both HUVECs and HCAECs[11]. In our study no correlation was observed between IL-33 and TF for both the diabetic obese and non-diabetic groups. In the other study, it was found that different layers of monocytes have different amounts of ST2 receptors, and the effect of IL-33 depends on ST2 receptor density[18].

In our study, IL-33 levels were measured in serum. It can be inferred that IL-33 concentration can modulate TF expression and activity in local tissue or the amount of ST2 receptor density in endothelial cells[11].

Upon examination of the HOMA-IR values, a twofold increase was observed in the diabetic obese group compared to the non-diabetic obese group. It was then deduced that compensatory hyperinsulinemia associated with T2D contributed to the increase in plasma TF expression in these patients.

TF was positively correlated with glucose ($r = 0.285$; $p < 0.05$) and HbA1c ($r = 0.226$; $p < 0.05$) ($p < 0.05$). There was a strong positive correlation between glucose and HbA1c ($r = 0.734$; $p < 0.01$) ($p < 0.05$). However, there was no correlation between TF and IL-33 **(Figure 3-4).**

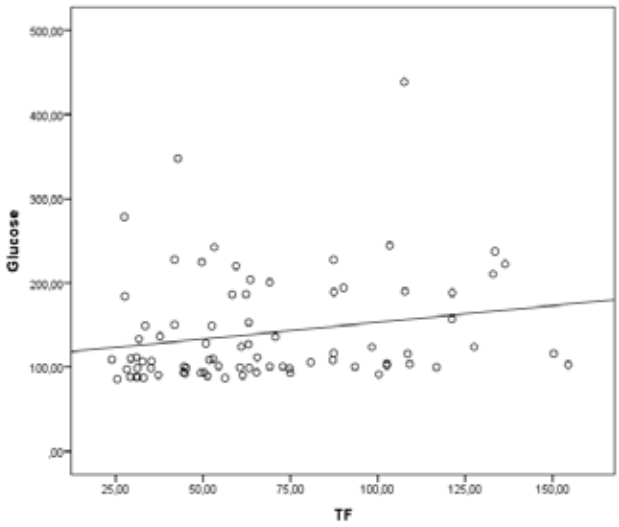


Figure 3: Correlation graph between TF and glucose. Serum levels of TF were correlated with serum levels of glucose ($r = 0.285$; $p < 0.05$).

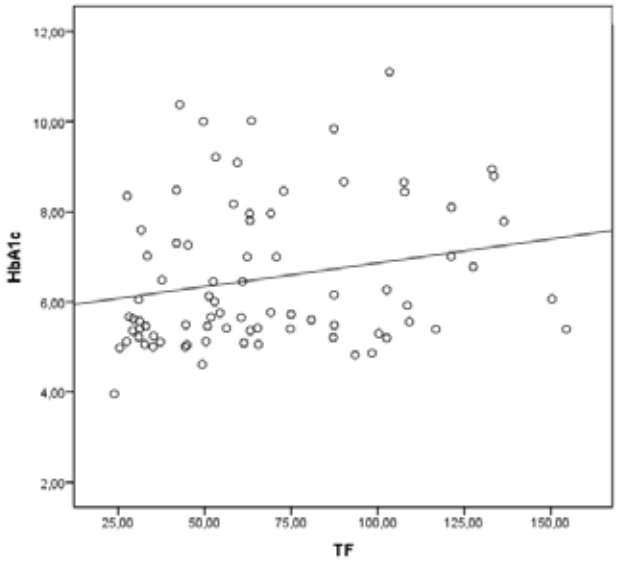


Figure 4: Correlation graph between TF and HbA1c. Serum levels of TF were correlated with serum levels of glucose ($r = 0.226$; $p < 0.05$).

Wang et al. study found a positive correlation between TF levels and fasting insulin, glucose, and free fatty acids, as well as a positive correlation between adipose TF mRNA and plasma free fatty acids[30]. In another study, it was found that increasing the amount of insulin for 24 hours by keeping glucose at normal levels led to increased circulating TF activity and increasing glucose and insulin levels together led to a much more significant increase in TF activity, which is associated with larger increases in the thrombin-antithrombin complex (TAT) and prothrombin fragment 1 + 2 (F1+2)[32]. In the current study, no significant correlation was observed between serum TF activity and BMI. This was interpreted to mean that the existence of obesity alone may have only a limited effect on increasing TF activity. The results of the current study also demonstrated a positive correlation between TF activity, glucose, and HbA1c (Figure 3-4), and also provided evidence that hyperglycemia increases TF procoagulant activity[32], and glycemic control leads to reduced circulating TF, especially in T2D[37]. Unlike the findings of the studies such as Vaidyula et al.[31] and Boden et al.[32], in recent studies demonstrated that hyperglycemia single-handedly influences TF. However, no correlation was found between TF and insulin levels. As for diabetic patients and diabetic patients with stroke, a positive correlation was found between hyperglycemia and TF[38,39]. Our study differs from these studies in that it reports a positive correlation between TF and HbA1c. It is known that the presence of advanced glycation end-products (AGE) increases TF[40].

In conclusion, a positive correlation was found between TF activity, and HbA1c and glucose levels. This suggests that TF may be predictive for diabetes which develop in the background of obesity, and that TF can be used as a prognostic value for diabetes.

REFERENCES

1. Yigitbasi T, Emekli N. Obezite Biyokimyası. In Yiğitbaşı T. and Emekli N. (Ed.), Klinik Biyokimya (pp.311-321). İstanbul, **2015:**Nobel Tıp Kitabevleri.

2. Servan PR. Obesity and Diabetes. *Nutr Hosp*, **2013;**28(5):138-43. https://doi.org/10.3305/nh.2013.28.sup5.6929.

3. Ortug G, Ignak S, Ortug, A. Characteristics of lingual papillae in diabetic rats. *Morphologie,* **2018;**102(339):250-254. https://doi.org/ 10.1016/j.morpho.2018.08.003.

4. Batirel S, & Emekli N. Diabetes Mellitus ve laboratuvar. In Yiğitbaşı T. and Emekli N. (Ed.), Klinik Biyokimya (pp.159-173). İstanbul, **2015:**Nobel Tıp Kitabevleri.

5. Li S, Wang J, Zhang B, Li X, Liu Y. Diabetes Mellitus and cause-specific mortality: a population-based study. *Diabetes Metab J*, **2019;**43(3):319-341.

6. https://doi.org/ 10.4093/dmj.2018.0060.

7. Rauch U, Nemerson Y. Circulating tissue factor and thrombosis. *Curr Opin in Hematol,* **2000**;7:273-277. https://doi.org/10.4093/dmj.2018.0060.

8. Hisada Y, Mackman N. Tissue factor and cancer: regulation, tumor growth, and metastasis. *Semin Thromb Hemost*, **2019;**45(4):385-395. https://doi.org/10.1055/s-0039-1687894.

9. Ruf W, Samad F. Tissue factor pathway linking obesity and inflammation. *Hamostaseologie*, **2015;**35(3):279-283. https://doi.org/10.5482/HAMO-14-11-0068.

10. Badeanlou L, Furlan-Ferguia C, Yang G, Ruf W, Samad F. Tissue factor-protease-activated receptor 2 signaling promotes diet-induced obesity and adipose inflammation. *Nat Med*, **2011;**17(11):1490-1497. https://doi.org/10.1038/nm.2461.

11. Cayrol C, Girard JP. Interleukin-33(IL-33): A nuclear cytokine from the IL-1 family. *Immunol Rev*, **2018;**281(1):154-168. https://doi.org/10.1111/imr.12619.

12. Stojkovic S, Kaun C, Rauscher S, Hell, L, Krychtiuk KA, Bonstingl C. et al. Tissue factor is induced by interleukin-33 in human endothelial cells: a new link between coagulation and inflammation. Sci Rep, **2016;**6:25171. https://doi.org/10.1038/srep25171.

13. Miller AM. Role of IL-33 in inflammation and disease. J Inflamm(Lond), **2011;**8(1):22. https://doi.org/10.1186/1476-9255-8-22.

14. Gutin I. In BMI we trust: reframing the body mass index as a measure of health. Soc Theory Health, **2018;**16(3):256-271. https://doi.org/10.1057/s41285-017-0055-0.

15. American Diabetes Association. Classification and diagnosis of diabetes: standards of medical care in diabetes-2019. Diabetes Care, **2019;**42(1):13-28. https://doi.org/10.2337/dc19-S002.

16. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and b-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia, **1985;**28:412-419. https://doi.org/ 10.1007/BF00280883.

17. Hasan A, Kochuman S, Al-Ozair, E, Tuomiletho J, Ahmad R. Association between adipose tissue ınterleukin-33 and immunometabolic markers in individuals with varying degrees of glycemia. Dis Markers, **2019;**2019:7901062. https://doi.org/ 10.1155/2019/7901062.

18. Griesenauer, B, Paczesny S. The ST2/IL-33 axis in immune cells during inflammatory diseases. Front Immunol, **2017;**8:475. https://doi.org/10.3389/fimmu.2017.00475.

19. Stojkovic S, Thulin A, Hell L, Thaler B, Rauscher S, Baumgartner J, et al. IL-33 stimulates the release of procoagulant microvesicles from human monocytes and differentially increases tissue factor in human monocyte subsets. Thromb Heamost, **2017;**117(7):1379-90. https://doi.org/10.1160/TH16-10-0784.

20. Abuyassin B, Laher I. Obesity-linked diabetes in the arab World: a review. EMHJ, **2015;**21(6):420-439. https://doi.org/10.26719/2015.21.420

21. Ayer JG, Song C, Steinbeck K, Celermajer DS, Freedman SB. Increased tissue factor activity in monocytes from obese young adults. Clin Exp Pharmacol Physiol, **2010;**37(11):1049-1054. https://doi.org/10.1111/j.1440-1681.2010.05430.x.

22. Ay L, Kopp HP, Brix JM, Ay C, Quehenberger P, Schernthaner GH, et al. Thrombin generation in morbid obesity: significant reduction after weight loss. J Thromb Haemost, **2010;**8(4):759-765. https://doi.org/10.1111/j.1538-7836.2010.03766.x.

23. Kopp CW, Kopp HP, Steiner S, Kriwanek S, Krzyzanowska K, Bartok A, et al. Weight loss reduces tissue factor in morbidly obese patients. Obes Res, **2003;**11(8):950-956. https://doi.org/10.1038/oby.2003.131.

24. Singh A, Foster GD, Gunawardana J, McCoy TA, Nguyen T, Veur SV, et al. Elevated circulating tissue factor procoagulant activity, factor VII, and plasminogen activator inhibitor-1 in childhood obesity: evidence of a procoagulant state. Br J Haematol, **2012;**158(4):523-527. https://doi: 10.1111/j.1365-2141.2012.09160.x.

25. 24- Samad F, Pandey M, Loskutoff DJ. Regulation of tissue factor gene expression in obesity. Blood, **2001;**98:3353–3358. https://doi.org/10.1182/blood.V98.12.3353.

26. Zeyda M, Wernly B, Demyanets S, Kaun C, Hammerle M, Schranz M, et al. Severe obesity increases adipose tissue expression of interleukin-33 and its receptor ST2, both predominantly detectable in endothelial cells of human adipose tissue. Int J Obes (Lond), **2013;**37(5):658-665. https://doi.org/10.1038/ijo.2012.118.

27. Hasan A, Al-Ghimlas F, Warsame S, Al-Hubai A, Ahmad R, Bennakhi A, et al. IL-33 is negatively associated with the BMI and confers a protective lipid/metabolic profile in non-diabetic but not diabetic subjects. BMC Immunology, **2014;**15:19. https://doi.org/ 10.1186/1471-2172-15-19.

28. Tang H, Liu N, Feng X, Yang Y, Zhuang S, Dai Y, et al. Circulating levels of IL-33 are elevated by obesity and positively correlated with metabolic disorders in Chinese adults. J Transl Med, **2021;**19(1):52. https://doi: 10.1186/s12967-021-02711-x.

29. Miller AM. Role of IL-33 in inflammation and disease. J Inflamm (Lond), **2011;**8(1):22. https://doi.org/10.1186/1476-9255-8-22.

30. Miller AM, Asquith DL, Hueber AJ, Anderson LA, Holmes WM, McKenzie AN, et al. Interleukin-33 induces protective effects in adipose tissue inflammation during obesity in mice. CircRes, **2010;**107(5):650-658. https://doi.org/10.1161/CIRCRESAHA.110.218867.

31. Wang J, Ciaraldi, TP, Samad F. Tissue factor expression in obese type 2 diabetic subjects and its regulation by antidiabetic agents. J Obes, **2015;**2015:291209. https://doi.org/ 10.1155/2015/291209.

32. Vaidyula VR, Rao AK, Mozzoli M, Homko C, Cheung P, Boden G. Effects of hyperglycemia and hyperinsulinemia on circulating tissue factor procoagulant activity and platelet CD40 ligand. Diabetes, **2006;**55(1):202–208. https://doi.org/ 10.2337/diabetes.55.01.06.db05-1026.

33. Boden G, Vaidyula VR, Homko C, Cheung P, Rao AK. Circulating tissue factor procoagulant activity and thrombin generation in patients with type 2 diabetes: effects of insulin and glucose. J Clin Endocrinol Metab, **2007;**92(11):4352-4358. https://doi.org/10.1210/jc.2007-0933.

34. Gerrits AJ, Koekman CA, van Haeften TW, Akkerman JWN. Increased tissue factor expression in diabetes mellitus type 2 monocytes caused by insulin resistance. J Thromb Haemos, **2011;**9(4):873-875. https://doi.org/10.1111/j.1538-7836.2011.04201.x.

35. Diamant M, Nieuwland R, Pablo RF, Sturk A, Smit JWA, Radder JK. Elevated numbers of tissue-factor exposing microparticles correlate with components of the metabolic syndrome in uncomplicated type 2 diabetes mellitus. Circulation, **2002;**106(19):2442-2447. https://doi: 10.1161/01.cir.0000036596.59665.c6.

36. Vambergue A, Rugeri L, Gaveriaux V, Devos P, Martin A, Fermon C. et al. Factor VII, tissue factor pathway inhibitor, and monocyte tissue factor in diabetes mellitus: influence of type of diabetes, obesity index, and age. Thromb Res, **2001;**101(5):367-374. https://doi: 10.1016/s0049-3848(00)00424-2.

37. Buchs AE, Kornberg A, Zahavi M, Aharoni D, Zarfati C, Rapoport M.J. Increased expression of tissue factor and receptor for advanced glycation end products in peripheral blood mononuclear cells of patients with type 2 diabetes mellitus with vascular complications. Exp Diabesity Res, **2004;**5(2):163-169. https://doi: 10.1080/15438600490424325.

38. Sambol, A, Osende J, Hathcock J, Degen M, Nemerson Y, Fuster V. et al. Role of risk factors in the modulation of tissue factors activity and blood thrombogenicity. Circulation, **2003;**107(7):973-977. https://doi.org/10.1161/01.CIR.0000050621.67499.7D.

39. Gentile NT, Vaidyula VR, Kanamalla U, DeAngelis M, Gaughan J, Rao AK. Factor VIIa and tissue factor procoagulant activity in diabetes mellitus after acute ischemic stroke: impact of hyperglycemia. Thromb Haemost, **2007;**98(5):1007–1013. https://doi.org/10.1160/TH06-12-0719.

40. Ichikawa K, Yoshinari M, Iwase M, Wakisaka M, Doi Y, Iino K, et al. Advanced glycosylation end products induced tissue factor expression in human monocyte like U937 cells and increased tissue factor expression in monocytes from diabetic patients. Atherosclerosis, **1998;**136(2):281–287. https://doi.org/10.1016/S0021-9150(97)00221-9.

41. Khechai F, Ollivier V, Bridey F, Amar M, Hakim J, de Prost D. Effect of advanced glycation end product–modified albumin on tissue factor expression by monocytes. Role of oxidant stress and protein tyrosine kinase activation. Arterioscler Thromb Vasc Biol **1997;**17(11):2885–2890. https://doi.org/10.1161/01.atv.17.11.2885.

Acta Pharm. Sci. Vol 59:(4), 2021
DOI: 10.23893/1307-2080.APS.05936

Evaluation of Medication and Herbal Product Use Among Pregnant Women

Rashida Muhammad UMAR[1], Rabia BEŞİROĞLU[1], Ayşe Kevser ŞAHİN[1], Emine Karataş KOÇBERBER[2], Cengizhan CEYLAN[3*]

1 İstanbul Medipol University, School of Pharmacy, Department of Clinical Pharmacy
2 Marmara University, Institute of Health Sciences, Department of Clinical Pharmacy
3 Selçuk University, Faculty of Pharmacy, Department of Clinical Pharmacy

ABSTRACT

The use of medications and herbal products among women increases after pregnancy. The purpose of the study is to evaluate the frequency of medication used and analyzed them based on the FDA pregnancy risk categories. This cross-sectional study was conducted on 300 adult volunteers attending antenatal care in a university-affiliated hospital. Medications were classified according to the FDA pregnancy risk classification. In 97,7% of women, the use of at least one medication during pregnancy. The median count for Category A medications was 2. Category C and B medications were reported by 100 (33,3%) and 53 women (17,7%). There was a significant difference between the education level and the use of herbal products (p=0.043). Pharmacists should take a greater role in educating pregnant women and women of childbearing age about the possible consequences of medications on the development of the fetus and guide healthcare practitioners in choosing the most appropriate medications.

Keywords: Medication use, herbal product use, pregnancy, FDA risk category

INTRODUCTION

The use of medications and herbal products is common among pregnant women. Medications may be needed for treatment in various conditions related to the mother and fetus during pregnancy. It has been reported that many pregnant women are prescribed drugs and they tend to use these prescription

*Corresponding author: e-mail: c.ceylan20@gmail.com
ORCIDs:
Rashida Muhammad UMAR : https://orcid.org/0000-0001-8902-7274
Rabia BEŞİROĞLU : https://orcid.org/0000-0003-3188-4278
Ayşe Kevser ŞAHİN : https://orcid.org/0000-0003-0223-2959
Emine Karataş KOÇBERBER : https://orcid.org/0000-0003-2803-0992
Cengizhan CEYLAN : https://orcid.org/0000-0003-4164-9212
(Received 21 February 2021, Accepted 3 June 2021)

drugs[1,2]. There is no doubt that the unjustifiable use of medications is common even during pregnancy[3]. In a multi-center study involving 9459 women, it was found that 81% of pregnant women used at least one medication (with or without prescription) during their pregnancy[4]. In another cross-sectional study, it was found that 56% of pregnant women used herbal supplement during their pregnancy[5].

The use of some medications and malnutrition during pregnancy have been associated with increase in the risk of some health conditions like schizophrenia, fertility disorder, metabolic problems, diabetes mellitus and cardiovascular diseases in developing fetus[6]. Teratogenic and embryo-fetotoxic effects of medications are related to the dose, route of administration, time of exposure, rate of exposure and genetic predisposition to the effect of a particular medication[7] and gestational age of the pregnancy. Before implantation, any abnormalities generally prevent implantation of the zygote. The most vulnerable period is the organogenesis stage where there is higher rate of cell division and development of organs[8]. A study revealed that the risk of congenital abnormalities is 2-3% and 2-5% of these abnormalities results from medication and chemical toxin exposure[7]. According to the 2014 European report, neurologic problems were present in 40% and other congenital malformations in 10% of newborns exposed to valproate[1]. As not all medications are studied in pregnant women, it is difficult to estimate the permeability of the medications into the placenta, its metabolism and elimination. It may also be unclear whether the teratogenic effect is caused by the main medication or its metabolites[6].

Anatomical and physiological changes such as decreased gastrointestinal motility, increased gastric pH, increased glomerular filtration rate, changes in the activities of liver enzymes that metabolize medications and changes in cardiac output are observed in pregnant women[9,10]. In the systematic review, it was stated that anticoagulants, antiretrovirals, antidepressants, antiepileptics, analgesics and anesthetics used during pregnancy should be re-dosed due to pharmacokinetic changes in their use during pregnancy[11]. The fetus is affected by these changes and this situation may cause anomalies[12].

There is not enough data about the use of herbal products during pregnancy. Therefore, the US Food and Medication Administration (FDA) does not recommend the use of herbal products during pregnancy[13]. According to the studies conducted on medications used in pregnancy in our country, it has been concluded that the most common medication groups are antidepressants and antibiotics. It is stated that pregnant women mostly use medications in the first trimester according to the gestation period. It is known that the use of over-

the-counter medications, which can be sold without a prescription, is 8.2% in our country, and unnecessary use is thought to be common[14]. The number of researches on the use of medications and herbal products in pregnant women is few in our country. It is necessary to determine the frequently used medications and risk categories during pregnancy. Guides should be prepared in the light of the available data[15].

The aim of this study is to identify the prevalence of medication use among pregnant women and categorize the medications based on the FDA pregnancy risk classification.

METHODOLOGY

The cross-sectional study was carried out in a university-affiliated teaching hospital. Adult volunteers attending antenatal care where included in the study. The study was carried out between November 2016 - February 2017. The socio-demographic characteristics of patients were recorded using a standard patient information form. Information on medication use and herbal supplements during pregnancy were also recorded. Patients were asked to specify the need for the medication/herbal supplement use and whether they informed their doctors. Patients were also asked to state their preferred source of medication information when they have the need to use a medication or supplement.

Classification of medications and herbal products

The medications and supplements recorded were classified in accordance with the FDA classification of medications in pregnancy. Active ingredients in combined medications and supplements were evaluated individually. The FDA classification was checked online from Medscape medication information website (accessed date Nov 2016 - Feb 2017). Daily caffeine limit was taken to be 300 mg as recommended by the World Health Organization (WHO)[16]. The amount of caffeine in 1 cup of Turkish coffee has been evaluated as 82 mg[17]. Only the number of herbal products used has been evaluated.

Statistical analysis

Descriptive statistics were given as mean ± standard deviation and median [interquartile range (IQR) of 25% to 75%] with minimum-maximum for continuous variables depending on their distribution. Numbers and percentages were used for categorical variables. Normality of the numerical variables was analyzed by the Kolmogorov-Smirnov test and checked by Q-Q plots and histograms. In comparing two independent groups, the Independent Samples t-test was used where numerical variables had a normal distribution. For variables

without normal distribution, the Mann Whitney U test was applied. For comparison of differences between categorical variables, Fisher Freeman Halton test was used in RxC tables. Spearman Rho correlation coefficient was used to analyze the associations between numerical variables.

For statistical analysis and figures, Microsoft Office Excel and "Jamovi project (2020), Jamovi (Version 1.2.24) [Computer Software] (Retrieved from https://www.jamovi.org) ve JASP (Version 0.13.1) (Retrieved from https://jasp-stats.org) were used. The significance level (p-value) was set at 0.05 in all statistical analyses.

RESULTS AND DISCUSSION

There were 300 participants with a mean age of 29,2 ± 4,3 years. The majority of women were with university education (59%). Most of the women (62,7%) were in the third trimester. Details of obstetric history is given in Table 1. The education status of patients was high as 62,7% of patients were university graduates.

Table 1. Demographic characteristics and obstetric history of the participants (n=300).

Variables	
Age (year) †	29.2 ± 4.3
Educational status ‡	
Primary	34 (11.3)
Secondary	78 (26.0)
University	177 (59.0)
Higher	11 (3.7)
Interview time for gestational period ‡	
During 1st trimester	40 (13.3)
During 2nd trimester	72 (24.0)
During 3rd trimester	188 (62.7)
Gravidity β	1.0 [1.0- 7.0]
Parity β	0.0 [0.0- 5.0]
Abortion β	0.0 [0.0- 3.0]

†: Mean ± standard deviation, ‡: n (%), β: median [range].

Medication and herbal product use

Data on overall medication use and medications stratified according to their categories are summarized in Table 2. In 97,7% of women, there was a history of at least one medication during pregnancy. The median count of medications was 2. Although 285 women (95%) reported prescription medication use, over-the-counter medication use was detected in 45 participants (15%). In this study, 219 women (73%) reported using herbal products during pregnancy.

Table 2. Details of medication use in the study group (n=300).

Variables	
Overall medication use ‡	293 (97.7)
Medication count β	2.0 [0.0- 7.0]
Prescription medication use ‡	285 (95.0)
Medication count β	2.0 [0.0- 7.0]
Over-the-counter medication use ‡	45 (15.0)
Medication count β	0.0 [0.0- 2.0]
Herbal product use ‡	219 (73.0)
Medication count β	1.0 [0.0- 6.0]
Use of Category A medication ‡	286 (95.3)
Use of Category B medication ‡	53 (17.7)
Use of Category C medication ‡	100 (33.3)
Use of Category D medication ‡	7 (2.3)
Use of Category X medication ‡	0.0 (0.0)
Medication count in category A β	2.0 [0.0- 5.0]
Medication count in category B β	0.0 [0.0- 2.0]
Medication count in category C β	0.0 [0.0- 3.0]
Medication count in category D β	0.0 [0.0- 1.0]
Medication count in category X β	0.0 [0.0- 0.0]
Medication use without knowing being pregnant ‡	43 (14.3)
Medication use without doctor recommendation ‡	17 (5.7)
Personal recommendation of a medication without doctors' recommendation ‡	
Byself	13 (76.5)
Friends	1 (5.9)
Family members	1 (5.9)
Unknown	2 (11.8)
Medication use without information about its category ‡	43 (14.3)
Current herbal tea and medication count β	1.0 [0.0- 6.0]

†: Mean ± standard deviation, ‡: n (%), β: median [range].

Category A medications were the most frequently used throughout the pregnancy in 286 patients (95,3%). The median count for Category A medications was 2. Category C and B medications were reported by 100 (33,3%) and 53 women (17,7%). Acetylsalicylic acid is in category D when used in the third trimester of pregnancy. Has been observed that the most frequently used drug in category D was acetylsalicylic acid.

In out of 17 women with over-the-counter medication use, 13 women (76,5%) used these medications by themselves.

The most commonly prescribed medications during pregnancy are antianemic medications (25,5%), multivitamin and mineral combinations (19%), vitamins (17%), antacids (11,4%), minerals and electrolytes (8%), nutraceuticals (4,8%), thyroid medications (3,5%), progestin (3,4%) and other (7,4%) respectively. Details are given in Figure 1.

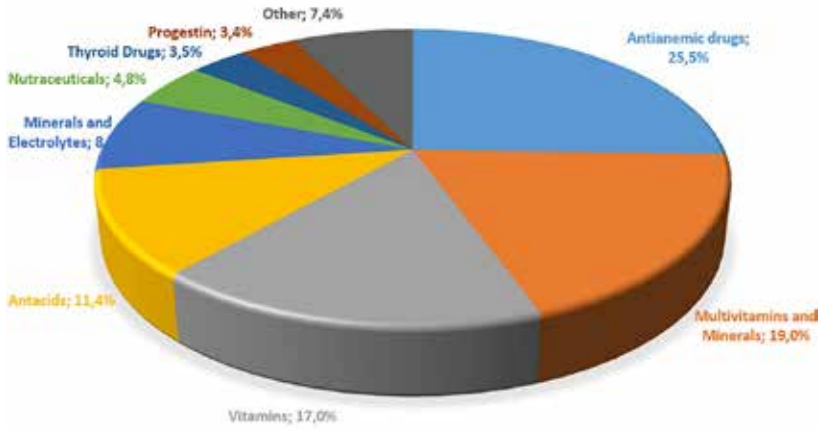


Figure 1: Prescription medications classification

There were no differences in age, educational status, interview time and gestational history between the participants with and without overall, prescription and over-the-counter medication uses, and use of medication without doctors' recommendation, ($p>0.05$ for all) details are given Table 3.

Table 3. Comparison of demographic characteristics and obstetric history of the participants with different types of medication use.

	Overall medication use			Use of medication without doctors' recommendations		
	No (n=7)	**Yes (n=293)**	**p**	**No (n=283)**	**Yes (n=17)**	**p**
Age (year) [β]	28.0 [25.0- 32.0]	29.0 [26.0- 32.0]	0.701	29.0 [26.0- 32.0]	31.0 [29.0- 32.0]	0.082
Educational status [‡]						
Primary	0 (0.0)	34 (11.6)	0.999	32 (11.3)	2 (11.8)	0.424
Secondary	2 (28.6)	76 (25.9)		76 (26.9)	2 (11.8)	
University	5 (71.4)	172 (58.7)		165 (58.3)	12 (70.6)	
Higher	0 (0.0)	11 (3.8)		10 (3.5)	1 (5.9)	
Interview time for gestational period [‡]						

During 1st trimester	1 (14.3)	39 (13.3)	0.999	40 (14.1)	0 (0.0)	0.201
During 2nd trimester	1 (14.3)	71 (24.2)		66 (23.3)	6 (35.3)	
During 3rd trimester	5 (71.4)	183 (62.5)		177 (62.5)	11 (64.7)	
Gravidity [β]	1.0 [1.0- 2.0]	1.0 [1.0- 2.0]	0.545	1.0 [1.0- 2.0]	2.0 [1.0- 2.0]	0.279
Parity [β]	0.0 [0.0- 1.0]	0.0 [0.0- 1.0]	0.864	0.0 [0.0- 1.0]	1.0 [0.0- 1.0]	0.088
Abortion [β]	0.0 [0.0- 0.0]	0.0 [0.0- 0.0]	0.257	0.0 [0.0- 0.0]	0.0 [0.0- 0.0]	0.297
	Use of medication without knowing being pregnant			Prescription medication use		
	No (n=139)	Yes (n=161)	p	No (n=15)	Yes (n=285)	p
Age (year) [β]	28.8 ± 4.3	29.5 ± 4.4	0.139	30.0 [27.5- 32.0]	29.0 [26.0- 32.0]	0.801
Educational status [‡]						
Primary	21 (15.1)	13 (8.1)	0.031	1 (6.7)	33 (11.6)	0.600
Secondary	43 (30.9)	35 (21.7)		5 (33.3)	73 (25.6)	
University	71 (51.1)	106 (65.8)		8 (53.3)	169 (59.3)	
Higher	4 (2.9)	7 (4.3)		1 (6.7)	10 (3.5)	
Interview time for gestational period [‡]						
During 1st trimester	23 (16.5)	17 (10.6)	0.163	3 (20.0)	37 (13.0)	0.729
During 2nd trimester	28 (20.1)	44 (27.3)		3 (20.0)	69 (24.2)	
During 3rd trimester	88 (63.3)	100 (62.1)		9 (60.0)	179 (62.8)	
Gravidity [β]	2.0 [1.0- 2.0]	1.0 [1.0- 2.0]	0.012	1.0 [1.0- 2.0]	1.0 [1.0- 2.0]	0.930
Parity [β]	1.0 [0.0- 1.0]	0.0 [0.0- 1.0]	0.001	0.0 [0.0- 1.0]	0.0 [0.0- 1.0]	0.961
Abortion [β]	0.0 [0.0- 0.0]	0.0 [0.0- 0.0]	0.885	0.0 [0.0- 0.0]	0.0 [0.0- 0.0]	0.377
	Over-the-counter medication use			Herbal product use		
	No (n=255)	Yes (n=45)	p	No (n=81)	Yes (n=219)	p
Age (year) [β]	29.1 ± 4.4	29.7 ± 4.0	0.407	28.5 ± 4.3	29.5 ± 4.4	0.086
Educational status [‡]						
Primary	27 (10.6)	7 (15.6)	0.258	14 (17.3)	20 (9.1)	0.043
Secondary	71 (27.8)	7 (15.6)		20 (24.7)	58 (26.5)	
University	147 (57.6)	30 (66.7)		47 (58.0)	130 (59.4)	
Higher	10 (3.9)	1 (2.2)		0 (0.0)	11 (5.0)	
Interview time for gestational period [‡g]						

During 1st trimester	37 (14.5)	3 (6.7)	0.134	12 (14.8)	28 (12.8)	0.899
During 2nd trimester	64 (25.1)	8 (17.8)		19 (23.5)	53 (24.2)	
During 3rd trimester	154 (60.4)	34 (75.6)		50 (61.7)	138 (63.0)	
Gravidity [β]	1.0 [1.0- 2.0]	1.0 [1.0- 2.0]	0.216	1.0 [1.0- 2.0]	1.0 [1.0- 2.0]	0.229
Parity [β]	0.0 [0.0- 1.0]	0.0 [0.0- 1.0]	0.396	0.0 [0.0- 1.0]	0.0 [0.0- 1.0]	0.475
Abortion [β]	0.0 [0.0- 0.0]	0.0 [0.0- 0.0]	0.418	0.0 [0.0- 0.0]	0.0 [0.0- 0.0]	0.363

[†]: Mean ± standard deviation, [‡]: n (%), [β]: median [range].

There was a significant difference between educational status and use of medication without knowing being pregnant (p=0.031). The median number of pregnancy and live births were significantly lower in women who use medications without knowing being pregnant (p=0.012 and p=0.001).

There were significantly more women who do not use herbal medications with primary education compared with those with herbal product use (p=0.043).

In Table 4, correlation analysis between age and the counts of different medications is given. There was a significant positive correlation between age and herbal medication use (r=0.127, p=0.028). Age was not correlated with other types of medications (p>0.05 for all).

Table 4. Correlation analysis of age and gestational week, and use of different types of medications.

	Age		Gestational age (week)	
	Spearman's rho	**p-value**	**Spearman's rho**	**p-value**
Overall medications	0.097	0.094	0.267	<0.001
Prescription medications	0.068	0.242	0.244	<0.001
Over-the-counter medications	0.062	0.286	0.089	0.124
Herbal products	0.127	0.028	-0.034	0.563
Medications in Category A	0.069	0.230	0.250	<0.001
Medications in Category B	0.059	0.311	-0.019	0.738
Medications in Category C	0.101	0.082	0.121	0.035
Medications in Category D	-0.014	0.805	0.007	0.905

Correlation analysis revealed significant differences between gestational age and the counts of overall medications, prescription medications, medications in Category A and Category C. As the gestational age increased, the number of

medications in these subgroups significantly increased ($p<0.05$ for all).

In Table 5, the medications' distribution in each the FDA pregnancy risk category on different uses of medications is given. Medications in Category A was the most common medication used in various uses. Compared with Category B, medications in Category C were more commonly used considering overall, prescription, over-the-counter uses, and cases without doctors' recommendations.

Table 5. Distribution of the FDA pregnancy risk categories based on different medication use.

	Overall medication use (n=293)	Prescription medication use (n=285)	Over-the-counter medication use (n=45)	Use of medication without doctors' recommendation (n=17)	Use of medication without knowing being pregnant (n=43)
Medications in Category A ‡	286 (97.6)	280 (98.2)	43 (95.6)	208 (95)	17 (100)
Medications in Category B ‡	53 (18.1)	52 (18.2)	8 (17.8)	39 (17.8)	4 (23.5)
Medications in Category C ‡	100 (34.1)	96 (33.7)	15 (33.3)	78 (35.6)	4 (23.5)
Medications in Category D ‡	7 (2.4)	7 (2.5)	1 (2.2)	5 (2.3)	0 (0)

‡*: n (%).*

The use of medications in Category C was significantly higher in the third trimester than the first and second trimesters ($p=0.002$). Herbal medications did not show any significant change between the trimesters($p=0.899$), details are given Table 6.

Table 6. Distribution of the FDA pregnancy risk categories based on different gestational periods.

	1st trimester (n=40)	2nd trimester (n=72)	3rd trimester (n=188)	p-value
Medications in Category A ‡	38 (95.0)	69 (95.8)	179 (95.2)	0.999
Medications in Category B ‡	9 (22.5)	10 (13.9)	34 (18.1)	0.504
Medications in Category C ‡	5 (12.5)	20 (27.8)	75 (39.9)	0.002
Medications in Category D ‡	0 (0.0)	0 (0.0)	7 (3.7)	0.204
Herbal products	28 (70.0)	53 (73.6)	138 (73.4)	0.899

‡*: n (%).*

Medication use during pregnancy depends on highly on the risk-benefit ration. The possible harmful effects of medications on fetus has been a matter of con-

cern for many decades. In some occasions medication use is inevitable especially in patients with chronic diseases or those who develop serious acute diseases that require immediate treatment. In these cases, the choice of the most appropriate medication becomes significant. This study is among a few carried out in Turkey to analyze the use of medications during pregnancy. The results reflect common use of medications among females before and during pregnancy. Although most medications were routinely used during pregnancy, the presence of chronic and the presentation of some health conditions that needed treatment in some patients may have contributed to the high rate of medication use in the study population. Folic acid is recommended to prevent neural tube defects in neonates and iron replacement is also recommended to prevent pregnancy-related anemia[18-20]. There were fewer patients using folic acid as most of the study population were in their 2nd and 3rd trimesters. The need for folic acid, on the other hand, increases prenatally through the 1st trimester[20]. Although the use of vitamin complexes is recommended in patients with obvious need for replacement, their use was common among the study population.

The FDA pregnancy risk category of 33,3% and 17,7% of the prescribed medications recorded in this study were C and B respectively, while the category of 95,3% of these medications was A. Similarly, most medications taken without doctor's advice nor prescription were in the C category. In a study involving a large patient population in the United States, it was reported that 50% of the medications used in pregnancy were in category B and 37% were in category C[21]. This is similar to our results, but there were fewer medications in the B category as we recorded more medications in category C. In a previous study, 6,3% and 3,3% of prescribed medications were reported to be in the D and X categories respectively[22]. In this study, only one patient was prescribed acetylsalicylic acid in her 3rd trimester. Acetylsalicylic acid has been associated with central nervous system, circulatory and skeletal anomalies when used in early pregnancy and prolongation of birth when used at high doses in the end stage of pregnancy[14]. So, it may be used to delay birth in patients with this indication and it was used for this indication in two patients with premature birth risk. There was only one category X herbal supplement that the patient used without her doctor's consent and it contains *Panax ginseng*.

The most commonly used medication without doctor's consent was acetaminophen. Although its use at the late stages of pregnancy was associated with breathing difficulties in infants, its FDA pregnancy category is B and it is the safest analgesic during pregnancy[14]. The use of omega 3 during pregnancy is on the rise as seen in the results. A study revealed that omega 3 reduced the risk

of teratogenesis in pregnant women with depression[23]. Although many positive effects are linked to omega 3, there is still need for precise data to certify its use during pregnancy.

About 52,6% of patients explained using some medications and supplements before they were confirmed pregnant. The most commonly used medication was folic acid. The use of folic acid prenatally was shown to effectively prevent neural tube defect risk[24]. This shows that most pregnancies were planned, and most women were aware of the positive effects of folic acid. Two patients who had failed contraception reported using norethisterone which is in the X category. The use of oral contraceptives in the 1st trimester was shown to have limited teratogenic effect on the embryo[25].

The FDA later decided that the categorical system was not sufficient to characterize and communicate risks to drug use in pregnancy, and that a 'narrated' narrative based on available animal and human data is more appropriate for such a purpose. Despite the changes, the old classification system is still used in various sources.

Herbal use was common in the study population. Most women use these products for no special reason. Some used some tea infusion as alternatives to medications to prevent nausea etc. Studies have shown that herbal products are often used to treat nausea, vomiting, anxiety, stress, cough, common cold, indigestion and constipation[26-28]. The caffeine limit was exceeded by ten patients. There are controversies on the limit of daily caffeine intake during pregnancy, while the WHO suggests 300 mg to be the maximum[16], the American College of Obstetricians and Gynecology suggests the consumption of less than 200 mg/day[29]. A meta-analysis study revealed a significant reduction in birth weight and placental weight in babies born to pregnant women who received high doses of caffeine during pregnancy. Therefore, it is recommended to limit the intake of caffeine during pregnancy[30]. Also the risk of low birth weight was reported to increases as caffeine intake increases[31]. Considering the consumption of black tea and coffee in Turkey, the presence of only a few women exceeding the caffeine limit suggest that pregnant women are conscious of the possible negative effects of caffeine on their baby.

Since the doses and time of herbal products used in the study are not included, larger-scale studies are required.

There are a few limitations of our study. Since the sample size is small, larger studies are needed. Limited data have been collected, especially on herbal products.

The new classification determined by the FDA should be used in future research.

ETHICAL COMMITTEE APPROVAL

Approval from Istanbul Medipol University Non-Interventional Clinical Research Ethics Committee (approval date: 16.11.2016 / approval no: 517).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept – R.M.U., R.B., A.K.Ş., E.K.K. C.C.; Design – R.M.U., E.K.K.; Supervision – R.M.U., E.K.K.; Resources – R.M.U., R.B., A.K.Ş.; Materials – R.M.U., E.K.K.; Data Collection and/or Processing –R.B., A.K.Ş.; Analysis and/or Interpretation – R.M.U., E.K.K., C.C.; Literature Search – R.M.U., E.K.K., R.B., A.K.Ş., C.C.; Writing – R.M.U., E.K.K., C.C., Critical Reviews – R.M.U., E.K.K., C.C., R.B., A.K.Ş.

ACKNOWLEDGEMENT

The authors thankful to Bağcılar Medipol Mega University Hospital for providing the opportunity to do this research.

DISCLAIMER

The authors alone are responsible for the content and writing of the paper.

REFERENCES

1. Thomas SH, Yates LM. Prescribing without evidence - pregnancy. Br J Clin Pharmacol, **2012;** 74(4): 691-697. https://doi.org/10.1111/j.1365-2125.2012.04332.x

2. Pariente G, Leibson T, Carls A, Adams-Webber T, Ito S, Koren G. Pregnancy-Associated Changes in Pharmacokinetics: A Systematic Review. PLoS Med, **2016;** 13(11): e1002160. https://doi.org/10.1371/journal.pmed.1002160

3. Kfuri TA, Morlock L, Hicks RW, Shore AD. Medication errors in obstetrics. Clin Perinatol, **2008;** 35(1): 101-ix. https://doi.org/10.1016/j.clp.2007.11.015

4. Mburu G, Ayon S, Mahinda S, Kaveh K. Determinants of Women's Drug Use During Pregnancy: Perspectives from a Qualitative Study. Matern Child Health J, **2020;** 24(9): 1170-1178. https://doi.org/10.1007/s10995-020-02910-w

5. Al Essa M, Alissa A, Alanizi A, Bustami R, Almogbel F, Alzuwayed O, et al. Pregnant women's use and attitude toward herbal, vitamin, and mineral supplements in an academic tertiary care center, Riyadh, Saudi Arabia. Saudi Pharm J, **2019;** 27(1): 138-144. https://doi.org/10.1016/j.jsps.2018.09.007.

6. Schaefer C. Medications during pregnancy and lactation, treatment options and risk assessment. 3th edition. USA: Elsevier; **2015.** pp. 2-24.

7. Sachdeva P, Patel BG, Patel BK. Drug use in pregnancy; a point to ponder!. Indian J Pharm Sci, **2009;** 71(1): 1-7. https://doi.org/10.4103/0250-474x.51941.

8. Radziejewska A, Chmurzynska A. Folate and choline absorption and uptake: Their role in fetal development. Biochimie, **2019;** 158: 10-19. https://doi.org/10.1016/j.biochi.2018.12.002.

9. Anderson GD. Pregnancy-induced changes in pharmacokinetics: a mechanistic-based approach. Clin Pharmacokinet, **2005;** 44(10): 989-1008. https://doi.org/10.2165/00003088-200544100-00001.

10. Koren G, Pariente G. Pregnancy- Associated Changes in Pharmacokinetics and their Clinical Implications. Pharm Res, **2018;** 35(3): 61. https://doi.org/10.1007/s11095-018-2352-2.

11. Feghali M, Venkataramanan R, Caritis S. Pharmacokinetics of drugs in pregnancy. Semin Perinatol, **2015;** 39(7): 512-519. https://doi.org/10.1053/j.semperi.2015.08.003.

12. John LJ, Shantakumari N. Herbal Medicines Use During Pregnancy: A Review from the Middle East. Oman Med J, **2015;** 30(4): 229-236. https://doi.org/10.5001/omj.2015.48.

13. Olukman M, Parlar A, Orhan CE, Orhan AE. Gebelerde İlaç Kullanimi: Son Bir Yillik Deneyim. J Turk Soc Obs Gynecol, **2006;** 3(4): 255-261.

14. Ayele Y, Mekuria AN, Tola A, Mishore KM, Geleto FB. Prescription drugs use during pregnancy in Ethiopia: A systematic review and meta-analysis. SAGE Open Med, **2020;** 8: 2050312120935471. https://doi.org/10.1177/2050312120935471.

15. WHO Recommendations. 2016. Retrieved from https://www.who.int/elena/titles/caffeine-pregnancy/en/ (accessed on November 1, **2016**).

16. Naser LR, Sameh A, Muzaffar I, Omar AR, Ahmed MA. Comparative evaluation of caffeine content in Arabian coffee with other caffeine beverages. African J Pharm Pharmacol, **2018;** 12(2): 19-26. https://doi.org/10.5897/AJPP2017.4880.

17. Karabulut A, Sevket O, Acun A. Iron, folate and vitamin B12 levels in first trimester pregnancies in the Southwest region of Turkey. J Turk Ger Gynecol Assoc, **2011;** 12(3): 153-156. https://

doi.org/10.5152/jtgga.2011.36

18. Açkurt F, Wetherilt H, Löker M, Hacibekiroğlu M. Biochemical assessment of nutritional status in pre- and post-natal Turkish women and outcome of pregnancy. Eur J Clin Nutr, **1995;** 49(8): 613-622.

19. Lumley J, Watson L, Watson M, Bower C. Periconceptional supplementation with folate and/or multivitamins for preventing neural tube defects. Cochrane Database Syst Rev, **2001;** (3): CD001056. https://doi.org/10.1002/14651858.cd001056.

20. Andrade SE, Gurwitz JH, Davis RL, Chan KA, Finkelstein JA, Fortman K, et al. Prescription drug use in pregnancy. Am J Obstet Gynecol, **2004;** 191(2): 398-407. https://doi.org/10.1016/j.ajog.2004.04.025.

21. Özbudak H, Ünal AZ, Sabuncuoğlu S. Gebelikte non-steroidal antiinflamatuvar ilaçların kullanımının değerlendirilmesi. Marmara Pharm J, **2016;** 20(2): 64-71. doi:10.12991/mpj.20162059818.

22. Su KP, Huang SY, Chiu TH, Huang KC, Huang CL, Chang HC, et al. Omega-3 fatty acids for major depressive disorder during pregnancy: results from a randomized, double-blind, placebo-controlled trial. J Clin Psychiatry, **2008;** 69(4): 644-651. https://doi.org/10.4088/jcp.v69n0418.

23. Greenberg JA, Bell SJ, Guan Y, Yu YH. Folic Acid supplementation and pregnancy: more than just neural tube defect prevention. Rev Obstet Gynecol, **2011;** 4(2): 52-59.

24. Savolainen E, Saksela E, Saxén L. Teratogenic hazards of oral contraceptives analyzed in a national malformation register. Am J Obstet Gynecol, **1981;** 140(5): 521-524. https://doi.org/10.1016/0002-9378(81)90227-1.

25. Kıssal A, Çevik Güner Ü, Batkın Ertürk D. Use of herbal product among pregnant women in Turkey. Complement Ther Med, **2017;** 30: 54-60. https://doi.org/10.1016/j.ctim.2016.11.001.

26. Muñoz Balbontín Y, Stewart D, Shetty A, Fitton CA, McLay JS. Herbal Medicinal Product Use During Pregnancy and the Postnatal Period: A Systematic Review. Obstet Gynecol, **2019;** 133(5): 920-932. https://doi.org/10.1097/aog.0000000000003217.

27. Costantine MM. Physiologic and pharmacokinetic changes in pregnancy. Front Pharmacol, **2014;** 5: 65. https://doi.org/10.3389/fphar.2014.00065.

28. American College of Obstetricians and Gynecologists Clinical Guidance Committee Opinion, **2016**. Retrived from. https://www.acog.org/clinical/clinical-guidance/committee-opinion/articles/2010/08/moderate-caffeine-consumption-during-pregnancy (accessed on November 1, 2016).

29. Chen LW, Wu Y, Neelakantan N, Chong MF, Pan A, van Dam RM. Maternal caffeine intake during pregnancy is associated with risk of low birth weight: a systematic review and dose-response meta-analysis. BMC Med, **2014;** 12: 174. https://doi.org/10.1186/s12916-014-0174-6.

30. Rhee J, Kim R, Kim Y, Tam M, Lai Y, Keum N, et al. Maternal Caffeine Consumption during Pregnancy and Risk of Low Birth Weight: A Dose-Response Meta-Analysis of Observational Studies. PLoS One, **2015;** 10(7): e0132334. https://doi.org/10.1371/journal.pone.0132334.

Acta Pharm. Sci. Vol 59:(4), 2021
DOI: 10.23893/1307-2080.APS.05937

Methanolic Leaf Extract of *Dissotis Rotundifolia* Alleviates Acetic Acid-Induced Ulcerative Colitis in Rats

Oduro Kofi YEBOAH[1], Newman OSAFO[1*], Aaron Opoku ANTWI[1], Leslie Brian ESSEL[2]

1 Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, College of Health Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

2 Division of Pharmacology/Toxicology, University of Missouri, Kansas City, USA.

ABSTRACT

Dissotis rotundifolia (Sm.) Triana has been used locally in the treatment of inflammatory conditions such as painful swellings and conjunctivitis. This study aimed at investigating the effect of *Dissotis rotundifolia* in acetic acid-induced ulcerative colitis. Sprague Dawley rats were administered with *Dissotis rotundifolia* extract at doses 30, 100 and 300 mg kg^{-1} or sulfasalazine 500 mg kg^{-1} for 8 days. On the 4th day of treatment, colitis was induced by intrarectal administration of 1 ml, 4 %v/v acetic acid. On day 8, animals were sacrificed and parameters such as microscopic and macroscopic colon damage assessed. The extract exhibited significant ($P < 0.0001$) reduction in microscopic and macroscopic colon damage. 30 and 300 mg kg^{-1} of the extract significantly ($P < 0.0001$) inhibited weight loss and colon oedema. The leaf extract of *Dissotis rotundifolia* showed significant amelioration of acetic acid-induced ulcerative colitis which may be attributable to its anti-inflammatory effect.

Keywords: Chronic inflammation, colon damage, *Dissotis rotundifolia*, oedema, ulcerative colitis.

*Corresponding Author:
Newman Osafo, PhD
Department of Pharmacology
Faculty of Pharmacy & Pharm. Sciences
Kwame Nkrumah University of Science & Technology
Kumasi, Ghana
Mobile: +233208286553
E-mail: nosafo.pharm@knust.edu.gh
ORCIDs:
Oduro Kofi Yeboah : https://orcid.org/0000-0001-7080-0730
Newman Osafo : https://orcid.org/0000-0001-8142-2368
Aaron Opoku Antwi : https://orcid.org/0000-0001-9713-9487
Leslie Brian Essel : https://orcid.org/0000-0002-7355-2530
(Received 21 January 2021, Accepted 3 June 2021)

INTRODUCTION

Ulcerative colitis is a chronic inflammatory disease of the gastrointestinal tract that usually affects the rectum and sometimes extends proximally within the colon[1]. The disease shares a number of similarities with Crohn's disease enough to warrant their description under the collective term Inflammatory Bowel Diseases[1]. However, notable differences exist between the two. Except in the case of backwash ileitis, ulcerative colitis is devoid of small bowel involvement, as opposed to Crohn's disease where patients present with small bowel involvement. Moreover, ulcerative colitis does not affect the upper gastrointestinal tract, and the presentation of hematochezia is common. However, upper gastrointestinal tract is affected in Crohn's disease and hematochezia is rarely seen on presentation[2]. In addition, patchy, segmental and typically transmural inflammation in the gut, characterized by macrophage aggregation that often form noncaseating granulomas are seen in Crohn's disease. Ulcerative colitis, on the other hand, is characterized by significant leukocytic infiltration of the lamina propria and crypts, where they form microabscesses as well as depletion of mucin by goblet cells[3]. Although the exact pathoetiology of ulcerative colitis is not completely understood, it is widely accepted that dysregulated interaction between the commensal enteric flora and gut-associated immune system plays a crucial role in this disease[3,4]. The dysfunction of the mucosal immune system triggers intestinal inflammation *via* the activation of both the innate and acquired immune systems in the gut[5]. Recent mortality data reveals a 10% increase in intermediate and long-term mortality among ulcerative colitis patients with an even higher percentage in patients diagnosed in childhood or adolescence[6], clearly highlighting the need for novel therapies[7].

Medicinal plants play a beneficial role in healthcare. According to Amani et al. (2013) treatment with products of natural origin produces promising results and fewer side effects. One such plant known for its beneficial effects is *Dissotis roundifolia* (Sm.) Triana[8]. The West African native, *Dissotis rotundifolia*, commonly called Pink Lady[9] belongs to the family Melastomataceae[10,11] and is a versatile perennial creeping herb that roots at the nodes[12]. The leaves are ovate to ovate-lanceolate or suborbicular and the leaves are modestly crenuate with an acute apex, and a truncate to short-attenuate base[12,13]. The herb is used traditionally in the treatment of inflammatory conditions such as conjunctivitis and painful swellings[14], asthma[15], sinusitis and bronchitis[16]. The leaf decoction is also used in the treatment of stomach ache and diarrhea[13]. Medicinal plants are excellent sources of lead compounds that may provide new and cost-effective treatment options with tolerable side effect profile which may as well induce

and maintain total remission and also improve upon the quality of life of patients with ulcerative colitis. The aim of this study is to determine the effect of *Dissotis roundifolia* leaf extract (DRE) on acetic acid-induced ulcerative colitis.

METHODOLOGY

Animals

A total of 30 male Sprague Dawley rats (180 – 210 g) were obtained from and maintained in the Animal Housing facility of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. The rats were housed in stainless steel cages (34 cm × 47 cm × 18 cm) with soft wood shavings as bedding, and fed with commercially available pellet diet (GAFCO, Tema, Ghana) and given water *ad libitum*.

Drugs and Chemicals

Sulfasalazine was purchased from Pfizer Inc, New York, USA; disodium monohydrogen phosphate, sodium chloride and formaldehyde were purchased from Sigma-Aldrich Chemical Co, St Louis, USA; hematoxylin and eosin stain was purchased from Abbey Color, Philadelphia, USA; glacial acetic acid was purchased from Eastman Chemical Company, Kingsport, Tennessee, USA.

Collection of Plant material

The fresh leaves of *Dissotis rotundifolia* was collected from the area around Kakum National Park, Cape Coast, Ghana in January 2012 and was authenticated by the Curator at the Herbarium of the Department of Environmental Science, School of Biological Sciences, University of Cape Coast. A voucher specimen (No. 107346) has been deposited at the herbarium. The leaves were thoroughly washed and dried in the shade for 3 weeks. Subsequently, the partially dried plant material was oven-dried at 40°C for 3 h and then pulverized into powder.

Preparation of Extract

The extraction of the crude extract was carried out as described by Rath et al. (1995) and Kweku et al. (2018) [17,18]. Briefly, 60 g of the powdered leaves was transferred into a 1 L Erlenmeyer flask followed by the addition of 210 ml of 70% methanol. The neck of the flask was plugged tightly with cotton wool, and the contents of the flask mixed by placement on an orbital shaker (IKA HS/KS260 basic orbital shaker-Werke-GmbH & Co. KG Germany) at a speed of 200 rpm for 72 h. The powdered leaves were allowed to be drenched in the methanol for 72 hours, after which the resulting mixture was then filtered using a Whatman No.1 filter paper into a 500 ml flat bottom flask, and the filtrate discarded appropriately. The obtained filtrate was subsequently concentrated us-

ing a rotary evaporator (BÜCHI rotavapor R-200, Germany). The concentrated crude preparation obtained was then dried in an oven at a temperature of 50°C to obtain 6.9% ($^{w}/_{w}$) of dried powdered extract, henceforth referred to as *Dissotis rotundifolia extract* (DRE). DRE was stored at −8°C until required for use.

$$\% \ Yield = \frac{A_1}{A_o} \times 100 \ \&$$

Where A_o was the mass of the leaf sample and A_1 was the mass of the crude extract.

Induction of colonic injury and body weight determination

Acetic acid-induced ulcerative colitis was induced in rats as described by Fabia et al. (1992) and Osafo et al. (2019)[19,20]. Male Sprague Dawley rats (180 – 210 g) were randomly divided into six groups (n = 5) and treated as follows:

Group I: normal saline (0.9% $^{w}/_{v}$) *p.o* for 8 days.

Group II: normal saline (0.9% $^{w}/_{v}$) *p.o* for 8 days and 1 ml, 4.0% acetic acid ($^{v}/_{v}$) intrarectally on day 4.

Group III: sulfasalazine (500 mg kg^{-1} *p.o.*) for 8 days and 1 ml 4.0% acetic acid intrarectally on day 4.

Groups IV-VI: *Dissotis rotundifolia* leaf extract (DRE) 30, 100 and 300 mg kg^{-1} *p.o.* respectively for 8 days and 1 ml 4% acetic acid intrarectally on day 4.

Intrarectal administration of 4.0% acetic acid was made under anaesthesia. Body weight changes were monitored every morning, before feeding, over the 8-day period and the effect of DRE on the overall percentage change in body weight in relation to body weight on day 1, was expressed as area under the time course curve (AUC).

Macroscopic and Microscopic colon damage assessment

Colonic injury with acetic acid was induced in Sprague Dawley rats as described above. At the end of the 8-day period, animals were euthanized by cervical dislocation before large bowels were excised. Colons were extirpated and macroscopic damage was assessed as described by Kimball et al. (2004) using a 5-point scale based on the weight, consistency of the stool found within as well as length, measured from 1 cm above the anus to the top of the cecum[21]. Disease activity index (DAI) for each group was calculated as a sum of the individual macroscopic damage score. To evaluate microscopic colon damage by light mi-

croscopy, samples of the distal colon were fixed immediately in 10% neutral buffered formalin solution, embedded in paraffin, cut into transversal sections and mounted on glass slides. Sections were deparaffinized and stained with hematoxylin and eosin stain (H&E). In each specimen, six random fields of view were analyzed for microscopic colonic damage and scored by two double-blinded trained observers as described by Dieleman et al. (1998)[22]. Three independent parameters were measured: inflammatory cell infiltration (0-3: none, slight, moderate, severe), extent of injury (0-3: none, mucosal, submucosal, transmural), and crypt damage (0-4: none, basal $^{1}/_{3}$ damage, basal $^{2}/_{3}$ damage, only surface epithelium lost, entire crypt and epithelium lost). DAI was computed as the sum of individual damage scores with 10 as the maximum possible score.

Hematological analysis

Colitis was induced as described in Section 2.2.3. At the end of the 8-day period, the rats were euthanized by cervical dislocation and blood samples were collected from the jugular vein. A full blood count was performed on the blood samples using hematology analyzer (BC-2800, Mindray, Shenzhen, China).

Colon oedema assessment

Acetic acid-induced colonic injury was induced in rats as described in Section 2.2.3. At the end of the 8-day period, colons were excised and cut open by longitudinal incisions. The resected colons were washed thoroughly with normal saline to remove all residual fecal matter. As described by Morteau et al. (2000), degree of oedema was assessed by calculating the colon weight to length ratio[23].

Statistical analysis

Data was presented as Mean ± SEM. One-way ANOVA followed by Dunnett's multiple comparison test was employed in analyzing obtained experimental data. Graphs were plotted using GraphPad Prism for Windows Version 5.01 (GraphPad, San Diego, CA, USA).

RESULTS AND DISCUSSION

The control rats with colitis showed a progressive decrease in body weight after the induction of colon damage (Figure 1A) with a significant ($P < 0.0021$) loss in total body weight when compared with the control rats without colitis (Figure 1B). Treatment with the DRE at doses 30 and 300 mg kg^{-1} resulted in no significant ($P > 0.05$) changes in weight compared to the control group without colitis (Figure 1A) with no significant ($P > 0.05$) differences between the total loss of body weight over the course of the study when compared with the con-

trol group without colitis (Figure 1B). At dose 100 mg kg^{-1} of DRE, a total body weight loss (Figure 1B) was observed. The total loss of body weight at 100 mg kg^{-1} when compared with the rats without colitis was significant ($P < 0.0021$) (Figure 1B). In sulfasalazine-treated rats, there was decrease in body weight after colonic injury (Figure 1A) with a significant ($P < 0.021$) loss in total body weight after the 8th day when compared to the control rats without colitis (Figure 1B).

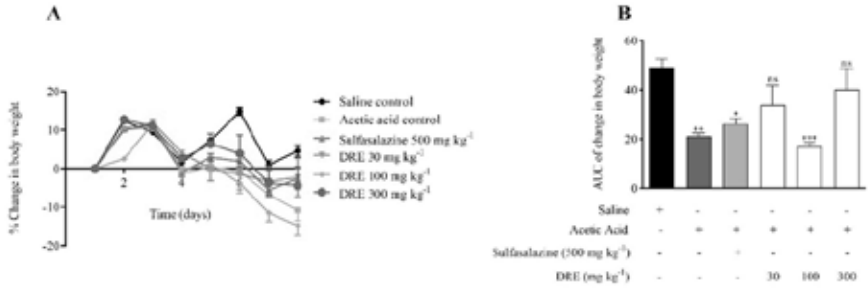


Figure 1: Effect of *Dissotis rotundifolia* on rat body weight in acetic acid-induced colitis.

The body weight was monitored as the percentage change in baseline body weight (A). Total body weight measured during the study period was calculated as area under the time course curves, AUC (B). ***$P = 0.009$, **$P = 0.0034$, *$P = 0.0185$, $^{ns}P > 0.05$ when compared with saline control group. AUC, area under the curve; DRE, *Dissotis rotundifolia* extract; Sulf, Sulfasalazine; AA, Acetic acid.

Clinically, toxins including acids, cause anorexia and induces vomiting by stimulating vagal afferent serotonergic nerves that connect with the chemoreceptor trigger zone in the floor of the fourth ventricle[24]. Owing to this, anorexia with pain can be regarded as a protective reflex that prevents absorption of toxins into the body by reducing the passage of chyme through diseased parts of the gastrointestinal tract[25]. This results in the avoidance of food and malabsorption with subsequent weight loss. The inhibition of weight loss by the extract indicates its ability to reduce anorexia, pain and malabsorption associated with mucosal and submucosal layer necrosis possibly by inhibiting afferent vagal nerve stimulation by ulcer-induced acid related pain.

The control rats with colitis macroscopically exhibited extensive colonic damage (Figure 2A) and significantly ($P < 0.0001$) high disease activity index compared to the control rats without colitis (Figure 2B). Macroscopic colonic damage (Figure 2A) and disease activity index (Figure 2B) significantly improved with sulfasalazine treatment when compared to the control rats with colitis. Treatment with DRE at doses of 30 and 300 mg kg^{-1} resulted in a significant improvement in mac-

roscopic scores (Figure 2A) and DAI (Figure 2B) compared to the control rats with colitis. However, a significant ($P < 0.01$) improvement in macroscopic scores (Figure 2A) and disease activity index (Figure 2B) was achieved at 100 mg kg^{-1} of DRE.

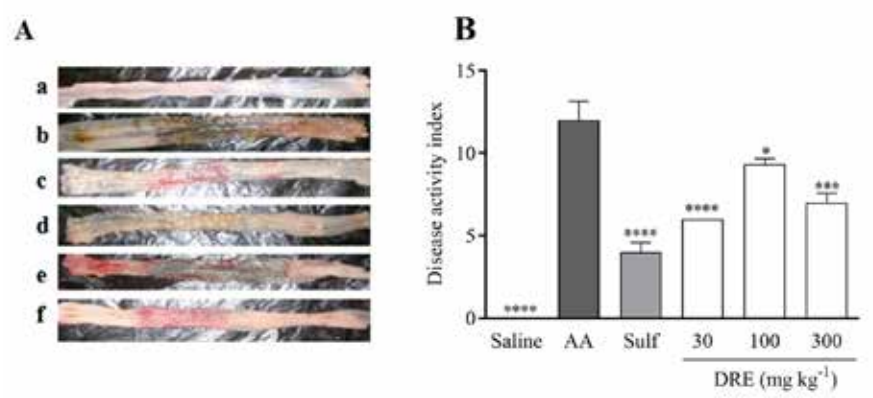


Figure 2: Effect of *Dissotis rotundifolia* on macroscopic acetic acid-induced colonic damage in rats.

Colons were extirpated and examined for: weight, the consistency of the stool found within as well as gross macroscopic appearance and length. (A) Representative slides of colon (a, untreated control; b, AA treatment only; c, AA + 500 mg kg^{-1} sulfasalazine; d, AA + 30 mg kg^{-1} DRE; e, AA + 100 mg kg^{-1} DRE; f, AA + 300 mg kg^{-1} DRE. (B) Disease Activity Index. ****P < 0.0001, ***P < 0.001 and *P < 0.01 when compared with the control rats with colitis. AA, acetic acid; DRE, *Dissotis rotundifolia* extract; Sulf, Sulfasalazine.

Intrarectal administration of acetic acid in the control rats with colitis caused massive loss of mucosal architecture characterized by massive mucosal ulceration (red arrow), loss of cellular detail, heavy inflammatory cell infiltration (green circle), slight thickening of the muscularis mucosa (red rectangle), crypt abscess formation (green arrow) and goblet cell depletion (Figure 3B). Treatment with the extract at 30 mg kg^{-1} resulted in a decrease in mucosal ulceration and the distortion of crypt architecture with no observable crypt abscess formation. At 30 mg kg^{-1} reduced infiltration of the mucosa, submucosa and lamina propria by inflammatory cells, chiefly neutrophils and lymphocytes, compared to the control rats with colitis was observed (Figure 3D). At 100 and 300 mg kg^{-1}, the extract reduced mucosal and submucosal inflammation with a slight loss of cellular detail, crypt distortion (green rectangle) and crypt abscess formation. Neutrophil and lymphocyte infiltration were reduced with less pronounce mucosal and submucosal ulceration compared to the control rats with colitis (Figure 3E and F). However, DRE at all concentrations significantly (*P*

< 0.0001) reduced the gross microscopic damage scores (Figure 4). Treatment with sulfasalazine 500 mg kg^{-1} resulted in a significant (P < 0.0001) reduction in microscopic damage score (Figure 4) by decreasing mucosal ulceration and the infiltration of inflammatory cells into the lamina propria, muscularis mucosa and submucosa and also by improving cellular detail (Figure 3C).

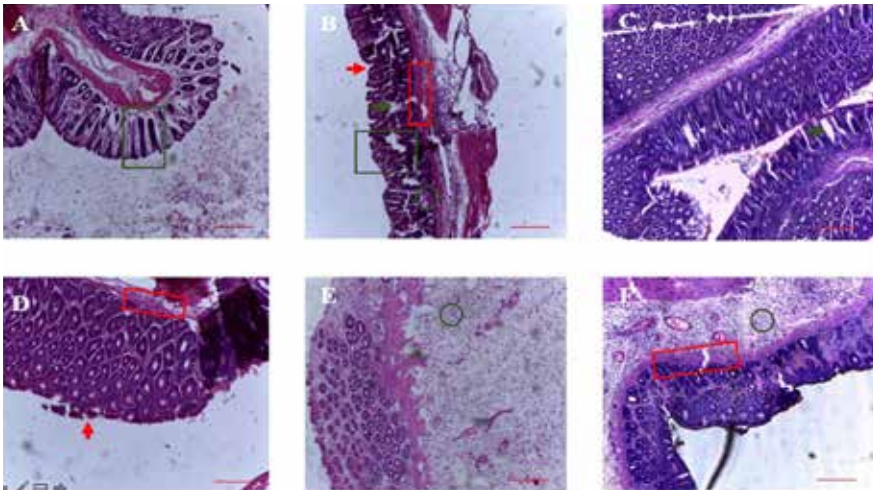


Figure 3: Histopathology of effect of *Dissotis rotundifolia* extract on acetic acid-induced ulcerative colitis in rats.

Control rats without colitis (A), control rats with colitis (B), sulfasalazine 500 mg kg^{-1} (C), DRE 30 mg kg^{-1} (D), DRE 100 mg kg^{-1} (E) and DRE 300 mg kg^{-1} (F). Scale bar represents 100 μm of tissue. DRE, *Dissotis rotundifolia* extract.

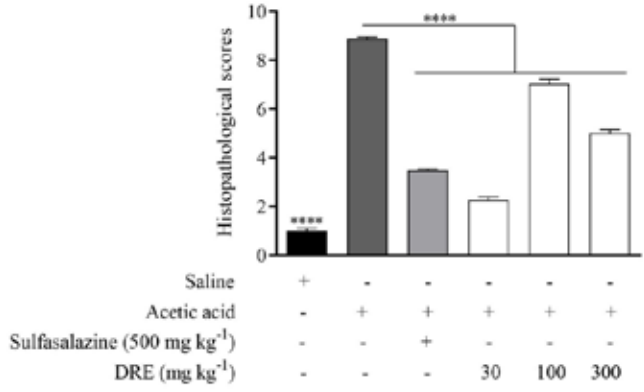


Figure 4: Histopathological score of effect of *Dissotis rotundifolia* extract on the colon.

****P < 0.0001 when compared with control rats with colitis. DRE, *Dissotis rotundifolia* extract.

Analysis of blood samples collected from the jugular vein of the animals showed significant hematological imbalances in the control rats with colitis (Figure 5). Treatment with sulfasalazine and DRE (30, 100 and 300 mg kg^{-1}) resulted in significant decrease in the number of WBCs compared to the control rats with colitis (Figure 5A). However, decrease in the number of WBCs upon treatment with DRE 30 and 300 mg kg^{-1} was significant compared to treatment with sulfasalazine ($P < 0.0001$) (Figure 5A). Significant increase in neutrophil number ($P < 0.0001$) as compared to the rats without colitis was observed in the acetic acid challenged group (Figure 5B). Neutrophil number was significantly reduced upon treatment with DRE (30, 100 and 300 mg kg^{-1}), however, treatment with sulfasalazine resulted in no significant decrease in neutrophil number when compared with the control rats with colitis (Figure 5B). Upon treatment with sulfasalazine and DRE (30, 100 and 300 mg kg^{-1}), significant ($P < 0.0001$) decrease in the lymphocyte number (Figure 5C) and percentage (Figure 5E) compared to the control rats with colitis was observed. Treatment with DRE 30 and 300 mg kg^{-1} resulted in a significant decrease in the number of platelets ($P < 0.0001$) while with sulfasalazine and DRE 100 mg kg^{-1} significant increase in platelets ($P < 0.0001$) was observed when compared to the control rats with colitis (Figure 5D). Treatment with sulfasalazine and DRE (30 and 100 mg kg^{-1}) resulted in a significant increase in mean cell hemoglobin ($P < 0.0001$) (Figure 5F). However, at a dose of 300 mg kg^{-1}, significant decrease in mean cell hemoglobin was observed ($P < 0.0001$) (Figure 5F). The effects observed at 300 mg kg^{-1} were indicative of myelosuppression.

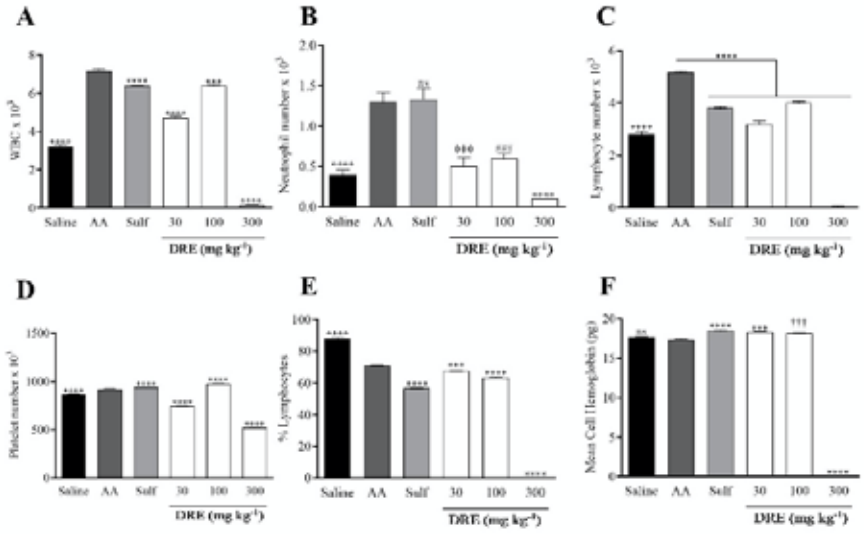


Figure 5: Effect of *Dissotis rotundifolia* extract on hematological parameters in acetic acid-induced colitis in rats.

$^{****}P < 0.0001$, $^{***}P = 0.0001$, $^{\phi\phi\phi}P = 0.0002$, $^{\#\#\#}P = 0.0007$, $^{\dagger\dagger\dagger}P = 0.003$, $^{ns}P > 0.05$ when compared with control rats with colitis. DRE, *Dissotis rotundifolia* extract; Sulf, Sulfasalazine; AA, Acetic acid.

The ability to inhibit necrosis of the colon, by DRE, is also seen in the reduction of intestinal hemorrhage evidenced by the decreased observation of melena and the subsequent increase in mean cell hemoglobin. Colonic damage led to the infiltration of the mucosa, submucosa and lamina propria by inflammatory cells chiefly neutrophils. The infiltrated neutrophils produce large amounts of reactive oxygen species which activates proteolytic enzymes causing endothelial cell damage. The extract was able to attenuate mucosal and submucosal necrosis by preventing oxidative stress-mediated tissue damage following initial tissue insult and also by promoting the recovery of mucosal integrity following mucosal damage.

After colons were extirpated and the colon weight to length ratio determined, the control rats with colitis showed significant ($P < 0.0001$) degree of oedema (Figure 6), compared to the control rats without colitis. Treatment with sulfasalazine resulted in a significant reduction ($P < 0.0001$) in oedema compared to the control rats with colitis. Significant reduction ($P < 0.0001$), in the degree of oedema, compared to the control group with colitis, resulted from the treatment with DRE at doses 30 mg kg^{-1} and 300 mg kg^{-1} respectively (Figure 6). However, treatment with DRE at 100 mg kg^{-1} resulted in no significant decrease ($P > 0.05$) (Figure 6), in total oedema compared to the control rats with colitis.

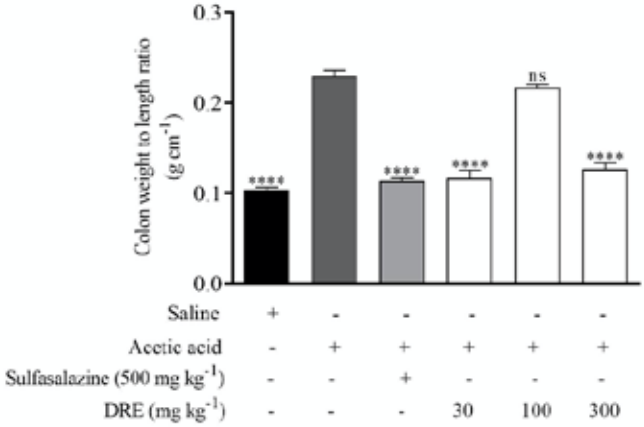


Figure 6: Effect of *Dissotis rotundifolia* extract on colon oedema in acetic acid-induced colonic damage in rats.

$^{****}P < 0.0001$, $^{ns}P > 0.05$ when compared with the control group with colitis. DRE, *Dissotis rotundifolia extract*.

Inflammation causes the activation of the components of the immune system both cellular and systemic, causing the recruitment and activation of immune cells such as leucocytes, neutrophils, etc. Chemical mediators such as chemokines, cytokines and free radicals released by the innate immune cells causes leucocyte recruitment to the injury site leading to elimination of the insult with activation of the adaptive immune response by natural killer and dendritic cells[26]. The inflammatory mediators generated can modulate cell proliferation, death and differentiation and amplify the response to the initial injury[27]. This implies that, inhibition of the proliferation of immune or inflammatory cells such as in myelosuppression, will lead to the attenuation of the inflammatory process and a decrease in disease activity. The decreased plasma cellular components by the extract indicates bone marrow suppression. Though inconclusive, it can however be inferred that DRE suppresses the proliferation, differentiation and maturation of inflammatory cells from the bone marrow reducing their recruitment to the site of injury thereby attenuating the inflammatory process.

Functional activation of mast cells during mucosal inflammation causes *de novo* synthesis and release Arachidonic acid metabolites such as prostaglandins and leukotrienes, PAF, chemokines and cytokines[28,29]. The increased production and release of these mediators alters vascular permeability and enhance vasodilation[30]. The increased vascular permeability as a result of acetic acid-induced colonic damage led to a decrease in intravascular oncotic pressure and an increase in the oncotic pressure of the interstitial fluid following the escape of protein-

rich fluid from plasma into the extravascular space. This, together with increased vascular hydrostatic pressure following vasodilation, resulted in the outflow of intravascular fluid and its subsequent accumulation in interstitial spaces. The net increase in the extravascular fluid volume resulted in oedema of the colon. Thus, the significant inhibition of colon oedema by DRE indicates the role of DRE in inhibiting intestinal vascular permeability and vasodilation by attenuating the activities of various vasoactive mediators such as prostaglandins and histamine on their respective receptors. Claudin-1 is one of the commonest proteins that enhances or maintains colonic epithelial barrier function[30] and reduction in its levels has been observed in the gut mucosa following induction of colon injury with acetic acid[31]. Reduction in claudin-1 levels leads to a decrease in tight junction formations with subsequent development of oedema following the escape of intravascular fluid into the interstitial spaces. The reduction in colon oedema by DRE signifies its ability to enhance claudin-1 formation in addition to its probable inhibitory effect on vasoactive mediators such as prostaglandin and histamine.

The aqueous methanol leaf extract of *Dissotis rotundifolia* is effective and useful in experimental ulcerative colitis. It decreases ulcerative colitis-associated weight loss, macroscopic and microscopic colon damage, and colon oedema due to its anti-inflammatory property.

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 Number DP-COL/2018/007. 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

OKY, Performed the experiment and drafted the manuscript; NO, Conceived the idea of the experimental design and analysed the obtained the data; AOA, Data analysis and interpretation; LBE, Drafted the manuscript.

FUNDING SOURCE

No funding was obtained for this study.

ACKNOWLEDGEMENTS

A special thank you to Mr. Thomas Ansah for his technical support in carrying out this work.

REFERENCES

1. Abraham C, Cho JH. Inflammatory bowel disease. N Engl J Med, **2009;** 361(21): 2066-2078.

2. Baumgart DC. The diagnosis and treatment of Crohn's disease and ulcerative colitis. Dtsch Arztebl Int, **2009;** 106(8): 123-133.

3. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. Nature, **2007;** 448(7152): 427-434.

4. Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. Nat Rev Immunol, **2003;** 3(7): 521-533.

5. Sartor RB. Mechanisms of disease: pathogenesis of Chrohn's disease and ulcerative colitis. Nat Clin Pract Gastroenterol Hepatol, **2006;** 3(7): 390–407.

6. Jess T, Frisch M, Simonsen J. Trends in overall and cause-specific mortality among patients with inflammatory bowel disease from 1982 to 2010. Clin Gastroenterol Hepatol, **2013;** 11: 43-48.

7. Torres J, Danese S, Colombel J-F. New therapeutic avenues in ulcerative colitis: thinking out of the box. Gut, **2013;** 62: 1642-1652.

8. Amani SA, Reham ME, Gamal AS. Natural products in the treatment of ulcerative colitis and peptic ulcer. J Saudi Chem Soc**, 2013;** 17(1): 101-124.

9. Loigier HA. Spermatophyta. Descriptive flora of Puerto Rico and Adjacent Islands. Editorial de la Universidad de Puerto Rico. **1994,** 1, 3.

10. Yeboah OK, Osafo N. Review of the ethno-medical, phytochemical, pharmacological and toxicological studies on *Dissotis rotundifolia* (Sm.) Triana. J Comp Alt Med Res, **2017;** 1-11.

11. Wagner WL, Herbst DR, Sohmer SH. Manual of the flowering plants of Hawaii. University of Hawaii press, Bishop Museum, Honolulu. **1990,** pp 210.

12. Luo Z, Zhang D, Renner SS. Why two kinds of stamens in buzz-pollinated flowers? Experimental support for Darwin's division-of-labour hypothesis. Func Ecol, **2008;** 22: 794-800.

13. Abere TA, Okoto PE, Agoreyo FO. Antidiarrhoea and toxicological evaluation of the leaf extract of *Dissotis rotundifolia* Triana (Melastomataceae). BMC Compl Alt Med, **2010;** 10: 71.

14. Gill LS. Ethnomedical uses of plants in Nigeria. University of Benin press. **1992,** pp 103.

15. Amri E, Kisangau DP. Ethnomedicinal study of plants used in villages around Kimboza forest reserve in Morogoro, Tanzania. J Ethnobiol Ethnomed, **2012;** 8: 1.

16. Okeri HA, Alonge PO. Determination of the ascorbic acid content of two medicinal plants in Nigeria. Pak. J Pharm Sci, **2006;** 19(1): 39-44.

17. Rath G, Toure A, Nianga M, Wolfender JL, Hostettann K. Characterization of C-glycosylflavones from *Dissotis rotundifolia* by liquid chromatography–UV diode array detection–tandem mass spectrometry. Chromatographia, **1995;** 41(5): 332-342.

18. Kweku DW, Adinortey M, Weremfo A. Phytochemical constituents and aromatic content of methanol and aqueous extracts of *Dissotis rotundifolia* whole plant. SARJNP, **2018;** 1(1): 1-5.

19. Fabia R, Ar'Rajab A, Andersson R, Ahrén B, Bengmark S. Acetic acid-induced colitis in the rat: A reproducible experimental model for acute ulcerative colitis. Eur Surg Res, **1992;** 24: 211-225.

20. Osafo N, Obiri DD, Danquah KO, Essel LB, Antwi AO. Potential effects of xylopic acid on

acetic acid-induced ulcerative colitis in rats. Turk J Gastroenterol, **2019;** 30(8): 732-744.

21. Kimball ES, Wallace NH, Schneider CR, D'Andrea MR, Hornby PJ. Vanilloid receptor 1 antagonists attenuate disease severity in dextran sulphate sodium-induced colitis in mice. Neurogastroenterol Motil, **2004;** 16: 811–818.

22. Dieleman LA, Palmen MJ, Akol H, Bloemena E, Pena AS, Meuwissen M, et al. Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. Clin Exp Immunol, **1998;** 114(3): 385-391.

23. Morteau O, Morham SG, Sellon R, Dieleman LA, Langenbach R, Smithies O, et al. Impaired mucosal defense to acute colonic injury in mice lacking cyclooxygenase-1 or cyclooxygenase-2. J Clin Invest, **2000;** 105(4): 469-478.

24. Spiller RC. ABC of the gastrointestinal tract: Anorexia, nausea, vomiting and pain. BMJ, **2001;** 323(7325): 1354-57.

25. Cetinkaya A, Bulbuloglu E, Kurutas EB, Ciralik H, Kantarceken B, Buyukbese MA. Beneficial effects of N-acetylcysteine on acetic acid-induced colitis in rats. Tohoku J Exp Med, **2005;** 206: 131–139.

26. Moore MM, Chua W, Charles KA, Clarke SJ. Inflammation and cancer: causes and consequences. Clin Pharmacol Ther, **2010;** 87: 504–508.

27. Xianli Y, Xiao P, Yan L, Mingcai L. Role of IL-38 and its related cytokines in inflammation. Mediators Inflamm, **2015;** 807976.

28. Galli SJ, Grimbaldeston M, Tai M. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. Nat. Rev. Immunol, **2008;** 8(6): 478-486.

29. Wood JD. Histamine, mast cells, and the enteric nervous system in the irritable bowel syndrome, enteritis, and food allergies. Gut, **2006;** 55: 445.

30. Howe KL, Reardon C, Wang A, Nazli A, McKay DM. Transforming growth factor-1 regulation of epithelial tight junction proteins enhances barrier function and blocks enterohemorrhagic *Escherichia coli* O157:H7-induced increased permeability. Am J Pathol, **2005;** 167: 1587–1597.

31. Wang Q, Hou Y, Yi D, Wang L, Ding B, Chen X, et al. Protective effects of N-acetylcysteine on acetic acid-induced colitis in a porcine model. BMC Gastroenterol, **2013;** 13: 133.

Acta Pharm. Sci. Vol 59:(4), 2021
DOI: 10.23893/1307-2080.APS.05938

In Vitro Quality Evaluation of Amoxicillin Trihydrate Capsules Marketed in Gaza Strip-Palestine

Nahed Diab HEGAZY*

College of Medicine and Health Science, Department of Pharmacy and Biotechnology, University of Palestine, Gaza Strip, Palestine

ABSTRACT

The objective of this Post-Marketing Pharmaceutical Quality Evaluation of Amoxicillin trihydrate 500mg capsules to evaluate the quality standards of seven different marketed brands with various price ranges, collected from retail drug stores of Gaza-strip, Palestine. The quality of amoxicillin trihydrate capsules was assessed through evaluation of identification, uniformity of weight, disintegration, dissolution and assay of content of active ingredient using spectrophotometric method. It was observed that six of seven brands of amoxicillin trihydrate capsules meet quality specifications in Pharmacopoeia. The spectrophotometric method which used in assay of content of active ingredient of amoxicillin trihydrate capsules is simple, inexpensive, easy to use and could be used in routine monitoring especially in the absence of high technology equipment. This study shows the needing for more market monitoring of all available brands of all drugs in the drug market of Gaza Strip-Palestine.

Keywords: Amoxicillin trihydrate, pharmaceutical quality evaluation, dissolution test, gaza strip, pharmacopoeial specifications.

INTRODUCTION

Currently, it is estimated that 10–15% of the global drugs supplied are counterfeit. The prevalence is higher in developing countries, in Africa, and in parts of Asia and Latin America where up to 30–60% of drugs on the market are counterfeit. Among the medicines, antibiotics account for 28% of global counterfeit medicines. These problems have resulted in a weak therapeutic efficiency and devel-

*Corresponding author:
E mail: n.hegazy@up.edu.ps
Tel: +972599171907
ORCID:
https://orcid.org/0000-0002-1207-3325
(Received 26 February 2021, Accepted 7 June 2021)

opment of dire resistant strains. There is, therefore, a need to routinely assess the pharmaceutical quality of drugs[1]. Amoxicillin is an oral semi-synthetic, ß-lactam antibiotic used to treat bacterial infections caused by susceptible microorganisms. It is usually the drug of choice within the class because it is better absorbed, following oral administration, then other ß -lactam antibiotics[2]. Amoxicillin (500 mg capsules or tablets) is categorized as a biopharmaceutics classification system (BCS) class 1 drug. A recently published biowaiver monograph on amoxicillin trihydrate recommends submission of either comparative in vitro dissolution data or in vivo bioequivalence data as evidence to establish therapeutic equivalence of generic solid oral amoxicillin products of 250 and 500 mg[3].

Post-Marketing monitoring of medicines has been performed to evaluate the quality of marketed pharmaceutical brands[4]. Quality of the drug according to the modern definition requires that the product contain the quantity of each active ingredient claimed on its label within the applicable limits of its specifications, contain the same quantity of active ingredient from one dosage unit to the next, be free from extraneous substances, maintain its potency, therapeutic availability and appearance until used, and upon administration release active ingredient for full biological availability[5]. Quality control is the part of Good Manufacture Practice (GMP) that is concerned with sampling, specifications, testing, documentation and release procedures which ensure that the necessary and relevant tests are actually carried out and that the materials are not released for use, not products released for sale or supply, until their quality has been judged to be satisfactory[6]. The safety and efficacy of a pharmaceutical dosage form can be guaranteed when its quality is reliable. The efficacy of pharmaceutical dosage forms generally depends on their formulation properties, and manufacturing methods, hence it is likely that the quality of dosage form may vary[7].

The increase in the number of generic drug products from different multiple sources has placed people and prescribers in a position of selecting one from among several seemingly equivalent products[8]. Many of these products are inexpensive and affordable, but with uncertainly about their quality[9]. Several studies showed that switching from branded to generic medicine might result in changes of pharmacokinetics/pharmacodynamics profile, leading to subtherapeutic concentration or therapeutic failure and or adverse reactions[10]. It is very essential to do bioequivalence studies for generic products on account of any significant difference in the rate and extent by which the therapeutic ingredients become available at the site of drug action, administered under uniform conditions in an adequately designed study[11]. To identify bioavailability prob-

lems dissolution testing serves as an indicator[12]. Biopharmaceutically as well as chemically equivalent drug products must have the same quality, strength, purity, content uniformity, disintegration and dissolution rates[13]. In vitro quality control (QC) of pharmaceutical products is a fixed set of investigation started during production by in-process quality control tests and after production by finished product quality control tests as per official pharmacopoeias and different regulatory agencies. QC tests help in avoiding the confusion regarding safety, potency, efficacy and stability of pharmaceuticals[14].

Regular control of drug products has long been an integral part of the pre-and post-marketing quality control to safeguard the public. Many developing countries do not have an effective means of monitoring the quality of generic drug products in the market. This results in a widespread distribution of substandard and/or counterfeit drug products[8].

There are a number of cases related to substandard and counterfeit drugs. Composition and ingredients of substandard drugs don't meet the correct scientific specifications for these reasons they are ineffective and often dangerous to the patient. Counterfeit drugs may include products with the correct ingredients but fake packaging, with the wrong ingredients, without active ingredients or with insufficient active ingredients[15]. Substandard and counterfeit drugs are a major cause of morbidity, mortality and loss of public confidence in drugs and health structures[16]. WHO has estimated that approximately 10% of the global pharmaceuticals market consists of counterfeit drugs, but this estimate increases to 25% in developing countries, and may exceed 50% in certain countries. FDA estimates that up to 25% of the drugs consumed in poor countries are substandard or counterfeit[15]. Substandard and counterfeit drugs are not only limited to poor and developing countries but also intensely noticeable in developed countries. In 2007–2008, due to the uses of adulterated blood thinner, heparin 149 Americans were dying that was legally imported. In 2012, contaminated steroids killed 11 people and sickened another 100 people in the US. In another case, vials of the cancer medicine, Avastin were found to contain no active ingredients[17]. In a study of WHO found that 28% of antibiotic and 20–90% of antimalarial drugs were failed quality specifications[18].

As amoxicillin is widely used antibiotic in Gaza Strip, the objective of this study was to assess the quality of different leading brands of amoxicillin trihydrate 500mg capsules formulation commercially available in the market of Gaza Strip. This study also used and validated an analytical method for the assay of content of amoxicillin trihydrate in the capsules, which will be easy to use, accurate, simple and inexpensive when compared with other methods.

METHODOLOGY

Materials

Amoxicillin trihydrate (Merck, Germany), Amoxicillin trihydrate capsules (500 mg): seven brands, Sodium hydroxide, Hydrochloric acid, Ferric sulphate (Merck, Germany).

Instruments

Analytical balance (YMC, Japan), Disintegration apparatus (Toyama sangyo, Japan), Dissolution test apparatus (Apparatus II "Paddle apparatus"), Electric-heating distilling apparatus, Magnetic stirrer (Heidolph, Germany), Micrometer (Mitutoyo, Japan), Micropipette (Nichiryo, Japan), UV-visible spectrophotometer (equipped with 1cm Shimadzu, 1601, matched quartz cells).

Methods

Identification test of active substance

Amoxicillin trihydrate capsules

20 capsules from each brand were weighed. A quantity of the powder from each brand containing 0.05 g of amoxicillin trihydrate was weighed, then 10 ml of 1 % ferric sulphate was added[19].

Amoxicillin trihydrate pure powder

A 0.05 g of amoxicillin trihydrate pure powder was weighed, then 10 ml of 1 % ferric sulphate was added.

Uniformity of weight determination

20 capsules from each brand were taken at random and brushed from dust using soft brush then were weighed individually using analytical balance. Each capsule was opened without losing any part of the shell and the contents was removed as completely as possible then the shell was weighed. The weight of the content is the difference between weighing's (weight of capsule ''content & shell" – weight of shell). The average weight of content, percentage deviation from the average weight and SD were calculated (BP 2018).

Disintegration test

Disintegration time of six units per brand was determined in distilled water at 37 ± 1°C using disintegration apparatus (BP 2018). Determination was done in triplicate, and then the mean and SD were calculated.

Dissolution test

According to official monograph, the dissolution was performed according USP 41 NF20. The dissolution rate was determined by using dissolution apparatus II, and 900 ml of distilled water. Six units were used from each brand. The dissolution medium was maintained at 37 ± 0.5°C, and the paddle was rotated at 75 rpm. Samples (10 ml) were withdrawn at different time intervals (10, 20, 30, 45, and 60 minutes). The samples were filtered and diluted appropriately with distilled water. The absorbance was measured using UV-visible spectrophotometer at 272 nm. The content of amoxicillin trihydrate capsules in each sample was determined based on the calibration curve and regression equation which was generated according to the following procedure:

- Accurately 100 mg of pure amoxicillin trihydrate powder was dissolved in distilled water and diluted with distilled water to the mark in a 50 ml volumetric flask.
- The resulting solution was filtered then different volumes were taken (1 ml, 2 ml, 3 ml, 4 ml and 5 ml) using pipette and dilution was done with the distilled water to the mark 50 ml volumetric flask.
- Different aliquots of standard solutions (40 μg/ml, 80 μg/ml, 120 μg/ml, 160 μg/ml and 200 μg/ml) were prepared.
- The absorbance of the resulting solutions was measured at the maximum 272 nm using UV-visible spectrophotometer.
- Procedure was done in triplicate, and the mean of the absorbance values was calculated.

A linear plot with concentration of amoxicillin trihydrate on X-axis and absorbance on Y-axis, and the regression equation were obtained using Microsoft excel. The dissolution profiles of the different brands of amoxicillin trihydrate capsules were generated from the graph of the percentage amount of the dissolved drug versus time.

Analytical method validation

The quantitative determination method for amoxicillin trihydrate in the capsules was validated through assessment of accuracy and precision. The accuracy and precision of the methods were assessed by performing recovery experiments. Recovery experiment was performed as following: to a fixed amount of drug in the dosage form (pre-analyzed), pure drug was added at three amount levels (each added amount was performed in triplicate), then total amount was found by Dibbern et al. method and % recovery of pure drug was calculated[20].

The detailed procedures were done as the following:

Standard preparing: A quantity of pure powder containing 22.5 mg of amoxicillin trihydrate was added to 50 ml volumetric flask then 0.1 M sodium hydroxide was added till reach the mark and shaken. The resulting solution (a) was filtered and 1 ml of the filtrate was diluted to 100 ml with 0.1 M sodium hydroxide to get solution (b) with concentration of 4.5 μg/ml of amoxicillin. The absorbance of the solution (b) was measured at the maximum at 247 nm using UV-visible spectrophotometer. The content was calculated taking 286 as the value of A (1 %, 1 cm). Volumes that contain (10 μg, 20 μg and 40 μg) were taken from the solution (b).

Sample preparing: 20 capsules were taken, the contents was removed as completely as possible and weighed accurately. A quantity of the powder from capsules containing 22.5 mg of amoxicillin trihydrate was added to 50 ml volumetric flask then 0.1 M sodium hydroxide was added till reach the mark and shaken. The resulting solution (c) was filtered and 1 ml of the filtrate was diluted to 50 ml with 0.1 M sodium hydroxide to get solution (d) with concentration of 9 μg/ml of amoxicillin. Suitable dilution was made to get solution (e) with concentration of 1.5 μg/m of amoxicillin. The absorbance of the solution (e) was measured at the maximum at 247 nm using UV-visible spectrophotometer. The content was calculated taking 286 as the value of A (1 %, 1 cm). Volume that contain 20 μg of drug was taken from solution (e).

Recovery experiment

After preparing the solutions of pure drug and sample of amoxicillin trihydrate the following steps were done for recovery:

- Each volume that contains (10 μg, 20 μg and 40 μg) of pure drug was taken and added to volume of sample containing 20 μg.
- The absorbance of the resulting solutions were measured using UV-visible spectrophotometer at the maximum at 247 nm.
- The total amount was calculated taking the value of A (1 %, 1 cm.) as 286 for amoxicillin trihydrate.
- The percentage recovery of pure drug was calculated as the following:

% recovery = total amount (pure drug & sample) – amount of sample/ amount of pure drug x 100

- Each added amount of pure drug to the sample was performed in triplicate.
- The mean, SD and Relative Standard Deviation % (RSD %) were calculated.

Assay of content of active ingredient

Application of validated method

20 capsules were taken, the contents was removed as completely as possible and weighed accurately. A quantity of the powder containing 22.5 mg of amoxicillin trihydrate was added to 50 ml volumetric flask then 0.1 M sodium hydroxide was added till reach the mark and shaken. The resulting solution was filtered and 1 ml of the filtrate was diluted to 50 ml with 0.1 M sodium hydroxide. The absorbance of the resulting solution was measured at the maximum at 247 nm using UV-visible spectrophotometer. The content was calculated taking 286 as the value of A (1 %, 1 cm)[21]. Determination was done in triplicate, and then the mean of percentage content and SD were calculated.

RESULTS AND DISCUSSION

Identification test of amoxicillin trihydrate

The identification test of the standard (pure amoxicillin trihydrate) and the various brands resulted as intense yellow color was produced in standard as well as in all brands. From the results it was observed that all brands contain the needed active substance by comparing the result with that of standard pure active substance.

The results of the physicochemical properties of the various brands of amoxicillin trihydrate capsules are presented in Table 1.

Table 1. Physicochemical properties of seven brands of amoxicillin trihydrate capsules

Brand	Weight uniformity (g) Mean ± SD	Disintegration time (minutes) Mean ± SD	Dissolution at 60 minutes (%) Mean ± SD	Assay (%) Mean ± SD
A1	0.597 ± 0.016	6.363 ± 0.172	90.956 ± 0.707	108.970 ± 0.586
A2	0.600 ± 0.005	4.630 ± 0.312	89.471 ± 1.179	107.390 ± 1.088
A3	0.622 ± 0.005	5.210 ± 0.518	94.906 ± 0.926	110.436 ± 0.391
A4	0.571 ± 0.019	3.863 ± 0.551	83.684 ± 1.345	107.729 ± 0.391
A5	0.587 ± 0.007	3.153 ± 0.252	92.238 ± 0.728	109.082 ± 0.391
A6	0.636 ± 0.015	4.473 ± 0.423	97.967 ± 0.192	107.390 ± 0.195
A7	0.593 ± 0.006	3.517 ± 0.180	91.651 ± 0.870	107.842 ± 0.517

Uniformity of weight determination

Weight variation is important to ensure good manufacturing practices (GMP) sustained by the manufacturers and the content uniformity of the formulation[22]. From results (Table 1) it was noticed that the uniformity of weight determination for all the brands showed compliance with the BP 2018 specification, as none of the brands deviated by up to ± 5.0 % from their mean values. This

indicates that the factors leading to weight variation were taken in consideration. Factors that affect tablet weight includes tooling of the compression machine, head pressure, machine speed and flow properties of the powder[23].

Disintegration test

It was observed that all brands of amoxicillin trihydrate capsules passed BP 2018 specification of disintegration test, as the disintegration time of amoxicillin trihydrate capsules was less than 30 minutes (Table 1).

Dissolution test

The dissolution test is the measurement of the proportion of drug dissolving in a stated time under standardized conditions in vitro[24]. The importance of the test is to ensure the availability of the drug for absorption and to predict in vivo bioavailability[9].

The calibration curve was shown in Figure 1, and the resulted regression equation as following:

$Y= 0.0028 x + 0.0092$, $R^2 = 0.999998$

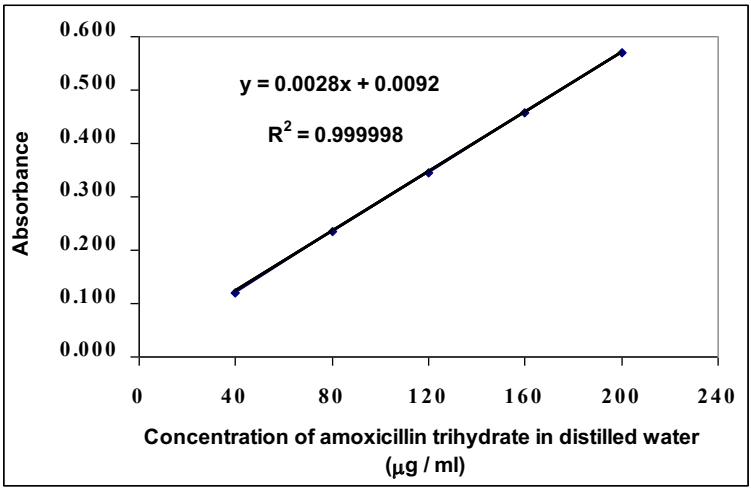


Figure 1: Calibration curve for amoxicillin trihydrate in distilled water.

The USP 41 stipulated that at 60 minutes, all capsules should have released into the dissolution medium an amount not less than 80.0% of the labeled amount of amoxicillin trihydrate. The percentage mean of the amount released at 60 minutes which are represented in Table 1 showed that all brands passed the dissolution test, that all brands released more than 80.0% of their content within 60 minutes.

It was observed that all brands meet pharmacopoeia specification of dissolution test. The results revealed that all brands exhibit good release of the drug to the site of absorption and may have good bioavailability. It is interesting to note that several authors have previously disagreed on the correlation between disintegration time and dissolution time. Some authors mention that disintegration and dissolution times are correlated, while others continue to disagree[9]. From the results, it was observed that no high range between times of the disintegration of brands, but it was noticed the differences in the dissolution profiles between brands as shown in Figure 2. Dissolution of drugs can be influenced by the physicochemical properties of the drug substance, the dosage form design, the manufacturing process, and the testing conditions[25]. As there are many factors affecting on the dissolution, this gives each product certain dissolution characteristics which varies from brand to another. So it is not surprising to observe variation in vitro dissolution among seven brands amoxicillin trihydrate capsules which were investigated in this study.

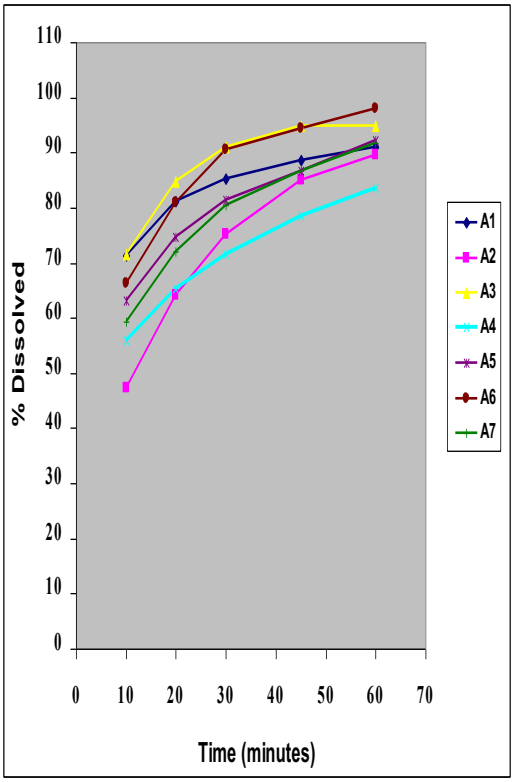


Figure 2: Dissolution profile of the seven different brands of amoxicillin trihydrate capsules in distilled water.

Analytical method validation

From the results (Table 2) it was observed that the recoveries percentage of the added pure amoxicillin trihydrate were in the range of (98.506 - 102.593 %), this indicated excellent accuracies and no interference from excipients was exhibited. SD is less than 2.0 and RSD % is less than 2.0 %, this indicated the high precision of the method.

Table 2. Results of recovery study of amoxicillin trihydrate using Dibbern et al. method

	Amount of amoxicillin trihydrate in formulation (µg)	Amount of pure amoxicillin trihydrate added (µg)	% Recovery of pure amoxicillin trihydrate*	SD	RSD %
A1	20.0	10.0	99.369	0.865	0.870
	20.0	20.0	101.249	0.330	0.326
	20.0	40.0	100.023	0.804	0.804
A2	20.0	10.0	99.952	0.158	0.158
	20.0	20.0	101.756	1.116	1.096
	20.0	40.0	99.577	0.000	0.000
A3	20.0	10.0	100.576	0.000	0.000
	20.0	20.0	101.118	1.175	1.162
	20.0	40.0	98.506	0.000	0.000
A4	20.0	10.0	100.570	0.000	0.000
	20.0	20.0	102.593	1.167	1.137
	20.0	40.0	99.886	0.819	0.820
A5	20.0	10.0	99.136	1.842	1.858
	20.0	20.0	101.054	0.000	0.000
	20.0	40.0	100.786	0.000	0.000
A6	20.0	10.0	100.424	0.000	0.000
	20.0	20.0	100.362	0.917	0.913
	20.0	40.0	100.608	0.845	0.839
A7	20.0	10.0	101.049	0.000	0.000
	20.0	20.0	101.152	1.897	1.876
	20.0	40.0	99.810	0.927	0.929

*mean value of three determinations**.**

Thus, the Dibbern et al[21]. method is simple, rapid, no laborious time consuming, inexpensive and no need for high cost instruments. The significant advantage is the possibility of using the method to assay the drug in complex dosage formulation in presence of the excipient without any interferences.

Assay of content of amoxicillin trihydrate

Assay of pharmaceutical products is a critical quality parameter required to confirm that the labeled amount of drug is available in a given dosage form and failure to meet the standard will result in poor quality medicines. Inadequate amounts of active pharmaceutical ingredient (API) will result in under-dosed medication, leading to poor treatment outcomes while excessive amounts of API cause over-dosage of medication, leading to increased adverse drug reactions and treatment failure[26].

The results showed that brands (A1, A2, A4, A5, A6, and A7) had values range (107.390-109.082% w/w), thus it lies within BP 2018 acceptable range (92.5-110.0% w/w), while brand (A3) had value 110.436 % w/w, thus not lies in BP 2018 acceptance range (Table 1).

It was observed that one of seven brands of amoxicillin trihydrate capsules (A3) failed to be within BP 2018 specification range of (92.5-110.0% w/w). This revealed that there is a problem in manufacturing of failed brands, while there is good manufacturing for accepted brands.

REFERENCES

1. Abraham W, Abuye H, Kebede S, Suleman S. In Vitro Comparative Quality Assessment of Different Brands of Doxycycline Hyclate Finished Dosage Forms: Capsule and Tablet in Jimma Town, South-West Ethiopia. Hindawi: Advances in Pharmacological and Pharmaceutical Sciences. **2021:**10 pages.

2. Kassaye L, Genete G. Evaluation and comparison of in-vitro dissolution profiles for different brands of amoxicillin capsules. African Health Sciences. **2013;** 13(2): 369-375.

3. Thambavita D, Jayathilake CM, Sandamali KDD, Galappatthy P, Jayakody RL. In Vitro Dissolution Testing to Assess Pharmaceutical Equivalence of Selected Amoxicillin Products Available in Sri Lanka: A Post-Marketing Study. Dissolution Technologies. **2019:** 56-61.

4. Hasin F, Hossain MdM, Ghosh MK, Paul S, Rahman M, Ahmad MdF. Pharmaceutical Quality Evaluation of Cefuroxime Axetil Tablets Available in Drug Market of BANGLADESH. World Journal of Pharmacy and Pharmaceutical Sciences. **2017;** 7(1): 1589-1599.

5. Banker GS. Drug Products: Their role in the treatment of disease, their quality and their status and future as drug-delivery systems. *Modern pharmaceutics*; New York: Marcel Dekker, Inc.; **2002.** 1-21.

6. World Health Organization and Cairo university, *Guidelines for good manufacturing practice of pharmaceuticals in Egypt*, Faculty of pharmacy, Cairo University, Central administration of pharmacy, WHO, Cairo, 19941

7. Odeniyi MA, Adegoke OA, Adereti RB, Odeku OA, Itiola OA. Comparative analysis of eight brands of sulfadoxinepyrimethamine tablets. Tropical Journal of Pharmaceutical Research. **2003;** 2(1): 161-167.

8. El Attug M, Ammar A, Ben Ahmed A, Alborawy H, Mashina A, Al Mug T, et al. Pharmaceutical Assessment Of Five Different Generic Brands of Prednisolone Tablets In Libyan Market. World Journal of Pharmaceutical Research. **2015;** 4(10): 1-18.

9. Nwodo N, Uzochukwu I, Omeje EO. Quality control assessment and the possibility of interchangeability between multisourced norfloxacin tablets marketed in Nigeria. Scientific Research and Essay. **2007;** 2(8): 348-352.

10. Crawford P, Feely M, Guberman A, Krämer G. Are there potential problems with generic substitution of antiepileptic drugs? A review of issues. Seizure. **2006;** 15: 165–176.

11. Shargel L, Andrew BC. Yu. Applied biopharmaceutics & pharmacokinetics. 7th edition. New York: McGraw-Hill; **2016.**

12. Shah V. Dissolution: a quality control test vs a bioequivalent test. Dissolution Technol. **2001;** 8(4): 1–2.

13. Hassali MA, Thambyappa J, Saleem F, Haq N, Aljadhey V. Generic substitution in Malaysia: recommendations from a systematic review. Journal of Applied Pharmaceutical Science. **2012;** 2(8): 159-164.

14. Sufian MA, Uddin MS, Islam MT, Zahan T, Hossain K, Uddin GMS, et al. Quality control parameters of parenteral pharmaceuticals based on pharmacopoeias. Indo American Journal of Pharmaceutical Sciences. **2016;** 3(12): 1624-1638.

15. Uddin MdS, Al Mamun A, Hossain MdS, Asaduzzaman Md, Sarwar MdS, Rashid M, et al. In vitro quality evaluation of leading brands of ciprofloxacin tablets available in Bangladesh. BMC Research Notes. **2017;** 10(185): 2-9.

16. Cockburn R, Newton PN, Agyarko EK, Akunyili D, White NJ. The global threat of counterfeit drugs: why industry and governments must communicate the dangers. Plos Med. **2005;** 2(4):100.

17. Anonymous. Counterfeit medications. https://en.wikipedia.org/wiki/Counterfeit_medications.

18. Glass BD. Counterfeit drugs and medical devices in developing countries. Dove Press Journal: Research and Reports in Tropical Medicine. **2014;** 5: 11–22

19. Issa MM, Nejem R, Al- Kholy M, El-Abadla N, Helles RS, Saleh AA. An indirect atomic absorption spectrometric determination of ciprofloxacin, amoxicillin and diclofenac sodium in pharmaceutical formulations. Journal of the Serbian Chemical Society. **2008;** 73 (5): 569-576.

20. Basavaiah K, Nagegowda P, Somashekar BC, Ramakrishna V. Spectrophotometric and titrimetric determination of ciprofloxacin based on reaction with cerium (IV) sulphate. Science Asia. **2006;** 32: 403-409.

21. Dibbern HW, Müller RM, Wirbitzki E. UV and IR spectra: Pharmaceutical substances (UV and IR) and pharmaceutical and cosmetic excipients (IR), Germany: Editio Cantor Verlag; **2002.**

22. Hegazy N, Abuwked E, Musallam A, El-Shanti A, Emghary M, AbdAlaziz I, et al. Comparative in vitro evaluation of various commercial brands of valsartan tablets marketed in Gaza Strip. Medcare Science Publisher, International Journal of Pharma Medicine. **2021;** 1: 5 Pages.

23. Abbirami V, Sainithya P, Shobana A, Devi DR, Hari BNV. A Review on In-vitro Bioequivalence Studies and its Methodologies. International Journal of ChemTech Research. **2013;** 5(5): 2295-2302.

24. Lachman L, Liberman HA, Kanig JL. The Theory and Practice of Industrial Pharmacy.; 3rd edition. Bombay: Varghese publication; **2008.** 300-804.

25. Kassahun H, Asres K, Ashenef A. In vitro quality evaluation of metformin hydrochloride tablets marketed in Addis Ababa. Bangladesh J. Sci. Ind. Res. **2019;** 54(2): 169-176.

26. Abebe S, Ketema G, Kassahun H. In vitro Comparative Quality Assessment of Different Brands of Furosemide Tablets Marketed in Northwest Ethiopia. Dove Press journal: Drug Design, Development and Therapy. **2020;** 14: 5119-5128.

Acta Pharm. Sci. Vol 59:(4), 2021
DOI: 10.23893/1307-2080.APS.05939

Flavonoids from Plant Source as Protein Tyrosine Phosphatase 1B Inhibitors: *In Silico* Update

Ihab ALMASRI[1], Halima OTHMAN[2], Bashaer ABU-IRMAILEH[3], Mohammad MOHAMMAD[4], Yasser BUSTANJI[5]

1 Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmacy, Al Azhar University-Gaza, Gaza Strip, Palestine. https://orcid.org/0000-0001-5988-9 5059.

2 Department of Biological Sciences, University of Jordan, Amman, Jordan.

3 Hamdi Mango Center for Scientific Research, The University of Jordan, Amman.

4 Department of Pharmaceutical Sciences, Faculty of Pharmacy, The University of Jordan.

5 Department of Basic Medical Sciences, College of Medicine, University of Sharjah, Sharjah 27272, UAE.

ABSTRACT

Protein Tyrosine Phosphatase 1B (PTP1B) enzyme, type 1 non-transmembrane protein tyrosine phosphatase, is extensively expressed in different organs in the body and is linked to different signal transduction pathways including insulin signaling pathway. PTP1B inhibition exhibits significant effects on glucose homeostasis and glucose balance in blood and tissue. Several types of synthetic/natural compounds have been tested. Among these compounds, flavonoids showed high potency as PTP1B inhibitors. Different studies revealed that flavonoids could be promising PTP1B inhibitors with antidiabetic and weight loss effects. In recent years, the discovery of new flavonoids with PTP1B inhibition activity has been facilitated by the application of molecular modeling and computational methods. This review highlights the research efforts conducted in the discovery of flavonoids as PTP1B inhibitors and the role of computer-aided drug design techniques utilized in the discovery process.

Keywords: PTP1B, flavonoids, inhibitory activity, *In Silico*, diabetes mellitus

*Corresponding Author:
Ihab Almasri, Mobile: +970 599 403 757; E-mail: ihabalmasri@yahoo.com
ORCIDs:
Ihab Almasri :https://orcid.org/0000-0001-5988-9 5059.
Halima Othman : https://orcid.org/0000-0001-5690-5317
Bashaer Abu-Irmaileh : https://orcid.org/0000-0003-1040-3156
Mohammad Mohammad : https://orcid.org/0000-0003-0025-365X
Yasser Bustanji : https://orcid.org/0000-0003-1315-0609
(Received 07 February 2021, Accepted 7 June 2021)

INTRODUCTION

For several millenniums, products derived from nature were considered a keystone in human strategies against diseases as a treatment and preventive agents. Recently, the interest in using medicinal plants for the treatment of diseases in many countries has increased. Natural products have wide structural and chemical diversity which continue to motivate novel discoveries in medicine. However, besides their biological and chemical diversity, the emergence of innovative technologies has reformed the screening of natural products methods used in discovering new drugs. They are evolutionarily optimized as drug-like molecules, and have therefore proved to be an important source for new drugs and bioactive leads. About 35% of approved drugs over the past two decades were plant based or derived from natural sources[1,2]. The discovery of novel, safer and more efficacious medicines continues to be the most significant goal regarding the development of new drugs. Therefore, researchers have recently reconsidered the use of bioactive compounds obtained from natural sources in folk medicine to treat diabetes mellitus (DM), cancer, and other diseases because of their natural origination[3].

Protein tyrosine phosphatase 1B (PTP1B)is an ubiquitously expressed phosphatase that has emerged as a relevant regulator of a variety of signaling pathways initiated by the activation of the tyrosine kinase receptor superfamily[4]. Therefore, several potential therapeutic uses of PTP1B inhibitors were reported in the literature. It was disclosed that increased PTP1B activity is associated with defective neuronal insulin signaling pathways that are impaired in Alzheimer's disease (AD), therefore PTP1B inhibition may represent an interesting therapeutic approach to modify abnormal signaling processes linked to AD[4]. Moreover, it was found that the expression of PTP1B is increased in inflammatory conditions where it plays a role in neuroinflammation[5]. PTP1B also acts as a negative regulator of interleukin-4-induced anti-inflammatory signaling, and thus it is considered a potential therapeutic target for neuroinflammatory and neurodegenerative diseases[5]. Additionally, PTP1B is an active player in several types of cancer including lung cancer and breast cancer, both as an oncogene and a tumor suppressor. The enzyme plays a role in the regulation of cell migration and adhesion in cancerous as well as normal cells[6]. Recently, PTP1B has received attention in liver diseases and represents an interesting target by modulating liver cells death, survival, and hepatic lipogenesis[7]. More notably is a decline in the number of apoptotic liver cells and the level of liver enzymes due to the interruption of PTP1B in mice model. Furthermore, PTP1B contributes to the cardiovascular disturbances at different molecular levels and

therefore, PTP1B inhibitors could be used in prevention and reversal of atherosclerosis development and thus reduces cardiovascular disease risk[8].

PTP1B has been identified as a negative regulator of both insulin and leptin signaling pathways leading to decreased sensitivity to both hormones. Studies have shown that insulin resistant conditions are associated with increased expression and activity of PTP1B and therefore is considered a valid therapeutic target for diabetes[8,9]. Diabetes mellitus type 2 (T2DM) is a chronic metabolic disorder manifested by high levels of blood sugar associated with a gradual decrease in insulin secretion and/or increase of insulin resistance. T2DM accounts for 90% of the cases of diabetes globally and about 90% of adults with T2DM are overweight or obese[10,11]. Therefore, it is not unexpected that substantial efforts have been made to identify medicines to treat T2DM. Several animal models, cell lines and clinical studies have shown that deficiency of PTP1B activity is accompanied by resistance to obesity with an increase in insulin sensitivity[8,9].

The significance of such target has increased the demand of substantial research in the discovery of PTP1B inhibitors for T2DM and obesity treatment[9,12]. Within the last few years, there has been a growing interest in the discovery of antidiabetic and weight loss agents from natural products. Among these natural products, flavonoids were found to be an important source for new antidiabetic drugs, whereas numerous cell and animal studies support the hypoglycemic activity of flavonoids including PTP1B inhibition[13].

In this review, we will provide a brief overview of flavonoids as natural products with anti-PTP1B activity, including a discussion of the results of many recent studies focused on the discovery of flavonoids as PTP1B inhibitors using computer-aided drug discovery techniques.

PTP1B structural biology and its mechanism as a target for obesity and diabetes

Protein tyrosine phosphatases (PTPs), signaling enzymes, are a group of enzymes which catalyze the dephosphorylation of tyrosine phosphorylated proteins. PTPs have a considerable effect on regulation of a number of cellular operations such as proliferation and differentiation, growth, cell-cell adhesion, metabolism, cell matrix contacts, and immune response. In particular, PTP1B,also known as protein tyrosine phosphatase non-receptor type 1, is a type 1 non-transmembrane protein that catalyzes tyrosine phosphorylated proteins. It is widely distributed indifferent organs in the human body and participated in different signal transduction pathways[14]. PTP1B participates

in insulin signaling pathway by dephosphorylation of insulin receptor and its downstream signaling components, which involved in glucose level regulation and resulted in diabetes and weight gain or as called diabesity; obesity resulted from diabetes[15,16]. Accordingly, targeting the activity of this enzyme is suggested to have therapeutic effect in diabetes. PTP1B inhibitors are attractive leads for the treatment of insulin resistance in diabetes. The discovery of the crystal structure of human PTP1B and its catalytic site by Barford and colleagues helped in understanding, illustrating, predicting, and designing potent inhibitors (Figure 1)[17].

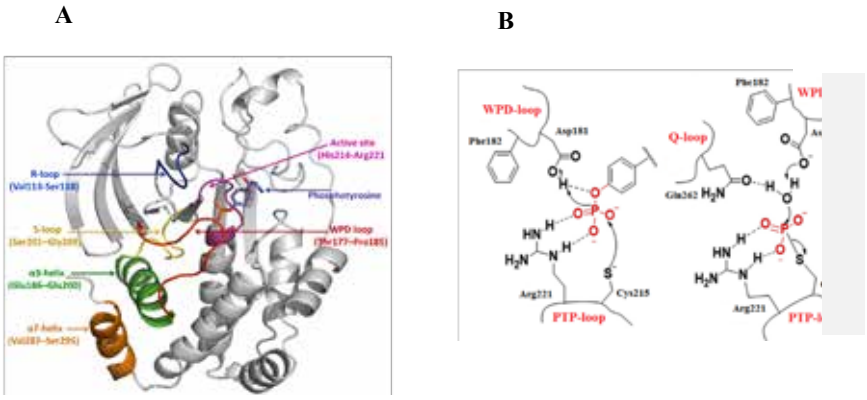


Figure 1: A. Cartoon representation of PTP1B protein structural elements18. **B.** The catalytic mechanism of tyrosine dephosphorylation within the active site.

The majority of PTP1B crystal structures consist of a single domain, arranged in eight α helices and eleven β strands (Figure 1). The catalytic site of PTP1B composed of amino acid residues His214-Arg221 and loops R (Val113-Ser118),WPD (Thr177-Pro185). WPD-loop, conserved protein loop, contains three highly conserved residues: tryptophan (W), proline (P) and aspartic acid (D). The other main elements of PTP1B are: R3-helix (Glu186-Glu200); R6-helix (Ala264-Ile281); and R7-helix (Val287-Ser295); and S loops (Ser201-Gly209) which play a role in the binding of substrate and take part in dephosphorylation of tyrosine moiety of substrate proteins[18,19]. Several X-ray crystallized structures revealed that Cys215, Arg22, Asp181, and Gln262 are the most vital residues in the enzyme catalytic activity[20]. Within the catalytic site Cys215 acts as a nucleophile in the primary separating phase. Moreover, a non-catalytic allosteric binding site for small molecule inhibitors was also identified (Figure 1)[21].

Selectivity is a very significant issue in the strategy of designing new PTP1B inhibitors. Targeting the catalytic active site of PTP1B,which is highly conserved among PTPs, raised substantial challenges regarding the selectivity and bioavailability of the enzyme inhibitors due to the highly charged character of the binding site. Therefore, researchers were encouraged to explore other mechanisms of enzyme inactivation.

In 2004, a newPTP1B allosteric site nearby the active binding pocket (20 Å) was discovered. It was revealed that allosteric inhibitors trap inactive conformation by inhibiting flexibility of the catalytic loop[22]. Moreover, Krishnan *et al.* (2014) have identified a new mechanism of allosteric inhibition throughout targeting the non-catalytic part of PTP1B at the C-terminal. It was demonstrated that targeting on-catalytic area adjacent to the active site would lock PTP1B in an inactive state as a result of the cooperative effects between the two sites[23]. Therefore, targeting the allosteric site would provide superior selectivity, less side effects, and lower toxicity because of its hydrophobic nature and no conserved sequence. Consequently, the allosteric site emerged as an interesting target in drug discovery.

Regulation of insulin signaling pathway

Insulin is a peptide hormone secreted by β-cell and is found to be negatively regulated by PTP1B. Upon binding to the extracellular α subunits of its receptor, insulin receptor (IR) is activated by autophosphorylation. Subsequently, phosphorylation of Tyrosine residues of insulin receptor substrates (IRS) mostly recruits and activates the phosphoinositide 3-kinases (PI3K) leading to the generation of second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3)(Figure 2)[24,25]. The membrane-bound PIP3 recruits and activates protein kinase-1 (PDK-1), which phosphorylates the protein kinase B (PKB). Pleckstrin homology (PH) domain, which is part of PDK-1, plays an important role in the activation of PDK-1 and the subsequent activation of Akt/PKB upon strong binding to membrane-bound PIP3. PDK-1 phosphorylates PKB/Akt at Thr-308. Therewith, phosphorylation at Ser-473 is essential for complete activation of PKB, and this is achieved by the mammalian target of rapamycin complex 2 (mTORC2)[23].

Activated PKB/Akt plays the main role in interceding insulin action and metabolic effects such as gluconeogenesis, glycogen synthesis, and glucose transport. The phosphorylation of the activated Akt substrate, AS160, is required for translocation of glucose transporter 4 (GLUT4) to the plasma membrane in a process known as GLUT4 translocation. PTP1B dephosphorylates the tyrosine residues on activated IR and IRS, thereby leading to their deactivation. The ac-

tion of PTP1B resulted in impediment of the activation of PI3K and PKB/Akt and subsequent downstream kinases and therefore turns off insulin signal. As a result, GLUT4 translocation is inhibited and therefore cellular glucose uptake is reduced, and insulin resistance occurred, which reduces glucose intake and increased hepatic glucose output, which finally cause increment in blood glucose level (Figure 2)[24,25].

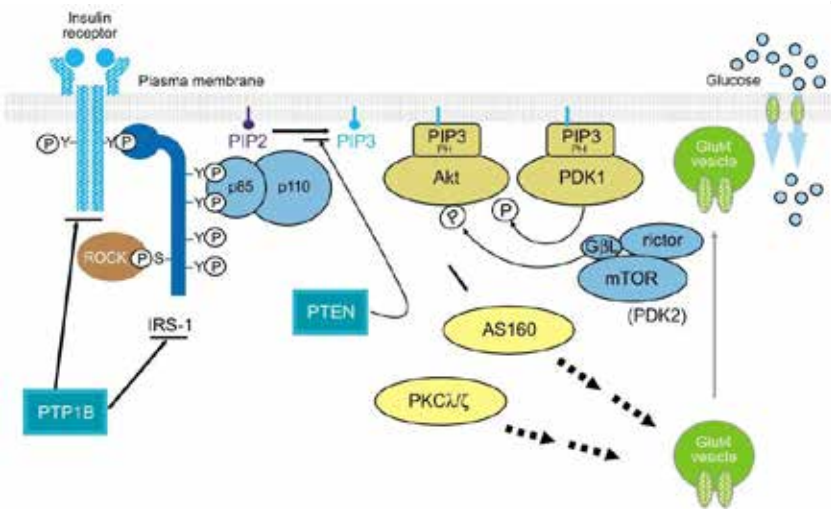


Figure 2: Negative regulation of insulin signaling pathway by PTP1B. Binding of insulin with its receptor tyrosine kinase (IR) results in the phosphorylation of insulin receptor substrates (IRS). Main IRS-mediated pathways include the PI3K/Akt pathway, which plays a central role in activation and regulation of several metabolic processes, including glycogen and protein synthesis glucose transport stimulation and adipogenesis. IR is dephosphorylated and inactivated by PTP1B which has negative effects on glucose metabolism and insulin action[25].

Flavonoids as PTP1B inhibitors

Flavonoids belong to a class of secondary plant metabolites having polyphenolic structure that exist extensively in nature and in the human diet. They are composed of 15 carbon atoms, having two aromatic rings connected by three carbon atoms. Flavonoids have several subgroups, which include chalcones, flavonols, flavones, flavanones, isoflavones, catechins, and anthocyanidins (Figure 3). Their broad spectrum of biological activity such as antidiabetic, cardioprotective, antitumor, anti-inflammatory, antiviral, antioxidant and antiplatelet activities, has attracted great interest in research on flavonoids[26].

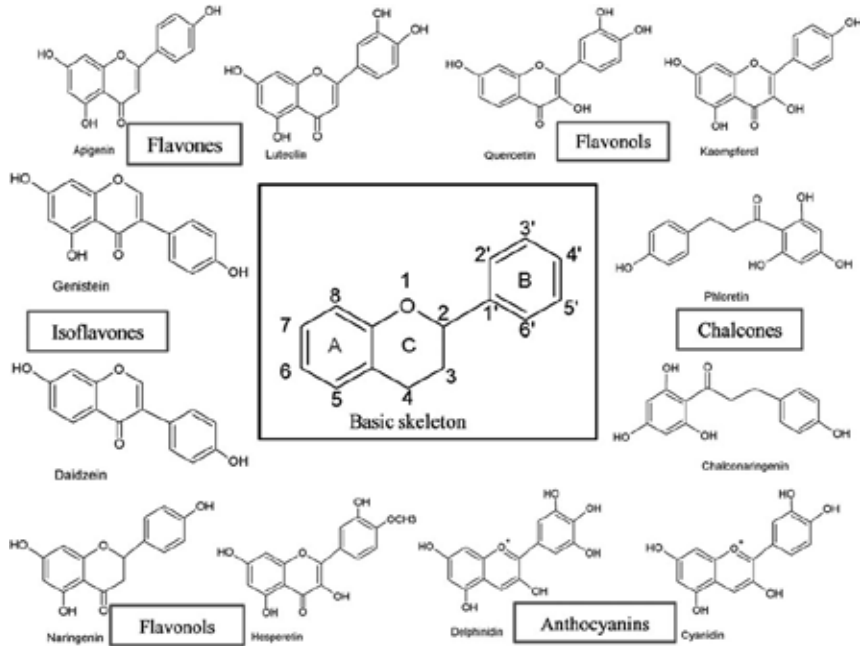


Figure 3: Basic skeleton structure of flavonoids and their classes[26].

In 2002, Ueda and colleagues were the first to study a natural derived inhibitor for PTP1B. They isolated 4-Isoavenaciolide from a fungal strain and was identified as an inhibitor of PTP1B with IC_{50} of 10.7 μM [27].

The interest in exploring the bioactivity of *Erythrina* species has led to the separation of many biologically active compounds that inhibit PTP1B *in vitro*. A bioassay-guided fractionation of the ethanolic extract of the stem bark of *E. addisoniae* (*Leguminosae*) has resulted in the identification of a group of prenylated isoflavonoids regarded as PTP1B inhibitors[28]. Three of these natural molecules showed potential inhibition against PTP1B with IC_{50} values ranging between 2.6 ± 0.5 to 10.1 ± 0.3 μM (compounds **1-3**, Figure 4). It was noticed that cyclization between a hydroxyl group at C-7 and one of the prenyl groups at C-6 or C-8 in the ring **A** and the presence of hydroxyl group in ring **B** at positions 2'and 4' could be significant for activity[28]. Further exploration for this plant resulted in the separation of six2-arylbenzofuran derivatives from the stem bark. Three of them (**4-6**, Figure 4) were new with relatively strong *in vitro* anti-PTP1B activity (IC_{50} between 13.6 ± 1.1 to17.5 ± 1.2)[29]. Moreover, 12 flavanones with 2,2-dimethylpyrano ring were isolated, six of them (**7-12**, Figure 4) showed *in vitro* PTP1B inhibition with IC_{50} values ranging between

13.9 ± 2.1 and 19.0 ± 1.8 μM[30]. The obtained results showed that the presence of methoxy groups and prenyl moiety on ring **B** boost the PTP1B inhibitory activity of isolated flavanones.

Nguyen *et al.*, reported the isolation of 15 pterocarpan derivatives and 6 prenylated isoflavonoids from the alcoholic extract of the root and the stem bark of *E. abyssinica* and *E. addisoniae*, respectively[31,32]. All the isolated compounds were evaluated for their PTP1B inhibition activity, as well as for their cytotoxic activities against a group of breast cancer cell lines. Pterocarpan derivatives (**13-20**, Figure 4) which have anti-PTP1B activity (IC_{50}) ranging from 4.2 ± 0.2 to 19.3 ± 0.3 μM, displayed potential cytotoxic activities (IC_{50} between 5.6 ± 0.7 to 28.0 ± 0.2 μM). Similarly, prenylated isoflavonoids (**21-25**, Figure 4) which have anti-PTP1B activity, between 4.6 ± 0.3 to 24.2 ± 2.1 μM, exhibited strong cytotoxic activities (3.97 ± 0.17 to 11.4 ± 1.9 μM). Structurally similar isoflavonoids derivatives of another *Erythrina* species (*E. lysistemon*, Hutch) were separated from the stem bark. Nine of them showed *in vitro* potential inhibition of PTP1B and the most potent compound was **26** (Figure 4) with an IC_{50} = 1.01 ± 0.3 μg/mL (2.4 μM)[33]. The obtained results supported the previous remarks, regarding the structural activity relationship (SAR) of prenylated isoflavonoids[28], which suggested that the presence of prenyl groups on pterocarpans could enhance their inhibitory activity especially at ring **A** and/or **D**. Furthermore, the presence of an aldehyde at C-8 and a hydroxyl group at C-6 may account for the decrease in activity[33].

A natural product library of 42 licorice flavonoids was screened against PTP1B. Several compounds with potential PTP1B inhibitory activity were identified, and the most potent compound was licoagrone (**27**, IC_{50}= 6.0 μM, Figure 5)[34]. Similarly, screening of another natural compounds library resulted in the discovery of five new inhibitors of PTP1B that were isolated from *Sophora flavescens*[35]. These compounds were derivatives of lavandulyl flavonoids, and the most potent compound was 2'-methoxykurarinone (**28**, IC_{50}=5.26 ± 0.24 μM, Figure 5), which is a noncompetitive PTP1B inhibitor (K_i = 3.15 μM)[35].

Luteolin (**29**, Figure 5), an abundant flavonoid existing in numerous vegetables and fruits, was reported to have potent PTP1B inhibitory activity (IC_{50} = 6.70 ± 0.03 μM). However, isoorientin and orientin, two C-glycosylated luteolin derivatives, were both inactive at the same concentration, which points out that C-glycosylation at various positions on luteolin could have a negative influence on luteolin-PTP1B inhibitory activity[36].

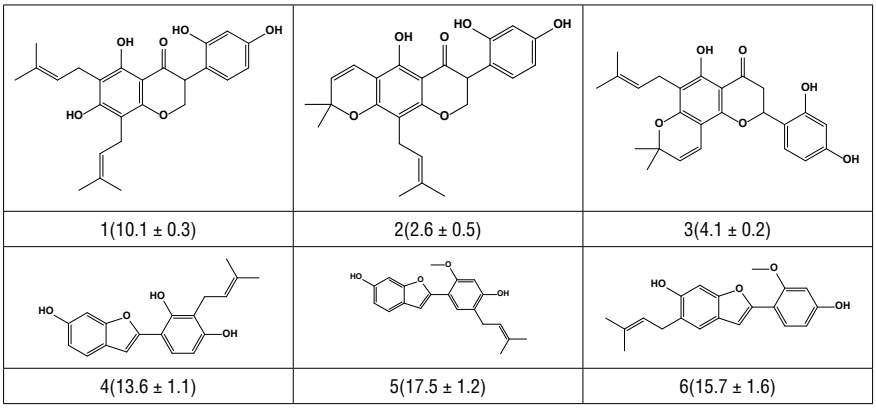
Investigation of the extract of *Cudrania tricuspidata* roots led to the separation of 16 compounds. Four of the isolated compounds were flavonoids (**30–33,** Figure 5) that showed significant PTP1B inhibitory activity in a dose-dependent manner, with IC_{50} ranging from 5.7–13.6 μM. Moreover, kinetic analyses revealed that the isolated flavonoid (**32**) was a noncompetitive PTP1B inhibitor, so it could be considered a valuable lead compound in designing antidiabetic and weight loss agents[37].

In another study, nine natural products were separated from *Ficus tikoua* (*Moraceae*). Two of these compounds were novel isoprenylated flavanones, while seven of them showed moderate *in vitro* PTP1B inhibition activities (IC_{50} range from 11.16 – 40.37 μM). The most potent one was the isoflavone derivative with IC_{50} = 11.16 μM (**34**, Figure 5)[38].

Three isolated flavonoids from *Agrimoni apilosa* showed a good inhibitory potency against PTP1B[39]. Kaempferol-3-*O*-α-L-rhamnoside (**35**) was the most potent flavonoid (12.16 ± 0.02 μM) while compound (**36**), apigenin-7-*O*-β-D-glucuronide-6"-methyl ester, showed strong PTP1B inhibition (IC_{50} = 14.35 ± 0.76 μM) (Figure 5).

In 2015, Jiang, *et al.*, evaluated the pharmacological activity of several flavonoids derived from *Hypericum scabrum* L. and they found that only quercetin (**37**, Figure 5) exhibited significant PTP1B inhibitory activity (IC_{50} = 2.19 ± 0.2 μM, Figure 5)[40].

Eight geranyl-substituted flavonoids with dual inhibition of α-glucosidase and PTP1B were separated from the methanolic extract of the fruits of *Paulownia tomentosa* (**38-45**, Figure 5). All of them exhibited effective mixed type I PTP1B inhibition activity (IC_{50} values between 1.9 – 8.2 μM) as shown by enzymatic kinetics study[41].



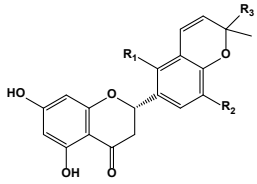
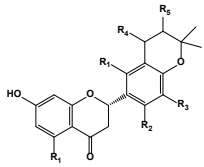
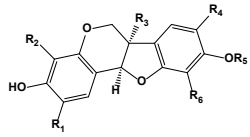
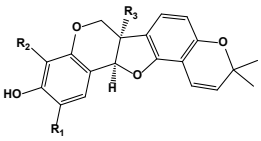
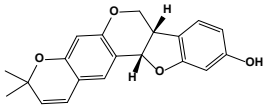
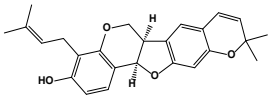
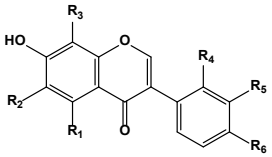
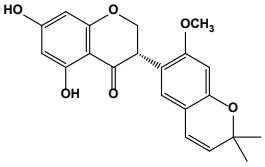
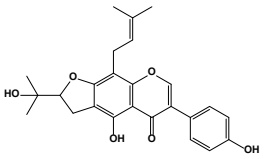
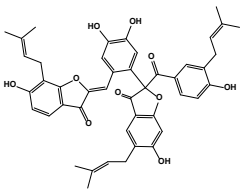
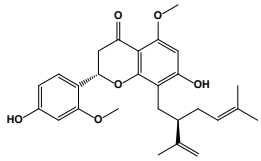
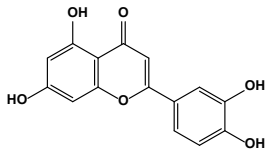
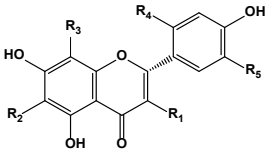
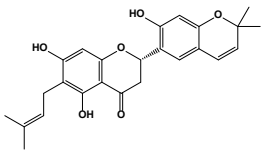
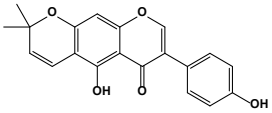
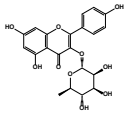
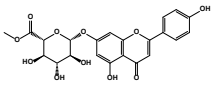
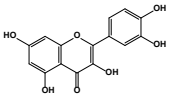
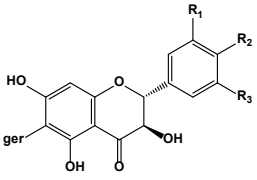
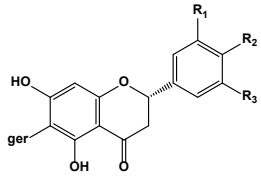
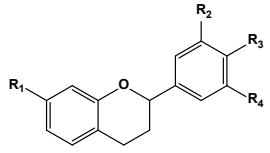
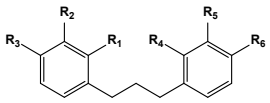
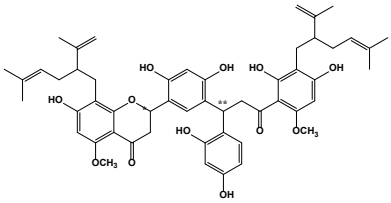
		
7 (13.9 ± 2.1) (R_1= prenyl, R_2= OH, R_3= CH_3) 8 (17.9 ± 1.7) (R_1= H, R_2= OCH_3, R_3= CH_2OH)	9 (14.9 ± 1.6) (R_1= R_5= OH, R_2= R_4= H, R_3= prenyl) 10 (18.2 ± 2.1) (R_1= R_5= OH, R_2= R_4= H, R_3= OCH_3) 11 (19.0 ± 1.8) (R_1= R_4=R_5= OH, R_2= H, R = prenyl) 12 (18.2 ± 1.2) (R_1= R_4= R_5= OH, R_2= prenyl, R_3= OH)	13 (19.3 ± 0.3) R_1=R_2=R_4=H,R_3=OCH_3,R_5=CH_3, R_6=prenyl 14 (19.5 ± 1.5) R_1=R_3=R_4=R_5 =R_6 =H, R_2= prenyl 15 (7.3 ± 0.1) R_1=R_3=R_5=R_6= H, R_2=R_4= prenyl 26 (2.4 μM) R_1= R_3=R_4=R_5= H, R_2=R_6= prenyl
		
16 (4.2 ± 0.2) R_1 = R_3 = H, R_2 = prenyl 17 (7.8 ± 0.5) R_1= prenyl, R_2= R_3 = H 18 (8.8 ± 0.5) R_1= prenyl, R_2 = H, R_3 = OH	19 (7.6 ± 0.9)	20 (6.4 ± 0.6)
		
21 (17.4 ± 1.1) R_1=R_5=H, R_2=R_3= prenyl, R_4=R_6=OH 22 (7.8 ± 0.5) R_1=R_2=R_4=H,R_3=R_5=prenyl, R_6=OH 23 (4.6 ± 0.3) *R_1=OH, R_2=R_3=R_4=H, R_5= prenyl, R_6=OCH_3	24 (13.8 ± 1.8)	25 (24.2 ± 2.1)

Figure 4. Structures of Natural flavonoids isolated from *Erythrinawith* their PTP1B inhibitory activities (IC_{50} in μM). * compound 23 is chromane derivative

		
27 (6.0 μM)	28 (5.26 ± 0.24 μM)	29 (6.70 ± 0.03 μM)
		
30 (13.6 ± 3.3 μM) R_1=R_3= prenyl, R_2=R_5= H, R_4=OH 31 (9.4 ± 2.9 μM) R_1=R_2= prenyl, R_3=R_5= H, R_4=OH 32 (5.7 ± 1.5 μM) *R_1=R_3= H, R_2=R_5= prenyl, R_4= OH	33 (12.3 ± 2.2 μM)	34 (12.3 ± 2.2 μM)
		
35 (12.16±0.02 μM)	36 (14.35±0.76 μM)	37 (2.19±0.2 μM)
		
38(4.9 ± 0.5 μM) R_1=OCH_3, R_2= OH, R_3=H 39(8.2 ± 0.6 μM) R_1=OH, R_2= OCH_3, R_3= H 40(6.6 ± 0.5μM) R_1=OH, R_2= OCH_3, R_3= OH ger:geranyl	41(1.9 ± 0.1μM) R_1=R_3=H, R_2= OH 42 (3.9 ± 0.3μM) R_1=OCH_3, R_2= OH, R_3=H 43(7.8 ± 0.6μM) R_1=OH, R_2= OCH_3, R_3= H 44(5.9 ± 0.4μM) R_1=OH, R_2= OCH_3, R_3= OH 45(3.8 ± 0.3μM) R_1= OCH_3, R_2= OH, R_3= OCH_3	46(20.00 ± 1.85μM) R_1=R_3=OH, R_2= OCH_3, R_4=prenyl 47(27.20 ± 3.61μM) R_1=R_3=OH, R_2= prenyl, R_4=H 48(54.20 ± 5.38μM) R_1=R_2= OCH_3, R_3= OH, R_4= H





49 (16.70 ± 1.20μM) R_1=OCH_3, R_2=prenyl,R_3=R_6=OH, R_4=R_5=H 50 (34.10 ± 10.54 μM) R_1=OCH_3, R_2=R_4=R_5=H,R_3=R_6=OH 51 (9.45 ± 0.93μM) R_1=R_3=R_6=OH, R_2=prenyl,R_4=R_5=H 52 (13.00 ± 0.25 μM) R_1=R_5=H, R_2=prenyl,R_3=R_6=OH, R_4=OCH_3	53 (*S,**R) (0.33μM) 54 (*R,**R) (0.35μM)

Figure 5: Chemical structure of natural PTP1B inhibitors. *compound 32 is chromane derivative

The aqueous extract of *Broussonetia kazinoki* has demonstrated a hypoglycemic effect in experimental diabetic rats[42]. Isoprenylatedflavan from *B. kazinoki*, Kazinol B, was effective in decreasing insulin resistance in 3 T3-L1 adipocytes *via* activation of AMPK and PKB[43]. According to the these results, it was proposed that *B. kazinoki* could be a potential source of compounds with antidiabetic activity. Interestingly, seven isolated compounds (**46-52**, Figure 5) exhibited *in vitro* PTP1B inhibitory activities with IC_{50} ranged between 9.45–54.20 μM[44]. The obtained results highlighted some points regarding the SAR of these natural compounds such as that compounds without prenyl group have weak or no activity, and glycosylation of the hydroxyl group decreases the activity.

Studying the ethanolic extract of *Sophora flavescens* resulted in the isolation of eight biflavonoids, consisting of a flavanone fused with a dihydrochalcone skeleton. Five of them were found to have potent *in vitro* PTP1B inhibitory activity (%inhibition at 10 μM was between 93.0–96.6%) and the IC_{50} for two of them (**53** and **54**, Figure 5) was determined to be 0.33 and 0.35 μM, respectively[45].

Molecular modeling approaches in the discovery of flavonoids as PTP1B inhibitors

The classical methods used in the discovery of new lead compounds and drugs are laborious, highly expensive, and time-consuming. A different surrogate strategy that could surmount such challenges is the utilization of computer-

aided drug design (CADD). For example, molecular docking simulations were acknowledged as an effective method for predicting the interaction as well as binding energies of target–ligand complexes and therefore are very valuable in rational structure-based drug design. Recent advances in lead discovery from nature using cheminformatics and *in silico* screening is widely reported in the literature[46-51].

Amentoflavone, a natural biflavonoid isolated from *Selaginella tamariscina*, was reported as PTP1B noncompetitive allosteric inhibitor (Ki = 5.2 μM)[52]. Molecular docking simulations between PTP1B-allosteric site and amentoflavone were performed, and docking model was determined using AutoDock software. Depending on this docking model, multiple five featured-pharmacophore maps were generated: one lipophilic feature, two hydrogen bonding acceptors and two hydrogen bonding donors. Applying receptor-guided pharmacophore-based virtual screening against a database containing 40 natural biflavonoids resulted in the identification of two biflavonoids: sumaflavone (**55**) and tetrahydroamentoflavone (**56**) as potent allosteric inhibitors (Figure 6). Based on the docking results, it was proposed that the hydroxyl group at position 4' is significant factor for allosteric inhibition[53].

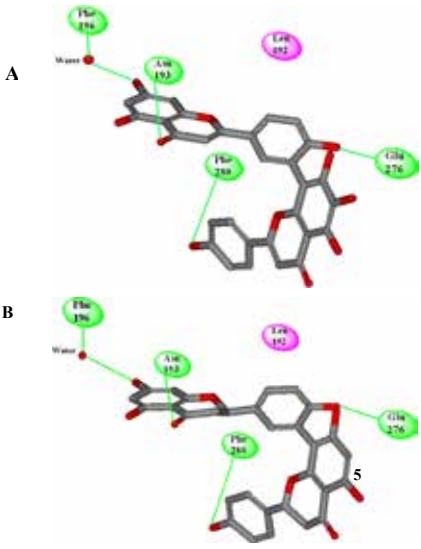


Figure 6: Docking models of biflavonoids and PTP1B. (A) Sumaflavone (55) (B) Tetrahydroamentoflavone (56)[53]. Hydrogen bonds between protein and ligands are shown in green while hydrophobic contacts are represented in magenta.

Screening of an in-house built database of 14 polyphenolic compounds against PTP1B revealed that morin and apigenin, two flavonoids widely abundant in herbs, vegetables, and fruits, are reversible PTP1B inhibitors[54]. Morin (**57**) was the most potent non-competitive PTP1B inhibitor (IC_{50} = 15.0 ± 0.8; K_i = 5.9 ± 0.4 μM). In order to identify the binding mode of morin which could support a non-competitive mechanism of binding, it was docked with PTP1B using SwissDock. The docking model showed that morin doesn't bind in the catalytic pocket of PTP1B but fits in neighboring site forming 3 hydrogen bonds, with Gln262, Arg254, and Asp28, and hydrophobic interactions with Met258 and Gly259 with predicted binding energy (ΔG) = – 6.827(kcal/mol) (Figure 7).

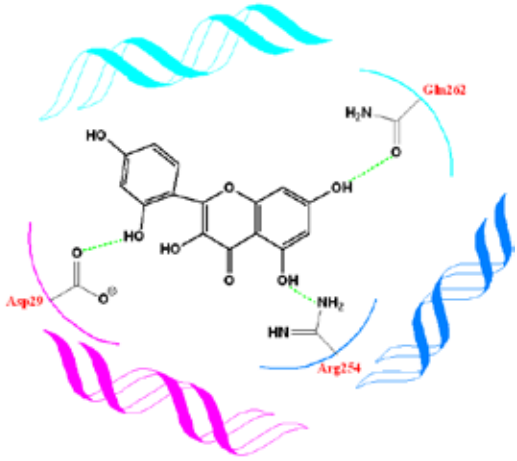


Figure 7: Graphical representation showing hydrogen bond network established by docked pose of Morin (57) and neighboring residues of PTP1B: Arg254, Gln262 and Asp29 (green lines)[54].

In another study, the interactions between PTP1B with three noncompetitive flavonoid inhibitors, morin (**57**, MOR), 6,8-diprenylorobol (**58**, DPO), and 2′-methoxykurarinone (**59**, MOK) were explored. The three flavonoids were docked withinPTP1B allosteric site using Autodock software followed by molecular dynamics simulations, using GROMACS package, in order to investigate the conformational changes of PTP1B. Results showed that DPO (**58**) fits within the PTP1B site with the highest docking score i.e., estimated binding energy = −8.3 kcal/mol (K_i =2 .5 μM). On the other hand, the predicted binding energies for MOK (K_i = 3.15 μM) and MOR (K_i = 5.9μM) were −6.5 and −5.3 kcal/mol, respectively, which is in a direct correlation with their K_i values (Figure 8)[55]. The dissimilarity in affinities of the 3 compounds was ascribed to non-bonded interactions and several hydrogen bonds using per-residue energy decomposition analysis.

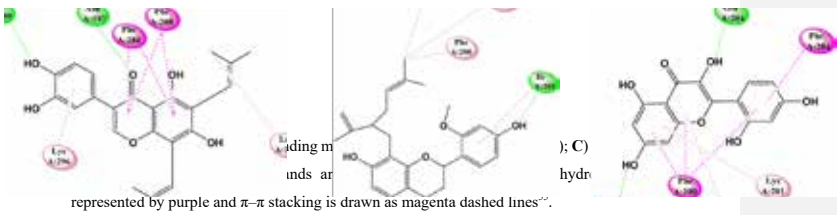


Figure 8. 2D representation of binding mode for: A) DPO (58); B) MOK (59); C) MOR (57). Hydrogen bonds between protein and ligands are drawn as green dashed lines, hydrophobic contacts are represented by purple and ϖ–ϖ stacking is drawn as magenta dashed lines[55].

A fractionation study conducted on the methanolic extract of rhubarb, *Rheum undulatum* L., resulted in the separation of 10 polyphenolic compounds including flavonoids. Three of them were stilbene derivatives (**60–62**) which exhibited significant PTP1B inhibition (IC_{50} between 4.25 to 6.78 μM)[56]. Further investigations using docking simulations and kinetic analysis were conducted to reveal the binding mode and the potential interactions of PTP1B with the isolated stilbene derivatives. The obtained findings demonstrated noncompetitive PTP1B inhibition for (**60**) and mixed-type inhibition for piceatannol (**61**) and δ-viniferin (**62**). Further investigation for the active stilbenes using molecular docking showed a strong binding with key residues within the binding site PTP1B with relatively high negative energies (Figure 9).

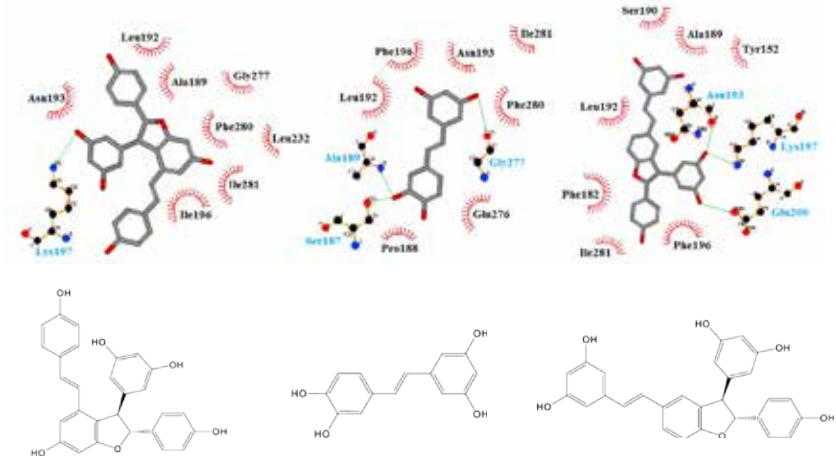


Figure 9: 2D ligand interaction diagram of 60 (A), 61(B) and 62(C) in the allosteric site of PTP1B enzyme. Green lines indicate H-bonds. Carbons are in black, nitrogens in blue, and oxygens in red. Hydrogen bonds between protein and ligands are drawn as green lines[56].

Fifteen compounds, isolated from *Anoectochilus chapaensis*, were investigated for their PTP1B inhibitory activity *in vitro*[57]. Four flavonoids; quercetin (**37**), isorhamnetin, isorhamnetin-3-*O*-β-D-glucoside, isorhamnetin-3-*O*-β-D-rutinoside (**63**), showed significant PTP1B inhibition with IC_{50} values ranging from 1.16 –5.63 μM. The results obtained by docking simulations revealed significant binding energies of -7.4 to -7.8 kcal/mol indicating tight binding and supporting the high affinity to the active catalytic site of PTP1B. Among the tested flavonoids, compound **63** showed the highest PTP1B estimated binding energy (-7.8 kcal/mol). Thorough analysis of the estimated binding mode revealed that the stability of the PTP1B-inhibitors complex could be sustained by the formation of multiple hydrogen bonds within the active site (Figure10). Structure–activity relationship study of the flavonoid glycosides derivatives revealed that the carbohydrate moiety at C-3 position has a great influence on PTP1B inhibitory activity[57].

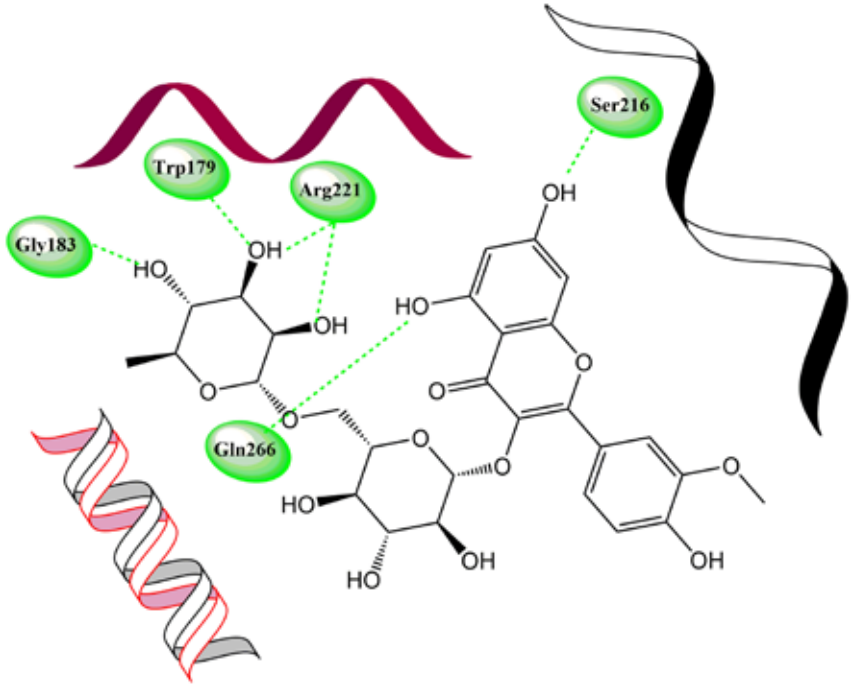


Figure 10: 2D ligand interaction diagram of binding mode of isorhamnetin-3-O-β-D-rutinoside (63, IC_{50} =1.20 ± 0.05 μM) within PTP1B active site. Hydrogen bonds between protein and ligands are drawn as green dashed lines[57].

Furthermore, twenty-six phenolic natural products were extracted from the root bark of *Morus alba*. Ten compounds showed significant PTP1B inhibition with IC_{50} ranged between 1.90 and 9.67 μM[58]. An enzymatic kinetic study showed that the three most potent compounds were noncompetitive allosteric PTP1B inhibitors, with K_i values between 0.33–1.09 μM. To investigate the binding modes and interaction of the most active compounds with PTP1B, docking simulations were conducted using AutoDock software. Docking results suggested that the three most potent compounds were noncompetitive allosteric inhibitors with negative binding energies range between −8.15 to −7.06 kcal/mol which reflect their tight binding and high affinity for PTP1B. Figure 11 shows the docking results of two of the most potent compounds (**64, 65**). As shown in figure 11, both compounds shared the same π-π stacked hydrophobic interaction with Phe196 and Phe280. Interestingly, hydrogen bonding was observed between the hetero oxygen of the ketalized ring in compound (**64**) and the allosteric residue Asn193 but not with non-ketalized compound (**65**). This might partially explain why compound (**64**) has the highest PTP1B inhibitory activity and the lowest binding energy (−8.15 kcal/mol).

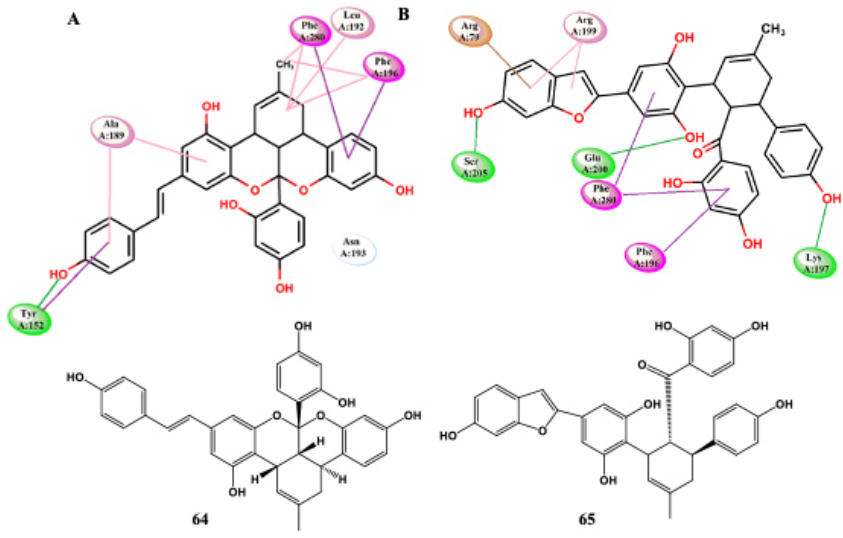


Figure 11: 2D diagram showing molecular docking results of PTP1B with: compound 64 (IC_{50} = 1.90 ± 0.12 μM) (A) and compound 65 (IC_{50} = 2.80 ± 0.19 μM) (B). Hydrogen bonds between protein and ligands are drawn as green lines, hydrophobic contacts are represented by purple, cation-π interactions in brown and π–π stacking in magenta[58].

CONCLUSION

Designing oral PTP1B inhibitors is a demanding task because of selectivity and bioavailability issues related to the homogeneity and the charged nature of the active site of PTP1B. Flavonoids have received much attention in the literature over the past decade, in which a variety of their potential therapeutic effects in several illnesses including diabetes, obesity, cancer, liver diseases, neurodegenerative diseases and cardiovascular diseases, have been verified. In fact, several natural flavonoids, acting as PTP1B inhibitors, have been proposed as potential antidiabetic drugs. These natural inhibitors, which possess interesting molecular architectures and potent activity, could be used as potential leads for developing promising drug candidates with better efficiency for the treatment of DM and its complications in the near future. This review focuses on summarizing the research efforts conducted in the discovery of flavonoids with PTP1B inhibitory activity, emphasizing the important role of molecular modeling and computer-aided drug design in the identification of these natural PTP1B inhibitors for the treatment of DM.

Unfortunately, despite the great efforts centered on the isolation and identification of many flavonoids as PTP1B inhibitors, and regardless of the diversity of the advanced molecular modeling techniques used in the discovery of these bioactive natural compounds, there were no further efforts exploited for optimization and development of these natural lead compounds into clinically effective drug candidates. Therefore, more research is required for the discovery and development of efficient flavonoids-based medicines with anti-PTP1B activity that could be used as antidiabetic drugs.

CONFLICT OF INTESEST

The authors do not have any conflict of interest.

REFERENCES

1. Thomford NE, Senthebane DA, Rowe A, Munro D, Seele P, Maroyi A, et al. Natural Products for Drug Discovery in the 21st Century: Innovations for Novel Drug Discovery. Int J Mol Sci. **2018;** 19(6): 1578. doi: 10.3390/ijms19061578.

2. Calixto JB. The role of natural products in modern drug discovery. An Acad Bras Ciênc. **2019;** 91(Suppl. 3): e20190105. doi: 10.1590/0001-3765201920190105.

3. Park J, Jang H. Anti-diabetic effects of natural products an overview of therapeutic strategies. Mol Cell Toxicol. **2017;** 13: 1-20. doi.org/10.1007/s13273-017-0001-1.

4. Vieira MN, Lyra E Silva NM, Ferreira ST, De Felice FG. Protein Tyrosine Phosphatase 1B (PTP1B): A Potential Target for Alzheimer's Therapy?. Front Aging Neurosci. **2017;** 9:7. doi:10.3389/fnagi.2017.00007.

5. Song GJ, Jung M, Kim JH, Park H, Rahman MH, Zhang S, et al. A novel role for protein tyrosine phosphatase 1B as a positive regulator of neuroinflammation. J Neuroinflammation, **2016;** 13(1): 86. doi: 10.1186/s12974-016-0545-3.

6. Kostrzewa T, Styszko J, Gorska-Ponikowska M, Sledzinski T, Kuban-Jankowska A. Inhibitors of Protein Tyrosine Phosphatase PTP1B With Anticancer Potential. Anticancer Res. **2019;** 39(7): 3379-3384. doi: 10.21873/anticanres.13481.

7. Kumar A, Rana D, Rana R, Bhatia R. Protein Tyrosine Phosphatase (PTP1B): A promising Drug Target Against Life-threatening Ailments. Curr Mol Pharmacol. **2020;** 13(1): 17-30. doi: 10.2174/1874467212666190724150723.

8. Abdelsalam SS, Korashy HM, Zeidan A, Agouni A. The Role of Protein Tyrosine Phosphatase (PTP)-1B in Cardiovascular Disease and Its Interplay with Insulin Resistance. Biomolecules, **2019;** 9(7): 286. doi: 10.3390/biom9070286.

9. Liu WS, Wang RR, Yue H, Zheng ZH, Lu XH, Wang SQ, et al. Design, synthesis, biological evaluation and molecular dynamics studies of 4-thiazolinone derivatives as protein tyrosine phosphatase 1B (PTP1B) inhibitors. J Biomol Struct Dyn. **2020;** 38(13): 3814-3824. doi. org/10.1080/07391102.2019. 1664333.

10. Jiang CS, Liang LF, Guo YW. Natural products possessing protein tyrosine phosphatase 1B (PTP1B) inhibitory activity found in the last decades. Acta Pharmacol Sin. **2012;** 33(10): 1217-1245.doi: 10.1038/aps.2012.90.

11. Bhupathiraju SN, Hu FB. Epidemiology of Obesity and Diabetes and Their Cardiovascular Complications. *Circ Res*. **2016;** 118(11): 1723-1735. doi: 10.1161/CIRCRESAHA.115.306825.

12. Xue W, Tian J, Wang XS, Xia J, Wu S. Discovery of potent PTP1B inhibitors via structure-based drug design, synthesis and in vitro bioassay of Norathyriol derivatives. Bioorg Chem. **2019;** 86: 224-234. doi.org/10.1016/j.bioorg.2019.01.059.

13. Yeon JY, Bae YJ, Kim EY, Lee EJ. Association between flavonoid intake and diabetes risk among the Koreans. Clin Chim Acta. **2015;** 439: 225-230.doi: 10.1016/j.cca.2014.10.042.

14. Bollu LR, Mazumdar A, Savage MI, Brown PH. Molecular Pathways: Targeting Protein Tyrosine Phosphatases in Cancer. Clin Cancer Res. **2017;** 23(9): 2136-2142. doi: 10.1158/1078-0432.CCR-16-0934.

15. Johnson TO, Ermolieff J, Jirousek MR. Protein tyrosine phosphatase 1B inhibitors for diabetes. Nat Rev Drug Discov. **2002;** 1(9): 696-709. doi.org/10.1038/nrd895.

16. Tamrakar AK, Maurya CK, Rai AK. PTP1B inhibitors for type 2 diabetes treatment: a patent review (2011-2014). Expert Opin Ther Pat. **2014;** 24(10): 1101-1115. doi: 10.1517/13543776.2014.947268.

17. Barford D, Flint AJ, Tonks NK. Crystal structure of human protein tyrosine phosphatase 1B. Science, **1994;** 263(5152): 1397-404.

18. Shinde RN, Kumar GS, Eqbal S, Sobhia ME. Screening and identification of potential PTP1B allosteric inhibitors using in silico and in vitro approaches. PLoS One, **2018;** 13(6): e0199020. doi: 10.1371/journal.pone.0199020.

19. Taha MO, Bustanji Y, Al-Bakri AG, Yousef AM, Zalloum WA, Al-Masri IM, et al. Discovery of new potent human protein tyrosine phosphatase inhibitors via pharmacophore and QSAR analysis followed by in silico screening. J Mol Graph Model. **2007;** 25(6): 870-84. doi: 10.1016/j.jmgm.2006.08.008.

20. Sharma B, Xie L, Yang F, Wang W, Zhou Q, Xiang M, et al. Recent advance on PTP1B inhibitors and their biomedical applications. Eur J Med Chem. **2020;** 199: 112376. doi.org/10.1016/j.ejmech.2020.112376.

21. Zargari F, Lotfi M, Shahraki O, Nikfarjam Z, Shahraki J. Flavonoids as potent allosteric inhibitors of protein tyrosine phosphatase 1B: molecular dynamics simulation and free energy calculation. J Biomol Struct Dyn. **2018;** 36(15): 4126-4142. doi: 10.1080/07391102.2017.1409651.

22. Wiesmann C, Barr KJ, Kung J, Zhu J, Erlanson DA, Shen W, et al. Allosteric inhibition of protein tyrosine phosphatase 1B. Nat Struct Mol Biol. **2004;** 11: 730-737. doi.org/10.1038/nsmb803.

23. Krishnan N, Koveal D, Miller DH, Xue B, Akshinthala SD, Kragelj J, et al. Targeting the disordered C-terminus of PTP1B with an allosteric inhibitor. Nat Chem Biol. **2014;** 10(7): 558–566. doi.org/10.1038/nchembio.1528.

24. Proença C, Freitas M, Ribeiro D, Sousa JLC, Carvalho F, Silva AMS, et al. Inhibition of protein tyrosine phosphatase 1B by flavonoids: A structure - activity relationship study. Food Chem Toxicol. **2018;** 111: 474-481.doi: 10.1016/j.fct.2017.11.039.

25. Choi K, Kim YB. Molecular mechanism of insulin resistance in obesity and type 2 diabetes. Korean J Intern Med. **2010;** 25(2): 119-129.doi:10.3904/kjim.2010.25.2.119.

26. Panche AN, Diwan AD, Chandra SR. Flavonoids: an overview. J Nutr Sci. 2016; 5: e47.doi: 10.1017/jns.2016.41.

27. Ueda K, Usui T, Nakayama H, Ueki M, Takio K. 4-Isoavenaciolide covalently binds and inhibits VHR, a dual-specificity phosphatase. FEBS Lett. **2002;** 525: 48-52.doi: 10.1016/s0014-5793(02)03065-x.

28. Bae EY, Na M, Njamen D, Mbafor JT, Fomum ZT, Cui L, et al. Inhibition of protein tyrosine phosphatase 1B by prenylated isoflavonoids isolated from the stem bark of Erythrina addisoniae. Planta Med. **2006;** 72(10): 945-948.doi: 10.1055/s-2006-946674.

29. Na M, Hoang DM, Njamen D, Mbafor JT, Fomum ZT, Thuong PT, et al. Inhibitory effect of 2-arylbenzofurans from Erythrina addisoniae on protein tyrosine phosphatase-1B. Bioorg Med Chem Lett. **2007;** 17(14): 3868-71.doi: 10.1016/j.bmcl.2007.05.005.

30. Cui L, Thuong PT, Lee HS, Ndinteh DT, Mbafor JT, Fomum ZT, et al. Flavanones from the stem bark of Erythrina abyssinica. Bioorg Med Chem. **2008;** 16(24): 10356-62. doi.org/10.1016/j.bmc.2008.10.012.

31. Nguyen PH, Le TV, Thuong PT, Dao TT, Ndinteh DT, Mbafor JT, et al. Cytotoxic and PTP1B inhibitory activities from Erythrina abyssinica. Bioorg Med Chem Lett. **2009;** 19(23): 6745-9. doi: 10.1016/j.bmcl.2009.09.108.

32. Nguyen PH, Sharma G, Dao TT, Uddin MN, Kang KW, Ndinteh DT, at al. New prenylated isoflavonoids as protein tyrosine phosphatase 1B (PTP1B) inhibitors from Erythrina addisoniae. Bioorg Med Chem. **2012;** 20(21): 6459-64.doi: 10.1016/j.bmc.2012.08.024.

33. Dao TT, Nguyen PH, Thuong PT, Kang KW, Na M, Ndinteh DT, et al. Pterocarpans with inhibitory effects on protein tyrosine phosphatase 1B from Erythrina lysistemon Hutch. Phytochemistry, **2009;** 70(17-18): 2053-7.doi: 10.1016/j.phytochem.2009.09.012.

34. Li W, Li S, Higai K, Sasaki T, Asada Y, Ohshima S, et al. Evaluation of licorice flavonoids as protein tyrosine phosphatase 1B inhibitors. Bioorg Med Chem Lett. **2013;** 23(21): 5836-9.doi: 10.1016/j.bmcl.2013.08.102.

35. Sasaki T, Li W, Higai K, Quang TH, Kim YH, Koike K. Protein Tyrosine Phosphatase 1B Inhibitory Activity of Lavandulyl Flavonoids from Roots of Sophoraflavescens. Planta Med. **2014;** 80(7): 557–560.doi: 10.1055/s-0034-1368400.

36. Choi JS, Islam MN, Ali MY, Kim YM, Park HJ, Sohn HS, et al. The effects of C-glycosylation of luteolin on its antioxidant, anti-Alzheimer's disease, anti-diabetic, and anti-inflammatory activities. Arch Pharm Res. **2014;** 37(10): 1354-63.doi: 10.1007/s12272-014-0351-3.

37. Quang TH, Ngan NT, Yoon CS, Cho KH, Kang DG, Lee HS, et al. Protein Tyrosine Phosphatase 1B Inhibitors from the Roots of Cudraniatricuspidata. Molecules, **2015;** 20(6):11173-83.doi: 10.3390/molecules200611173.

38. Wu LQ, Lei C, Gao LX, Liao HB, Li JY, Li J, et al. Isoprenylated Flavonoids with PTP1B Inhibition from Ficus tikoua. Nat Prod Commun. **2015;** 10(12): 2105-7.

39. Na B, Nguyen PH, Zhao BT, Vo QH, Min BS, Woo MH. Protein tyrosine phosphatase 1B (PTP1B) inhibitory activity and glucosidase inhibitory activity of compounds isolated from Agrimoniapilosa. Pharm Biol. **2016;** 54(3): 474-80.doi: 10.3109/13880209.2015.1048372.

40. Jiang L, Numonov S, Bobakulov K, Qureshi MN, Zhao H, Aisa HA. Phytochemical profiling and evaluation of pharmacological activities of Hypericum scabrum L. Molecules, **2015;** 20: 11257-11271.doi: 10.3390/molecules200611257.

41. Song YH, Uddin Z, Jin YM, Li Z, Curtis-Long MJ, Kim KD, et al. Inhibition of protein tyrosine phosphatase (PTP1B) and α-glucosidase by geranylated flavonoids from Paulownia tomentosa. J Enzyme Inhib Med Chem. **2017;** 32(1): 1195-1202.doi: 10.1080/14756366.2017.1368502.

42. Cha JY, Kim YT, Kim HS, Cho YS. Antihyperglycemic effect of stem bark powder from paper mulberry (Broussonetia kazinoki Sieb.) in type 2 diabetic Otsuka Long-Evans Tokushima fatty rats. J Med Food, **2008;** 11(3): 499-505.doi: 10.1089/jmf.2007.0028.

43. Lee H, Li H, Jeong JH, Noh M, Ryu JH. Kazinol B from Broussonetiakazinoki improves insulin sensitivity via Akt and AMPK activation in 3T3-L1 adipocytes. Fitoterapia, **2016;** 112: 90-96.doi: 10.1016/j.fitote.2016.05.006.

44. Xue JJ, Lei C, Wang PP, Kim KY, Li JY, Li J, et al. Flavans and diphenylpropanes with PTP1B inhibition from Broussonetia kazinoki. Fitoterapia, **2018;** 130: 37-42. https://doi.org/10.1016/j.fitote.2018.08.001.

45. Yan HW, Zhu H, Yuan X, Yang Y-N, Feng Z-M, Jiang J-S, et al. Eight new biflavonoids with lavandulyl units from the roots of Sophoraflavescens and their inhibitory effect on PTP1B.

Bioorg Chem. **2019**; 86: 679-685.doi: 10.1016/j.bioorg.2019.01.058.

46. Mohammad M, Almasri I, Issa A, Khdair A, Bustanji Y. Inhibition of pancreatic lipase by berberine and dihydroberberine: an investigation by docking simulation and experimental validation. Med Chem Res. **2013;** 22: 2273-2278. doi.org/10.1007/s00044-012-0221-9.

47. Abu-Gharbieh E, Shehab NG, Almasri IM, Bustanji Y. Antihyperuricemic and xanthine oxidase inhibitory activities of Tribulusarabicus and its isolated compound, ursolic acid: In vitro and in vivo investigation and docking simulations. PLoS One, **2018;** 13(8): e0202572. doi: 10.1371/journal.pone.0202572.

48. Almasri IM. Computational approaches for the discovery of natural pancreatic lipase inhibitors as antiobesity agents. Future Med Chem. **2020;** 12(8): 741-757.doi: 10.4155/fmc-2019-0284.

49. Bustanji Y, Taha MO, Yousef AM, Al-Bakri AG. Berberine potently inhibits protein tyrosine phosphatase 1B: investigation by docking simulation and experimental validation. J Enzyme Inhib Med Chem. **2006;** 21(2): 163-71.doi: 10.1080/14756360500533026.

50. Bustanji Y, Taha MO, Almasri IM, Mohammad MK. Docking simulations and in vitro assay unveil potent inhibitory action of papaverine against protein tyrosine phosphatase 1B. Biol Pharm Bull. **2009;** 32(4): 640-5.doi: 10.1248/bpb.32.640.

51. Harb A, Bustanji Y, Almasri I, Abdalla SS. Eugenol Reduces LDL Cholesterol and Hepatic Steatosis in Hypercholesterolemic Rats by Modulating TRPV1 Receptor. Sci Rep. **2019;** 9:14003. https://doi.org/10.1038/s41598-019-50352-4.

52. Na M, Kim KA, Oh H, Kim BY, Oh WK, Ahn JS. Protein tyrosine phosphatase 1B inhibitory activity of amentoflavone and its cellular effect on tyrosine phosphorylation of insulin receptors. Biol Pharm Bull. **2007;** 30(2): 379-381.doi: 10.1248/bpb.30.379.

53. Lee JY, Jeong KW, Woo ER, Kim Y. Docking Study of Biflavonoids, Allosteric Inhibitors of Protein Tyrosine Phosphatase 1B. Bull Korean Chem Soc. **2008;** 29(8): 1479-1484. doi: 10.5012/bkcs.2008.29.8.1479.

54. Paoli P, Cirri P, Caselli A, Ranaldi F, Bruschi G, Santi A, et al. The insulin-mimetic effect of Morin: a promising molecule in diabetes treatment. BiochimBiophys Acta. **2013;** 1830(4): 3102-11.doi: 10.1016/j.bbagen.2013.01.017.

55. Zargari F, Lotfi M, Shahraki O, Nikfarjam Z, Shahraki J. Flavonoids as potent allosteric inhibitors of protein tyrosine phosphatase 1B: molecular dynamics simulation and free energy calculation. J Biomol Struct Dyn. **2018;** 36(15): 4126-4142. doi: 10.1080/07391102.2017.1409651.

56. Ha MT, Park DH, Shrestha S, Kim M, Kim JA, Woo MH, et al. PTP1B inhibitory activity and molecular docking analysis of stilbene derivatives from the rhizomes of Rheum undulatum L. Fitoterapia, **2018;** 131: 119-126. https://doi.org/10.1016/j.fitote.2018.10.020

57. Cai J, Zhao L, Tao W. Potent protein tyrosine phosphatase 1B (PTP1B) inhibiting constituents from Anoectochiluschapaensis and molecular docking studies. Pharm Biol. **2015;** 53(7): 1030-4.doi: 10.3109/13880209.2014.957781.

58. Ha MT, Seong SH, Nguyen TD, Choe W-K, Ah KJ, Mae JY, et al. Chalcone derivatives from the root bark of Morusalba L. act as inhibitors of PTP1B and α-glucosidase. Phytochemistry, **2018;** 155: 114-125.doi:10.1016/j.phytochem.2018.08.001.

Acta Pharm. Sci. Vol 59:(4), 2021
DOI: 10.23893/1307-2080.APS.05940

Comparative *in vitro* Dissolution Study of Clonazepam Tablets of Bangladesh by UV-Visible Spectrophotometry

Sadman S.B. RASHED[1], Razwanur R. TUSHAR[2], Fairuza AHMED[1], Nusrat J. VABNA[3], Laila JAHAN[4], Mohammad M. BILLAH[2*]

1 Department of Pharmacy, Brac University, Bangladesh
2 Department of Pharmaceutical Technology, Institute for Pharmaceutical Skill Development and Research, Bangladesh
3 Department of Pharmacy, University of Asia Pacific, Bangladesh
4 Department of Pharmacy, State University of Bangladesh

ABSTRACT

This study was aimed to assess the pharmaceutical equivalence of four brands of clonazepam tablets available in Bangladesh using *in vitro* dissolution study by UV spectrophotometry. Dissolution study in water was carried out using USP type-2 paddle apparatus. Other quality control tests like weight variation, disintegration time and assay were also performed according to the established methods. Almost all the samples attained 85% drug dissolution within 15 minutes. The assay revealed that all brands contained around 94-107% (w/w) of labeled chemical content. All brands complied with the official specifications for disintegration time. Dissolution results of all the test products were compared to that of the reference product with difference factor (*f1*) and similarity factor (*f2*). Apart from brand B, the dissolution profiles of other brands showed no significant variations. The study indicates that brands C, D and E were equivalent to the reference brand A so they may be prescribed interchangeably.

Keywords: Clonazepam, dissolution, disintegration, pharmaceutical equivalence

*Corresponding Author:
Mohammad Mustakim Billah
Department of Pharmaceutical Technology, Institute for Pharmaceutical Skill Development and Research, Bangladesh
Email: mustakimbillah@hotmail.com
ORCIDs:
Sadman S.B. Rashed[1] (https://orcid.org/0000-0002-5897-6044),
Razwanur R. Tushar[2]. (https://orcid.org/0000-0002-2557-3150),
Fairuza Ahmed[1] (https://orcid.org/0000-0003-1908-3048),
Nusrat J. Vabna[3] (https://orcid.org/0000-0002-4108-7881),
Laila Jahan[4] (https://orcid.org/0000-0001-6618-9633),
Mohammad M. Billah[2*] (https://orcid.org/0000-0002-2393-6085)
(Received 30 December 2020, Accepted 26 August 2021)

INTRODUCTION

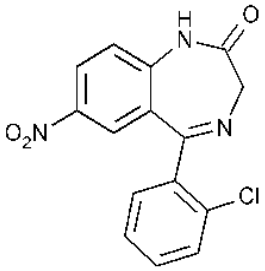
Clonazepam is an extremely potent benzodiazepine that is predominantly used to treat panic disorders, different types of seizures like myotonic or atonic seizures, absence seizures, mania[1,2]. It has also been reported to possess broad spectrum of activity against different types of epilepsy like photosensitive epilepsy and also appears to be highly effective in patients having proved resistance against other antiepileptic drugs[3]. This agent is also indicated to use for the treatment of parasomnia in children[4]. Pharmacodynamically, clonazepam exhibits manifestation of anticonvulsant, anxiolytic, sedative as well as central muscle relaxation action[5,6]. It is available in oral, parenteral dosage forms and pediatric drops[7].

The process of extracting drug from its solid-state matrix dosage form into solution within the gastrointestinal tract is referred as dissolution in pharmacokinetics. This process performs a significant role in releasing drug substance from the drug product in the body for its subsequent absorption[8]. Dissolution largely depends on the physicochemical properties of drugs as well as the formulation and process of manufacturing of the drug product[9]. Hence, it is essential to undertake constant dissolution analysis of marketed drug products to assure the quality of medicines[10].

Multiple pharmaceutical companies manufacture Clonazepam under different brand names in Bangladesh. When the generic products are bioequivalent with the innovator brand, only then generics are altered with the branded version[11]. *In vivo* bioequivalence research is conducted on animals and human whereas *in vitro* research is performed in dissolution equipment, in simulated biological conditions. Bioavailability depends on drug dissolution and its permeability across GIT, pharmaceutical equivalence of these drugs is determined by *in vitro* dissolution[12].

According to Biopharmaceutics Classification System (BCS), clonazepam belongs to Class II where drug permeability is high but solubility of drug is low[7]. This is used as the basis for the setting of *in vitro* dissolution specifications. The comparative dissolution study has been accomplished for many drugs in different countries. However, no studies were found to report a comparative dissolution profile on selected brands of clonazepam. Therefore, the present study was executed to evaluate the quality attributes of five brands of "clonazepam 2 mg tablets" in Bangladesh by *in vitro* investigation. To analyze clonazepam, different methods are available which might contain limitations to some extent. So, an economic, rapid UV Visible scheme was used for analysis of dissolution. Product information of Clonazepam is given at Table 1.

Table 1. Product Information

Product Name	Clonazepam 2 mg tablets
Molecular Weight	315.71 g/mol
Molecular Formula	$C_{15}H_{10}ClN_3O_3$
Chemical Name	5-(2-chlorophenyl)-7-nitro-1,3-dihydro-1,4-benzodiazepin-2-one
Chemical Structure	

METHODOLOGY

Drugs and Chemicals

Five brands of clonazepam 2mg tablets were purchased from a local drug store of Dhaka, Bangladesh. To maintain the confidentiality among the brands and restrict further use of the data of comparative study for commercial purposes, the brands' and manufacturers' names were kept undisclosed and thus the brands were randomly coded with alphabets. The drugs were checked for the manufacturer license no., DAR No., batch number, manufacturing and expiry dates (Table 2).

Table 2. List of Brands of Clonazepam 2mg tablets used *in vitro* analysis

Brand	Manufacturer	Batch No.	Mfg. Date	Exp. Date
A	'A' Manufacturing Company, Bangladesh	10003	June, 20	May, 22
B	'B' Manufacturing Company, Bangladesh	20004	March, 20	February, 22
C	'C' Manufacturing Company, Bangladesh	20D0224	May, 20	April, 23
D	'D' Manufacturing Company, Bangladesh	9004	April, 20	March, 22
E	'E' Manufacturing Company, Bangladesh	0E02409	August, 20	July, 22

Standard API of Clonazepam was collected from Sigma-Aldrich. Methanol (RCI LABSCAN Limited, Thailand) and Acetonitrile (SAMCHUN, Korea) obtained were of HPLC grades.

Instruments and Devices

BK-RC3 Dissolution Tester (Biobase, China), BK-BJ2 Disintegration Tester (Biobase, China), BA2004N Analytical Balance (Biobase, China), BK-UV 1800 Spectrophotometer (Biobase, China), PH-10S pH Meter (Biobase, China), UC-20A Water Bath Sonicator (Biobase, China) were used for the experiments.

Calibration of Dissolution Apparatus and UV- Spectrophotometer

UV/VIS spectrophotometer was internally calibrated and the calibration process was carried out to evaluate for absorbance and wavelength controls, stary light limit, photometric linearity, resolution power and appropriateness of baseline and sample cells. The calibration of the dissolution apparatus was performed half-yearly on a routine basis following the standard procedure recommended by United States Pharmacopoeia (USP) as per the General Chapter <711>[13]. As Reference Standard, USP Prednisone Tablets RS and USP Prednisone RS were used for the performance verification test. The dissolution test was performed according to Table 3. After manual sampling, the release of prednisone was determined through UV/VIS spectrophotometer.

Table 3. Specifications for calibration.

Parameters	Specifications
Medium	Degassed purified water
Volume	500 mL
Rotation Speed	50 rpm
Bath Temperature	37 ± 0.5 °C
Time Point	30 minutes
Absorbance Wavelength	242 nm

Method Validation

In this study the dissolution method was validated for Clonazepam 2 mg Tablets with official medium (water) by UV spectrophotometer at a wavelength of 254 nm using 10 mm cell. The validation was done according to ICH Q2 (R1) guideline[14]. Method validation characteristics were system suitability, specificity, linearity, accuracy, precision, intermediate precision and filter compatibility. Summary of the method validation is given at Table 4.

Table 4. Method validation report summary

Validation parameters	Acceptance criteria		Results
System suitability & Specificity	RSD (%) of five replicate absorbance of standard should be ≤ 2.0		≤ 2.0
	Interference of diluent and placebo		No interference
Linearity	R^2 value should be ≥ 0.999	50%, 80%, 100% 120% and 150% (0.011 mg/mL as 100%)	0.999
Accuracy	Average Recovery (%) of three sample at each recovery level within 98.0 to 102.0	50% (0.0055 mg/mL)	99.2
		100% (0.011 mg/mL)	99.9
		150% (0.0165 mg/mL)	100.2
Precision	RSD (%) six tablets after 45 min should be ≤ 2.0		1.1
Intermediate precision	RSD (%) six tablets after 45 min in different day with different analyst should be ≤ 2.0		1.0
	RSD (%) 12 tablets of both Precision Intermediate precision should be ≤ 2.0		1.1
Filter Compatibility	There should be no interference of filter paper		Filter paper does not adsorb the active.

Determination of Weight Variation

Twenty tablets of each brand were weighed individually with the analytical balance. The average weights and relative standard deviations were calculated and compared[15].

Disintegration test

Six tablets from each brand were taken and placed in disintegration chamber containing distilled water at 37°C within the tester. The time required for total disintegration and passing of the tablet entirely through the sieve was recorded[16].

Dissolution test

All dissolution tests in this study were performed using USP Apparatus 2 with official dissolution medium (water) as recommended in USP monograph for Clonazepam tablets. Three tablets of each brand were simultaneously placed in

900 mL of demineralized water at 37±0.5°C and allowed a rotation at 75 rpm. 10 ml of solution was withdrawn at 5, 10, 15, 20, 25, 30, 35, 40 and 45 minutes with replacement of equal volume of media. After each withdrawal, samples were filtered through #41 Whatman filter paper and assayed by UV visible spectrophotometer with a 10 mm quartz cell. A standard curve of pure API was derived at 254 nm, which was determined first through spectrum mode[17,18].

Assay

Ten clonazepam tablets were weighed and crushed; equivalent weight of tablet was weighed out and dissolved in methanol. This solution was then further diluted to get the desired concentration of 0.2mg/ml (working standard). The absorbance was determined through UV Visible spectrophotometer at 254 nm (90-110%)[18].

Drug release kinetics

In order to evaluate the drug release kinetics from the tablets, results of the dissolution study were fitted with different kinetic equations including:

Zero-order kinetics:

First-order kinetics:

Higuchi model:

Korsmeyer–Peppas kinetics: $Q_t/Q_o = Ktn$

Hixson-crowell model:

where, K_o, K_1, K_H, K_{HC} and K_{kp} indicates zero-order, first-order, Higuchi, Hixson-crowell and Korsmeyer–Peppas rate constants respectively, Q_t/Q_o means fraction of drug released at time t, K means rate constant and n means release exponent. The kinetics that gives the highest regression coefficient (R^2) value is considered as the best fit model[19].

Data analysis: Data were expressed as Mean ± standard deviation. The dissolution profiles were analyzed with difference factor (*f1)* and similarity factor (*f2*).

RESULTS AND DISCUSSION

Table 5 represents results obtained from the evaluation of tablets including weight variation, disintegration time and assay. The homogeneity of the tablet weight of each unit is evaluated to measure weight uniformity. An average weight of twenty tablets of the different brands was found in the range of 82-162 mg. All the clonazepam tablets were within the acceptable range (± 10%) of

the average weight (for tablets that weigh below 130mg), which is set by United States Pharmacopeia (USP)[7].

Table 5. Summary of quality attributes of Clonazepam 2mg tablets

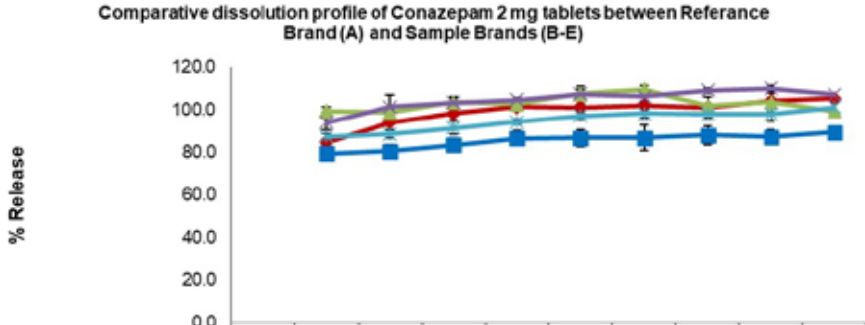
Brand	Weight variation (mg)		Disintegration Time (sec.) Mean ± SE (n=6)	Assay %
	RSD	Mean ± SE (n=20)		
A	1.2	135.08 ± 0.36	79 ± 4.32	107.3
B	1.8	81.53 ± 0.33	23 ± 1.45	98.7
C	1.0	102.95 ± 0.22	30 ± 2.17	98.0
D	0.7	162.65 ± 0.26	37 ± 3.54	106.8
E	2.3	83.07 ± 0.43	20 ± 0.80	94.1

Disintegration times of all tablet formulations were within the acceptable limit. USP compounding compendium states that uncoated and plain-coated tablets should disintegrate within 30 minutes. The use of different disintegrating agents in different formulation or products may result in different disintegration times[20]. Moreover, the production process of the tablets has discrete influence on disintegration of dosage form such as granulation techniques, compression force used for preparing tablets.

Dissolution Profiles

According to USP, the oral solid dosage form of clonazepam (tablet) should exhibit 85% dissolution in 60 minutes[7]. The result of the dissolution test of marketed clonazepam tablets were within the specification of pharmacopoeia. Almost all the brands released more than 85% of API within 15 minutes, except for brand B.

Dissolution process is influenced by various factors. For tablet formulation, nature of excipients used and the disintegration rate are very crucial points. Generally, four primary factors are involved in the intestinal absorption of drug substance from a solid oral dosage form. These include intestinal transit, membrane permeability, available surface area and the concentration profile of drug in the lumen. The solubility attributes of a drug can be predicted from the operation of dissolution test in a selected medium. Therefore, the drug readily disintegrates and exerts its therapeutic effect.



Time (min)	5	10	15	20	25	30	35	40	45
Reference Brand (A)	84.7	94.0	98.3	101.3	101.0	101.7	101.0	104.3	105.3
Brand (B)	79.4	80.4	83.4	86.4	86.7	86.7	88.0	87.0	89.4
Brand (C)	99.3	98.7	103.6	102.7	107.6	109.6	101.7	103.6	99.3
Brand (D)	94.0	101.3	103.3	104.6	107.3	106.3	109.0	110.0	107.0
Brand (E)	87.2	88.4	91.4	94.3	97.0	98.3	98.0	98.0	101.0

Figure 1: Comparative dissolution profile of Clonazepam 2 mg tablets between Reference Brand (A) and Sample Brands (B-E). Data were presented as mean ± standard error mean. Dissolution profiles (% dissolution) of brand B-E (n=3) were analyzed in five minutes intervals up to 45 minutes and compared with the reference brand A.

Dissolution profiles for marketed clonazepam tablets exhibit behavior according to the recommendation of USP (Figure 1). The fact that clonazepam is a BCS class II compound can explain the observation. The rate-limiting step for absorption of clonazepam is dissolution. The presence and the amount of disintegrating agents at varied extent such as sodium starch glycolate or povidone in different formulations may affect the dissolution attributes of tablets manufactured by different manufacturer. In addition, usage of different coating materials such as polyethylene glycol, ethylene cellulose etc. can also have significant effect on disintegration as well as dissolution or drug release in aqueous medium. The hydrophobic nature of clonazepam limits its dissolution in water.

Table 6. Results of Different models in terms of r^2, slope and intercept.

Model	Parameters	A	B	C	D	E
Zero order model	R^2	0.51491	0.46293	0.46922	0.47364	0.29488
	Slope	2.4643	2.005	2.4957	2.4914	1.8971
	Intercept	46.036	41.782	51.35	50.743	54.97
First order model	R^2	0.84179	0.61266	0.34487	0.58214	0.29915
	Slope	-0.0653	-0.0226	-0.0415	-0.054	-0.0333
	Intercept	1.5791	1.6547	0.9017	1.2065	1.2799
Higuchi model	R^2	0.79136	0.74178	0.74307	0.7542	0.54528
	Slope	17.557	14.586	18.049	18.068	14.826
	Intercept	22.25	21.388	26.333	25.597	32.129
Korsmeyer-Peppas model	R^2	0.86429	0.81524	0.81252	0.82982	0.60949
	Slope	66.824	55.69	68.739	69.024	57.087
	Intercept	15.688	15.76	19.545	18.586	25.925
Hixson-Crowell model	R^2	0.91225	0.55219	0.80154	0.71639	0.01256
	Slope	0.1956	0.0562	0.2035	0.1968	0.0218
	Intercept	0.7298	0.9764	1.6455	1.9144	2.753

From the comparison shown in Table 6, it was found that all the brands were well fitted to the Hixson-Crowell model, except brand E. This model assumes that the release rate is limited by the dissolution rate of drug particles and not by diffusion[21]. Hixson-Crowell equation is used to interpret the dissolution data of dispersible or immediate release dosage formulations. Therefore, a higher correlation coefficient indicates that change in surface area and diameter of particles during the process of dissolution have an effect on drug release[22].

Table 7. Calculated difference factor *(f1)* and similarity factor *(f2)* for all Clonazepam 2mg tablets

Samples	*f1*	*f2*
Brand (B)	14	36
Brand (C)	5	54
Brand (D)	6	54
Brand (E)	4	58

For clonazepam, difference factor *(f1)* and similarity factor *(f2)* were used to evaluate the dissolution profiles and pharmaceutical equivalence (Table 7). The difference factor *(f1)* is proportional to the percentage (%) difference be-

tween the two profiles at each time point and is a measurement of the relative error between the two curves, whereas the similarity factor *(f2)* is a logarithmic reciprocal square root transformation of the sum of squared error and is a measurement of the similarity between the two curves. The following equations were used to perform the calculation for clonazepam comparison. Here, *Rt* represents the percentage value of drug dissolved at time t obtained with the reference drug, n depicts the number of collection times considered for the calculation of *f2* and *Tt* is the dissolved percentage value of test drug (similar) at time *t*[23].

$$f_1 = \{[\sum_{t=1}^{n} |R_t - T_t|]/[\sum_{t=1}^{n} R_t]\} \cdot 100$$
$$f_2 = 50 \cdot \log\{[1+(1/n)\sum_{t=1}^{n} (R_t - T_t)^2]^{-0.5} \cdot 100\}$$

All the *f1* values are within the range of 1 to 15. Apart from brand B, *f2* values of other brands were above 50 thereby demonstrating an acceptable dissolution profile according to the established criterion. The rate-limiting step of drug absorption from the gastrointestinal tract is dissolution from the tablet; other factors are related to the manufacturing process. The evaluated brands were formulated in a way that enhances the solubility of the drug, releasing 80% of API in 5 minutes. Therefore, a large portion of clonazepam is absorbed in the stomach, remaining is absorbed in the small intestine. The absorption may vary in other parts of the gastrointestinal tract. Since the value of *f2* is greater than 50 for brands C, D and E, it can be concluded that these products show similar dissolution to that of reference brand A[23].

The results obtained in this study indicate that except brand B, all the other brands of Clonazepam (2mg) tablets complied with the USP specifications and can be considered to be equivalent to the reference product. It can be assumed that these tablet formulations may have similar bioavailability, however further *in vivo* study is required to support this presumption.

ACKNOWLEDGMENT

The present study was supported and carried out in the Pharmaceutical technology lab of Institute for Pharmaceutical Skill Development and Research, Bangladesh. Authors are grateful to the institution for providing such opportunity to contribute to science.

STATEMENT OF ETHICS

The paper is exempt from ethical committee approval.

CONFLICT OF INTEREST STATEMENT

All authors agreed on the article before submission and had no conflict of interests.

FUNDING SOURCES

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

AUTHOR CONTRIBUTIONS

This work was carried out in collaboration between all authors. Authors Mohammad Mustakim Billah and Razwanur Rahman Tushar designed, coordinated and supervised the project and also performed the statistical analysis. Sadman Sakib Bin Rashed performed in vitro experiments and participated in acquisition of data. Nusrat Jahan Vabna drafted the manuscript and Fairuza Ahmed analyzed the data, performed the drug release kinetics and critically revised the manuscript. Laila Jahan provided technical support for the experiments. All authors read and approved the final manuscript.

REFERENCES

1. J.K. Aronson. 16th ed.; Meyler's Side Effects of Drugs, **2015.**

2. A.E. Nardi; S. Machado; L. Ferreira Almada; F. Paes; A.C. Silva; R.J. Marques, et al. Clonazepam for the Treatment of Panic Disorder. Curr Drug Targets. **2013;** 14: 353–364.

3. R.M. Finder; R.N. Brogden; T.M. Speight; G.S. Avery. Clonazepam: A review of its Pharmacological Properties and Therapeutic Efficacy in Epilepsy. Evaluation of New Drugs. **2012;** 12: 321-365.

4. K. Guion; K. Johnson; KA. Freeman. Sleep Disturbance in Children with Intellectual and Neurodevelopmental Disabilities. In: Encyclopedia of Sleep, **2013;** 618–24.

5. R.Vardanyan. 1st ed.; Synthesis of Essential Drugs. **2006.**

6. P. Jenner; JA. Pratt; CD. Marsde. Mechanism of action of clonazepam in myoclonus in relation to effects on GABA and 5-HT. Advances in neurology, **1986;** 43: 629–643.

7. Office of Training and Communications Division of Communications Management The Drug Information Branch. **1997,** 20857.

8. In Vitro Dissolution Testing for Solid Oral Dosage; Particle Sciences, **2010.**

9. GL. Amidon; H. Lennernäs; Vp. Shah; JR. Crison. A Theoretical Basis for a Biopharmaceutic Drug Classification: The Correlation of in Vitro Drug Product Dissolution and in Vivo Bioavailability. Pharm Res An Off J Am Assoc Pharm Sci. **1995;** 12: 413–420.

10. T.S. Oishi; M.A Haque; I. Dewan; Smai. Comparative in vitro dissolution study of some ciprofloxacin generic tablets under biowaiver conditions by RP-HPLC. International Journal of Pharmaceutical Sciences and Research. **2011;** 2: 3129-3135.

11. E.M. Ahmed; M.E. Ibrahim; F.F Magbool. Comparative In-vitro Dissolution Study of Some Metronidazole Generic Tablets Under Bio-waiver Conditions by UV-Visible Spectrophotometry. Austin J Anal Pharm Chem. **2019;** 6: 1114.

12. A.S. Tajani; A. Haghighizadeh; V. Soheili; S. Mirshahi; O. Rajabi. In vitro bioequivalence study of 8 generic and 3 brands of sertraline-HCl tablets in Iran market. Biomed Pharmacol J. **2017;** 10: 1109–1116.

13. General Chapters: <711> Dissolution. Accessed June 21, **2021,** http://ftp.uspbpep.com/v29240/usp29nf24s0_c711.html

14. Tietje C, Brouder A, eds. International Conference On Harmonisation Of Technical Requirements For Registration Of Pharmaceuticals For Human Use. In: Handbook of Transnational Economic Governance Regimes. Brill | Nijhoff; **2010;** 1041-1053. doi:10.1163/ej.9789004163300.i-1081.897

15. Lachman L, Lieberman HA, Lieberman HH, Kanig JL. The Theory and Practice of Industrial Pharmacy. CBS Publishers and Distributor Pvt Ltd. **1987.**

16. Aodah AH, Fayed MH, Alalaiwe A, Alsulays BB, Aldawsari MF, Khafagy ES. Design, optimization, and correlation of in vitro/in vivo disintegration of novel fast orally disintegrating tablet of high dose metformin hydrochloride using moisture activated dry granulation process and quality by design approach. Pharmaceutics. **2020** Jul 1; 12(7): 1–20.

17. Hamed R, Awadallah A, Sunoqrot S, Tarawneh O, Nazzal S, AlBaraghthi T, et al. pH-Dependent Solubility and Dissolution Behavior of Carvedilol—Case Example of a Weakly Basic BCS Class II Drug. AAPS PharmSciTech. **2016** Apr 1;17(2):418–26.

18. USP Monographs: Clonazepam Tablets [Internet]. [cited 2021 Mar 16]. Available from: http://www.pharmacopeia.cn/v29240/usp29nf24s0_m18570.html

19. R KA. Mathematical Models of Drug Dissolution: A Review. Sch Acad J Pharm. **2014;** 3(5): 388–96.

20. USP Compounding Compendium; Clonazepam, 2012.

21. Padmaa Paarakh M, Ani Jose P, Setty CM, Christoper GVP. Release kinetics-concepts and applications. International Journal of Pharmacy Research & Technology, **2018;** 8: 12-20.

22. Baishya H. Application of Mathematical Models in Drug Release Kinetics of Carbidopa and Levodopa ER Tablets. J Dev Drugs. **2017;** 06(02): 1–8.

23. V.P. Shah; Y. Tsong; P. Sathe; R.L. Williams. Dissolution profile comparison using similarity factor, f2. Dissolution Technologies Inc. **1999;** 6.

REVIEW ARTICLES

Acta Pharm. Sci. Vol 59:(4), 2021
DOI: 10.23893/1307-2080.APS.05935

Review Article: Pitavastatin: Similarities and Differences Compared With Other Statins

Francesco FICI[1], Bahar Arican TARIM[2], Gokhan FAIKOGLU[3], Elif Ilkay YUCE[4] , Baris GUNGOR[5], Nicolas Roberto ROBLES[6]

1 Catedra de Riesgo Cardiovascular, Universidad de Salamanca, Salamanca, Spain

2 Department of Internal Medicine, Kartal Research and Education Hospital, Istanbul, Turkey

3 Department of Medical Pharmacology, Beykent University- Beykent Faculty of Medicine, Istanbul, Turkey

4 Department of Cardiology, Kelkit State Hospital, Gumushane, Turkey

5 Department of Cardiology, University of Sciences and Research Hospital, Istanbul, Turkey 6 Hospital Universitario de Badajoz. Badajoz. Spain

ABSTRACT

Dyslipidemia is the leading cause of cardiovascular mortality and morbidity. Reduction of lipids, particularly low- density lipoprotein cholesterol (LDL-C), with statins, significantly decreases the risk of cardiovascular events. Among different statins, pitavastatin, exhibits a peculiar pharmacokinetic and pharmacological profile. Indeed, differently from other statins, pitavastatin: a) is not metabolized by hepatic cytochrome CYP3A4 isoenzyme, therefore has a very low drug-drug interaction; b) has a similar or greater effect on LDL-C, c) is not associated with glucose metabolism impairment, and the risk of new onset diabetes is very low, d) increases high density lipoproteins (HDL) levels and, particularly, improves cholesterol efflux capacity of HDL, d) decreases cardiovascular outcome in primary and secondary prevention.

Keywords: Dyslipidemia, statins, pitavastatin, new-onset diabetes, HDL

Corresponding Autor:
Nicolas Roberto ROBLES
Hospital Universitario de Badajoz. Badajoz. Spain
nrrobles@yahoo.es
ORCID's:
Francesco Fici : 000-0002-5366-7082
Bahar Arican : 0000-0002-6017-3259
Gokhan Faikoglu : 0000-0002-8980-4499
Elif Bakır : 0000-001-9208-7972
Baris Gungor : 0000-0002-8883-117X
Nicolas Roberto Robles : 0000-003-4984-6691
(Received 08 February 2021, Accepted 3 June 2021)

INTRODUCTION

Dyslipidemia remains the leading cause of cardiovascular (CV) morbidity and mortality and aggressive reduction of lipids significantly improve cardiovascular outcome. Reduction of low density lipoproteins cholesterol (LDL-C) remains the cornerstone for a significant prevention of cardiovascular events[1,2]. Lowering LDL-C by 2–3 mmol/L is associated with a significant 40%-50% risk reduction, also in patients with diabetes or at low (<10%) CV risk[1]. Given the relationship between lipids lowering and CV protection, current guidelines recommend a LDL-C cut-off < 55 mg/dl, (or at least 50% reduction, from baseline) and < 70 mg/dL (or at least 50% reduction from baseline), in patients at very high or high CV risk respectively; while, in subjects at low-moderate risk, LDL-C must be decreased to < 116 and 100 mg/dL respectively[3]. Within the dyslipidemia treatment, statins are the most preferred medications. Therefore, this review mainly highlights the pharmacological, pharmacokinetic and therapeutic similarities and differences between the most prescribed pitavastatin and other available statins.

For this purpose, a literature search was conducted in PubMed, using the terms "pitavastatin", "dyslipidemia", "new onset diabetes", "adiponectin", "ApoI" and "cardiovascular prevention", to identify eligible articles and review in English language, published in peer reviewed journal. We did not considered short communications, editorials and posters. The resulting articles were evaluated by the authors for suitability for this review.

Pitavastatin and mechanism of action

Pitavastatin, a new-generation lipophilic statin, is indicated for the treatment of primary and mixed dyslipidemia and also prevention of cardiovascular disease. The pharmacological mechanism is similar to that of other statins that is inhibition of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, with subsequent reduction of cholesterol synthesis.

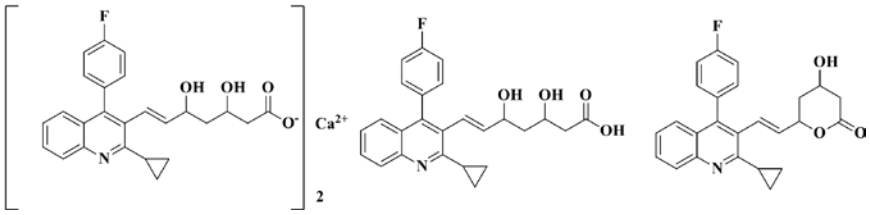


Figure 1: Pivastatin calcium salt and main metabolites[4]

Pharmacokinetic and metabolic aspects

Pitavastatin mainly inserted as calcium salt forms in pharmaceutical formulations. The quinoline ring and side chains that include fluorophenyl and cyclopropyl moieties provide improved pharmacokinetics, in the chemical structure of pitavastatin, in terms of lipid-water solubility balance. After oral administration, pitavastatin, is largely absorbed (80%), with an absolute bioavailability >60%, higher than that of other lipophilic statins[4]. The peak plasma level is achieved after 0.5-1.2 h, without difference between single and multiple doses[4–6]. Plasma concentrations and area under the curve (AUC) are proportional to the dose and reach the steady state after 4 days, without drug accumulation[4–6]. The elimination half-life, after single and multiple dose, is 9-13 hours respectively. Pitavastatin is excreted unchanged in the bile and then reabsorbed through the enterohepatic circulation. This finding explains the long elimination half-life. The drug is minimally metabolized at hepatic level, where, through a process of glucuronidation, is converted to pitavastatin lactone, the main inactive metabolite, which in turn is reversibly reconverted in pitavastatin acid[4,7]. The excretion is in large part with the feces, while a very low amount (<5%) is eliminated with urine, therefore dose reduction is not required in patients with kidney disease. (Figure1)

Differently from lovastatin, simvastatin, atorvastatin and fluvastatin, pitavastatin is not metabolized by hepatic cytochrome CYP3A4 isoenzyme, and, differently from rosuvastatin and fluvastatin, is minimally metabolized by CYP2C9 isoenzymes, consequently the risk of clinically significant drugdrug interaction is very low[4,6]. This finding has relevant clinical implications, because, unlike other statins, avoid high plasma levels, when pitavastatin is co-administered with cardiovascular drugs, such as verapamil, diltiazem, digoxin, amiodarone, warfarin, clopidogrel or amlodipine[6]. Such characteristic improves tolerability and patient's adherence, particularly in case of polytherapy. The concomitant administration of pitastatin with ciclosporin is contraindicated, while the dose of pitavastatin must be reduced (1mg/daily) in patients taking erythromycin or clarithromycin[6,7]. Pharmacokinetic properties are not affected by food, does not differ between Caucasian and Asian, young and elderly subjects[4,8,9]. This is an important aspect, considering that subjects aged ≥65 years have a higher prevalence of cardiovascular morbidity.

Therapeutic activity

The lipid lowering of pitavastatin is either similar, or even greater than that of other statins, with a high prevalence of patients which achieve LDL-C target[10–15]. (Figure 2)

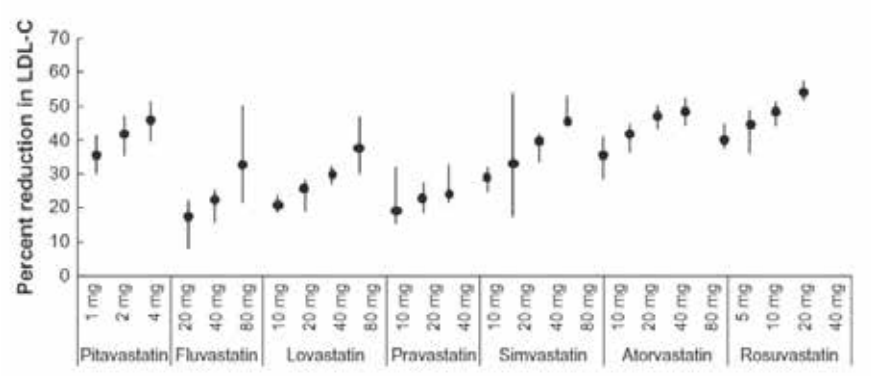


Figure 2: Comparison of percent reduction in LDL-C levels for different doses of statins. Taken from Saito Y Treatment Options for Hypercholesterolemia and Combined Dyslipidemia: Focus on Pitavastatin Clinical Medicine Insights: Therapeutics 2011:3 517-525[10]

Globally pitavastatin, decreases total cholesterol (TC 29% -33%), LDL-C, (42% - ~50%), non-HDL-C (-41%) and triglycerides (TG 30-32%), according to the dose[10,16,17]. (Figure 3)

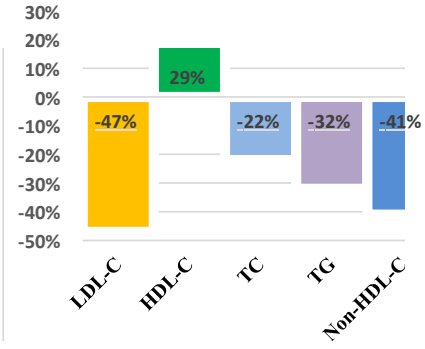


Figure 3: Effects of pitavastatin monotheraphy on lipid profile (HDL-C; High-density lipoprotein cholesterol, LDL-C; Ligh-density lipoprotein cholesterol, TC; Total cholesterol, TG; Total glyceride[17]

Compared to other statins, pitavastatin is about 6-fold more potent than atorvastatin, 1.7-fold more potent than rosuvastatin, 77-fold more potent than fluvastatin in reducing LDL-C[13].

Pitavastatin is equally effective, in elderly, in subjects with type 2 diabetes or metabolic syndrome and in people at high CV risk or with coronary artery disease[13,15,18–22].

However, pitavastatin, compared with other statins, differs in some pharmacological properties, concerning glucose metabolism and high-density lipoproteins (HDLs) plasma levels.

Glucose metabolism

The JUPITER trial raised concerns about the relationship between statins and new-onset diabetes (NOD), because 25% of patients treated with rosuvastatin developed NOD[23]. Moreover different meta-analyses and a recent cohort study, revealed that statins use is significantly associated with risk of NOD[24–27]. The incidence shows a remarkably variability (12%-61.7%), supporting the concept that, statin pharmacological properties, dosage, treatment duration and method to evaluate diabetes, play a major contributory role in the risk of NOD. Observational and comparative randomized clinical trials, have shown that pitavastatin, at variance of other statins, has a neutral, or even a favourable effect on glucose metabolism[28,29].

Pitavastatin has been compared with other statins in patients with and without diabetes. The LIVESstudy subanalysis, performed in 1197 diabetic patients, untreated with antidiabetic drugs, revealed a significantly decrease of glycosylated hemoglobin (HbA1c), during 2 years of pitavastatin treatment[30]. The CHIBA study sub-analysis has shown that pitavastatin, differently from atorvastatin, did not increase glycoalbumin plasma levels and had a neutral effect on fasting plasma glucose, insulin and HOMA-IR[31].

Furthermore a meta-analysis, involving non-diabetic patients shown that pitavastatin, compared with placebo or other statins, did not adversely affect glucose metabolism and decreased the risk of incident diabetes[32].

These findings are in agreement with a, randomized, double-blind, controlled trial and particularly with the PATROL and PAPAGAO –T studies, which have shown that atorvastatin and rosuvastatin, differently from pitavastatin, have significantly increased HbA1c plasma concentration[12,18,33]. A retrospective study, compared pitavastatin with atorvastatin, pravastatin and pitavastatin in type 2 diabetic subjects with stable antidiabetic therapy[34]. Blood glucose and

HbA1c increased with atorvastatin, but not with pitavastatin and pravastatin, suggesting the lack of difference between pravastatin and pitavastatin. However a recent meta-analysis, failed to show this similarity, because atorvastatin, rosuvastatin and also pravastatin, differently from pitavastatin, raised HbA1c and fasting blood glucose level[35]. Glicemic control during pitavastatin treatment was also evaluated in patients with coronary artery disease. In the Lamis II trial, no significant changes of HbA1c and blood glucose were observed in patients with acute myocardial infarct, after 1 year of treatment[36]. On the contrary pitavastatin significantly improved glucose metabolism in patients with acute coronary syndrome[37]. However some studies have reported a variable incidence of NOD during pitavastatin treatment. In the REAL-CAD trial incident diabetes was observed in 4.5% of patients with stable coronary artery disease, during 3 years follow-up and in patients at high risk of cardiovascular events NOD has been reported in 1.3% of subjects[13,20]. These results deserve some comments: a) both trials were large outcomes trials and were not adequately powered to assess the rate of NOD; b) in both studies, 42% and 20% of patients, respectively, were treated with beta-blockers, some of which are associated with a high risk of NOD[38]. However in patients with acute myocardial infarct, who did not have diabetes, the incidence of NOD with pitavastatin was 3%, significantly lower than 8.4% and 10.4% observed with atorvastatin and rosuvastatin respectively[39]. This finding has been confirmed by a network metaanalysis and by a recent retrospective cohort study in nondiabetic patients, which indicate that the likelihood to develop diabetes was largest with atorvastatin, rosuvastatin, simvastatin, pravastatin and lovastatin, whereas it was significantly decreased with pitavastatin[24,27].

Taken together, these findings show that pitavastatin, even at high doses, has a neutral effect on glucose metabolism, while the occurrence of new-onset diabetes cannot be ruled out, but the incidence rate is very limited. The pharmacological mechanism involved in this favourable aspect of pitavastatin is not yet fully understood. However accumulating evidence indicates that pitavastatin could stimulate adiponectin secretion, which, is involved in different biological process, such as insulin-sensitizing, anti-diabetic, anti-inflammatory and anti-atherosclerosis activities, through the increases fatty acid oxidation and glucose utilization in skeletal muscle and liver[28,40]. In spite of some discrepancies between different studies, pitavastatin, differently from other statins, significantly rises adiponectin plasma levels [27.2%, vs 17.3%, 14.7% and 7.2% with rosuvastatin, pravastatin and atorvastatin respectively][28,41,42].

Effect on high-density lipoprotein cholesterol (HDL C)

High plasma levels of HDLs seems to be related with low risk of CV events and, therefore, with atheroprotective properties[43,44]. Although statins usually lead to a minimal and variable change in HDLs, pitavastatin, differently from atorvastatin, pravastatin, fluvastatin and simvastatin, led to a significant increase in HDLs concentration (13.4% -29.0%), particularly in patients with low HDLs (<40 mg/dl) at baseline[17,30,42,45,46]. (Figure 4)

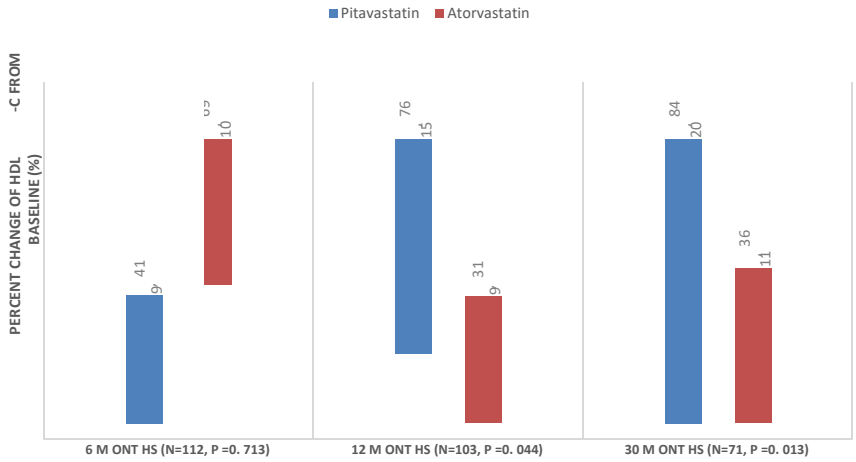


Figure 4: Time dependent percent change of HDL-C levels from baseline with Pitavastatin and Atorvastatin treatments[46]

Nevertheless, some concerns have been raised about the correlation between high HDLs level and CV protection, because pharmacological intervention, with drugs which increase HDLs, failed to show a significant reduction in CV outcome[47,48].

There is evidence that HDLs, promote the "cholesterol efflux capacity" which correlates with the antiatherogenic effect of HDLs[49,50].

Although a paucity of data, some studies provided evidence that pitavastatin, unlike other statins, was significantly associated with improved cholesterol efflux capacity of HDLs, [Fig 5] and suggest that such effect is mostly attributable to capacity of pitavastatin, differently from other statins, to increase apolipoprotein A1 plasma levels which improves HDL-C functionality through different biochemical mechanisms[41,42,46,51].

Pitavastatin and coronary artery disease

Statins are a mainstay in the primary and secondary prevention of atherosclerotic cardiovascular disease.

Different studies have assessed the therapeutic efficacy of pitavastatin in primary and secondary cardiovascular prevention. In patients with hypercholesterolemia and concomitant high cardiovascular risk factors, pitavastatin, compared with atorvastatin, provided a greater significant reduction of CV events (2.9% vs 8.1%) and coronary revascularization for stable angina, (4.5% vs 12.9%), during 5 years of treatment[13].

The REAL-CAD, a multicenter study, which involved 13054 patients with stable coronary artery disease, has shown that pitavastatin significantly decreased the risk of CV mortality and morbidity by 19%[20]. This result confirms the findings of the LAMIS and CIRCLE studies, performed in patients with acute myocardial infarct (AMI)[21,52]. Major cardiovascular events occurred in a small percentage of subjects (7.3%-8.3% respectively), lower than that observed with atorvastatin and pravastatin (19.3% and 27.2% respectively). However the risk of repeated coronary revascularization, either for new coronary lesions, or at target lesion was significantly reduced with pitavastatin compared to atorvastatin. In addition, in patients with acute coronary syndrome pitavastatin, was associated with stabilization of atherosclerotic plaque with increase fibrous-cap thickness and reduction of fibro-fatty volume index[22,45,53,54]. These effects were not inferior or significantly greater compared with atorvastatin[22,53]. Although in most studies high dose of pitavastatin, (4 mg/day), significantly protected patients from recurrent major CV event, the LAMIS II study (36) did not show significant difference between 2 and 4 mg/day in patients with AMI (incidence of CV adverse events, 9.07% vs 9.13% respectively), confirming the results of LAMIS, CIRCLE and TOGETHAR studies, showing that even low dose of pitavastatin may decreases the incidence of major CV events, also in primary prevention13,21,36,45,52.

However, as the lipids lowering of pitavastatin is dose dependent, 4 mg/daily, would be the suitable dosage for secondary CV prevention[4,5].

Future investigations

While the role of pitavastatin in patients with dyslipidemia, associated or not with coronary artery disease, is well established, its efficacy in subjects with immunodeficiency virus (HIV) infections, deserve further investigations. However, the absence of pharmacokinetic interaction between antiretroviral drugs and pitavastatin, provides a strong rationale for a systemic use of pitavastatin

to manage dyslipidemia and avoid CV outcome in these patients, The results of the REPRIEVE trial, now in progress will define the therapeutic place of pitavastatin in this group of patients[6,55,56].

Safety and tolerability

The most frequent adverse events (AEs) induced by statins are related to myopathy (in rare cases rhabdomyolysis) and liver injury.

Numerous studies have shown that pitavastatin is associated with a very low rate of AEs, also with high doses and during prolonged treatment[20]. Even if the rate of AEs differs across trials, overall 10.4% of patients have reported AEs in the LIVES study, performed in approximately 20000 patients, treated with pitavastatin (1-4 mg/day) for 2 years[57]. The most common, mild in severity, AEs were myalgia, muscle spams or weakness, experienced by 1.08%, 0.18% of patients respectively. Overall, no clinically significant changes in laboratory parameters have been observed during the study. A mild increase of creatinine phosphokinase (CK), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyltransferase (γ-GTP), incidence was found in 2,7%, 1.8%, 1.5% and 1.0% of subjects respectively. Furthermore concomitant administration of pitavastatin with agents that, rising statins plasma levels, lead to AEs, was not associated with significant incidence of AEs[8].

RESULTS AND DISCUSSION

Statins have become a cornerstone treatment in patients with dyslipidemia and in secondary prevention of atherosclerotic CV disease.

Pitavastatin, a new-generation lipophilic statin, indicated for the treatment of patients with dyslipidemia, shows similarities and differences compared with other statins.

Several studies have demonstrated that the lipid lowering of pitavastatin is similar, or even greater, than that of other statins, with a more significant effect in decreasing triglycerides and remnant lipoprotein cholesterol. The therapeutic efficacy has been documented in a wide range of patients with primary or combined dyslipidemia, also associated with type 2 diabetes or metabolic syndrome, in Asian and Caucasian subjects, in young and elderly patients, as well in subjects at high CV risk or with coronary artery disease. In this last group pitavastatin significantly decreased the rate of major adverse cardiovascular events and coronary plaque volume.

Unlike other statins, pitavastatin has a very low drug-drug interactions, because not metabolized by the CYP3A4 pathways. This aspect has important clinical

implications, because avoid the risk of high plasma level when co-administered with other cardiovascular drugs.

There is evidence that statins might increase the risk of NOD or may deteriorate glycemic homeostasis. Pitavastatin has a neutral or beneficial effect on glucose metabolism, by increasing adiponectin plasma levels and the incidence of NOD is very low, in comparison to other statins.

In addition, differently from some available stains, pitavastatin rises plasma levels of HDLs, improving their function, e.g the "reverse cholesterol efflux capacity" by stimulating the hepatic secretion of ApoA1.

Pitavastatin is well tolerated, also during long term administration, with very low incidence of dug related side effects, showing, in this way, a favorable risk-benefit profile.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

REFERENCES

1. Baigent C, Blackwell L, Emberson J, Holland LE, Reith C, Bhala N, et al. Efficacy and safety of more intensive lowering of LDL cholesterol: A meta-analysis of data from 170 000 participants in 26 randomised trials. The Lancet, **2010;** 376(9753): 1670-1681. doi:10.1016/S01406736(10)61350-5

2. Navarese EP, Robinson JG, Kowalewski M, Koodziejczak M, Andreotti F, Bliden K, et al. Association between baseline LDL-C level and total and cardiovascular mortality after LDL-C lowering a systematic review and meta-analysis. JAMA, **2018;** 319(15): 1566-1579. doi:10.1001/jama.2018.2525

3. Mach F, Baigent C, Catapano AL, Koskinas KC, Casula M, Badimon L, Chapman MJ. **(2020).** 2019 ESC/EAS Guidelines for the management of dyslipidaemias: Lipid modification to reduce cardiovascular risk (Vol. 41, pp. 111-188).

4. Catapano AL. Pitavastatin - pharmacological profile from early phase studies. Atherosclerosis Supplements, **2010;** 11(3): 3-7. doi:10.1016/S1567-5688(10)71063-1

5. Luo Z, Zhang Y, Gu J, Feng P, Wang Y. Pharmacokinetic properties of single- and multiple-dose pitavastatin calcium tablets in healthy chinese volunteers. Ther Res Clin Exp, **2015;** 77: 52-57. doi:10.1016/j.curtheres.2015.02.001

6. Corsini A, Ceska R. Drug–drug interactions with statins: will pitavastatin overcome the statins' Achilles' heel?. Curr Med Res Opin, **2011;** 27(8): 1551–1562.doi:10.1185/03007995.2011.589433

7. Hougaard Christensen MM, Bruun Haastrup M, Øhlenschlaeger T, Esbech P, Arnspang Pedersen S, Bach Dunvald AC, et al. Interaction potential between clarithromycin and individual statins-A systematic review, Basic Clin Pharmacol Toxicol, **2020;** 126(4): 307-317. doi:10.1111/bcpt.13343

8. Warrington S, Nagakawa S, Hounslow N. Comparison of the pharmacokinetics of pitavastatin by formulation and ethnic group: An open-label, single-dose, two-way crossover pharmacokinetic study in healthy caucasian and Japanese men. Clin Drug Investig, **2011;** 31(10): 735-743. doi:10.2165/11592480-000000000-00000

9. Baker W, Chamberlin K. Benefit\risk assessment of pitavastatin for the treatment of hypercholesterolemia in older patients. Clin Interv Aging, **2015;** 10: 733. doi:10.2147/CIA.S67532

10. Saito Y. Treatment options for hypercholesterolemia and combined dyslipidemia: Focus on pitavastatin. Clin Med Insights Ther, **2011;** 3: 517-525.

11. Saku K, Zhang B, Noda K. Randomized head-to-head comparison of pitavastatin, atorvastatin, and rosuvastatin for safety and efficacy (quantity and quality of LDL) - The PATROL trial-. Circ, **2011;** 75(6): 1493-1505. doi:10.1253/circj.CJ-10-1281

12. Moroi M, Nagayama D, Hara F, Saiki A, Shimizu K, Takahashi M, et al. Outcome of pitavastatin versus atorvastatin therapy in patients with hypercholesterolemia at high risk for atherosclerotic cardiovascular disease. Int J Cardiol, **2020;** 305: 139-146. doi:10.1016/j.ijcard.2020.01.006

13. Adams SP, Alaeiilkhchi N, Wright JM. Pitavastatin for lowering lipids. Cochrane Database of Systematic Reviews, **2017;** (7): 1-8. doi:10.1002/14651858.CD012735

14. Sponseller CA, Morgan RE, Kryzhanovski VA, Campbell SE, Davidson MH. Comparison of the lipid-lowering effects of pitavastatin 4 mg versus pravastatin 40 mg in adults with primary

hyperlipidemia or mixed (Combined) dyslipidemia: A phase IV, prospective, US, multicenter, randomized, double-blind, superiority trial. Clin Ther, **2014;** 36(8): 1211-1222. doi:10.1016/j.clinthera.2014.06.009

15. Stender S, Budinski D, Gosho M, Hounslow N. Pitavastatin shows greater lipidlowering efficacy over 12 weeks than pravastatin in elderly patients with primary hypercholesterolaemia or combined (mixed) dyslipidaemia. Eur J Prev Cardiol, **2013;** 20(1): 40-53. doi:10.1177/2047487312451251

16. Chan P, Shao L, Tomlinson B, Zhang Y, Liu ZM. An evaluation of pitavastatin for the treatment of hypercholesterolemia. Expert Opin Pharmacother, **2019;** 20(1): 103-113. doi:10.1080/14656566.2018.1544243

17. Tokgzoglu L, Zamorano JL. Current perspectives on the use of statins in the treatment of dyslipidaemic patients: Focus on pitavastatin. Drugs Context. **2020;** 12 (9): 2020-4 doi:10.7573/DIC.2020-4-4

18. Gumprecht J, Gosho M, Budinski D, Hounslow N. Comparative long-term efficacy and tolerability of pitavastatin 4 mg and atorvastatin 20-40 mg in patients with type 2 diabetes mellitus and combined (mixed) dyslipidaemia. Diabetes, Obesity and Metabolism, **2011;** 13(11): 1047-1055. doi:10.1111/j.1463-1326.2011.01477.x

19. Eriksson M, Budinski D, Hounslow N. Comparative efficacy of pitavastatin and simvastatin in high-risk patients: A randomized controlled trial. Adv Ther, **2011;** 28(9): 811-823. doi:10.1007/s12325-011-0056-7

20. Taguchi I, Iimuro S, Iwata H, Takashima H, Abe M, Amiya E, et al. High-dose versus low-dose pitavastatin in Japanese patients with stable coronary artery disease (REAL-CAD) a randomized superiority trial. Circ, **2018;** 137(19): 1997-2009.

21. Suh SY, Rha SW, Ahn TH, Shin EK, Choi CU, Oh DJ, et al. Long-term safety and efficacy of pitavastatin in patients with acute myocardial infarction (from the Livalo acute myocardial infarction study LAMIS). Am J Cardiol, **2011;** 108(11): 1530-1535. doi:10.1016/j.amjcard.2011.07.009

22. Hiro T, Kimura T, Morimoto T, Miyauchi K, Nakagawa Y, Yamagishi M, et al. Effect of Intensive Statin Therapy on Regression of Coronary Atherosclerosis in Patients With Acute Coronary Syndrome. A Multicenter Randomized Trial Evaluated by Volumetric Intravascular Ultrasound Using Pitavastatin Versus Atorvastatin. **2009;** J Am Coll Cardiol, 54(4): 293-302. doi:10.1016/j.jacc.2009.04.033

23. Ridker PM, Danielson E, Fonseca FAH, Genest J, Gotto AM, Kastelein JJP, et al. Rosuvastatin to Prevent Vascular Events in Men and Women with Elevated C-Reactive Protein. NEJM, **2008;** 359(21): 2195-2207. doi:10.1056/NEJMoa0807646

24. Thakker D, Nair S, Pagada A, Jamdade V, Malik A. Statin use and the risk of developing diabetes: a network meta-analysis. Pharmacoepidemiol Drug Saf, **2016;** 25(10): 1131-1149. doi:10.1002/pds.4020

25. Casula M, Mozzanica F, Scotti L, Tragni E, Pirillo A, Corrao G, Catapano AL. Statin use and risk of new-onset diabetes: A meta-analysis of observational studies. Nutr Metab Cardiovasc Dis, **2017;** 27(5): 396-406. doi:10.1016/j.numecd.2017.03.001

26. Navarese EP, Buffon A, Andreotti F, Kozinski M, Welton N, Fabiszak T, et al. Meta-analysis of impact of different types and doses of statins on new-onset diabetes mellitus. Am J Cardiol, **2013;** 111(8): 1123-1130. doi:10.1016/j.amjcard.2012.12.037

27. Na E, Cho S, Kim DJ, Choi J, Han E. Time-varying and dose-dependent effect of long-term statin use on risk of type 2 diabetes: A retrospective cohort study. Cardiovasc Diabetol, **2010;**19(1). doi:10.1186/s12933-020-01037-0

28. Arnaboldi L, Corsini A. Could changes in adiponectin drive the effect of statins on the risk of new-onset diabetes? The case of pitavastatin. Atherosclerosis Supplements, **2015;** 16: 1-27. doi:10.1016/S1567-5688(14)70002-9

29. Chapman MJ, Orsoni A, Robillard P, Hounslow N, Sponseller CA, Giral P. Effect of high-dose pitavastatin on glucose homeostasis in patients at elevated risk of new-onset diabetes: Insights from the CAPITAIN and PREVAIL-US studies. Curr Med Res Opin, **2014;** 30(5): 775-784. doi:10.1185/03007995.2013.874989

30. Teramoto T, Shimano H, Yokote K, Urashima M. New evidence on pitavastatin: Efficacy and safety in clinical studies, Expert Opin Pharmacother, **2010;** 11(5): 817-828.

31. Yokote K, Saito Y, Bujo H, Hanaoka H, Shinomiya M, Mikami K, et al. Influence of statins on glucose tolerance in patients with type 2 diabetes mellitus: Subanalysis of the collaborative study onhypercholesterolemia drug intervention and their benefits for atherosclerosis prevention (CHIBA Study). J Atheroscler Thromb, **2009;** 16(3): 297-298. doi:10.5551/jat.E1008

32. Vallejo-Vaz AJa. Effect of pitavastatin on glucose, HbA1c and incident diabetes: A meta-analysis of randomized controlled clinical trials in individuals without diabetes. Atherosclerosis, **2015;** 241(2): 409-418. doi:10.1016/j.atherosclerosis.2015.06.001

33. Liu PY, Lin LY, Lin HJ, Hsia CH, Hung YR, Yeh HI, et al. Pitavastatin and Atorvastatin Double-Blind Randomized ComPArative Study among HiGh-Risk Patients, Including ThOse with Type 2 Diabetes Mellitus, in Taiwan (PAPAGO-T Study). PLoS ONE, **2013;** 8(10), e.0076298. doi:10.1371/journal.pone.0076298

34. Yamakawa T, Takano T, Tanaka SI, Kadonosono K, Terauchi Y. Influence of pitavastatin on glucose tolerance in patients with type 2 diabetes mellitus. J Atheroscler Thromb, **2008;** 15(5): 269-275. doi:10.5551/jat.E562

35. Cui JY, Zhou RR, Han S, Wang TS, Wang LQ, Xie XH. Statin therapy on glycemic control in type 2 diabetic patients: A network meta-analysis. J Clin Pharm Ther, **2018;** 43(4): 556-570. doi:10.1111/jcpt.12690

36. Hong YJ, Jeong MH, Bae JH, Oh SK, Rha SW, Hur SH, et al. Efficacy and safety of pitavastatins in patients with acute myocardial infarction: Livalo in Acute Myocardial Infarction Study (LAMIS) II. Korean Journal of Internal Medicine,**2017;** 32(4): 656-667. doi:10.3904/kjim.2016.016

37. Wang YB, Fu XH, Gu XS, Fan WZ, Jiang YF, Hao GZ, et al. Effects of intensive pitavastatin therapy on glucose control in patients with non-ST elevation acute coronary syndrome. Am J Cardiol, **2017;** 7(4): 89-96.

38. Bangalore S, Parkar S, Grossman E, Messerli FH. A Meta-Analysis of 94,492. Patients With Hypertension Treated With Beta Blockers to Determine the Risk of New-Onset Diabetes Mellitus. Am J Cardiol, **2007;** 100(8):1254-62.

39. Choi SH, Lim S, Hong ES, Seo JA, Park CY, Noh, JH, et al. PROPIT: A PROspective comparative clinical study evaluating the efficacy and safety of PITavastatin in patients with metabolic syndrome. Clin Endocrinol, **2015;** 82(5): 670-677. doi:10.1111/cen.12580

40. Drew BG, Duffy SJ, Formosa MF, Natoli AK, Henstridge DC, Penfold SA, et al. High-den-

sity lipoprotein modulates glucose metabolism in patients with type 2 diabetes mellitus. Circ, **2009;** 119(15): 2103-2111. doi:10.1161/CIRCULATIONAHA.108.843219

41. Sasaki J, Ikeda Y, Kuribayashi T, Kajiwara K, Biro S, Yamamoto K, et al. A 52Week, Randomized, Open-Label, Parallel-Group Comparison of the Tolerability and Effects of Pitavastatin and Atorvastatin on High-Density Lipoprotein Cholesterol Levels and Glucose Metabolism in Japanese Patients with Elevated Levels of Low-Density Li. Clin Ther, **2008;** 30(6): 1089-1101. doi:10.1016/j.clinthera.2008.05.017

42. Pirillo A, Catapano AL. Pitavastatin and HDL: Effects on plasma levels and function(s). Atheroscler Suppl, **2017;** 27: e1-e9.

43. Nakazawa M, Arashi H, Yamaguchi J, Ogawa H, Hagiwara N. Lower levels of highdensity lipoprotein cholesterol are associated with increased cardiovascular events in patients with acute coronary syndrome. Atherosclerosis, **2020;** 303: 21-28. doi:10.1016/j.atherosclerosis.2020.05.005

44. Acharjee S, Boden WE, Hartigan PM, Teo KK, Maron DJ, Sedlis SP, et al. Low levels of high-density lipoprotein cholesterol and increased risk of cardiovascular events in stable ischemic heart disease patients: A post-hoc analysis from the COURAGE trial (clinical outcomes utilizing revascularization and aggressive drug evaluat. J Am Coll Cardiol, **2013;** 62(20): 1826-1833. doi:10.1016/j.jacc.2013.07.051

45. Kodama K, Komatsu S, Ueda Y, Takayama T, Yajima J, Nanto S, et al. Stabilization and regression of coronary plaques treated with pitavastatin proven by angioscopy and intravascular ultrasound: The TOGETHAR trial. Circ, **2010;** 74(9): 1922-1928. doi:10.1253/circj.CJ-10-0038

46. Kurogi K, Sugiyama S, Sakamoto K, Tayama S., Nakamura S, Biwa T, et al. Comparison of pitavastatin with atorvastatin in increasing HDL-cholesterol and adiponectin in patients with dyslipidemia and coronary artery disease: The COMPACT-CAD study. J Cardiol, **2013;** 62(2): 87-94. doi:10.1016/j.jjcc.2013.03.008

47. Niacin in Patients with Low HDL Cholesterol Levels Receiving Intensive Statin Therapy. NEJM, **2011;** 365(24): 2255-2267. doi:10.1056/nejmoa1107579

48. Schwartz GG, Olsson AG, Abt M, Ballantyne CM, Barter PJ, Brumm J, et al. Effects of Dalcetrapib in Patients with a Recent Acute Coronary Syndrome. NEJM, **2012;** 367(22): 2089-2099. doi:10.1056/nejmoa1206797

49. Khera AV, Cuchel M, de la Llera-Moya M, Rodrigues A, Burke MF, Jafri K, et al. Cholesterol Efflux Capacity, High-Density Lipoprotein Function, and Atherosclerosis. NEJM, **2011;** 364(2): 127-135. doi:10.1056/nejmoa1001689

50. Rohatgi A, Khera A, Berry JD, Givens EG, Ayers CR, Wedin KE, et al. HDL Cholesterol Efflux Capacity and Incident Cardiovascular Events. NEJM, **2014;** 371(25): 2383-2393. doi:10.1056/nejmoa1409065

51. Miyamoto-Sasaki M, Yasuda T, Monguchi T, Nakajima H, Mori K, Toh R, Ishida T, Hirata KI. Pitavastatin Increases HDL Particles Functionally Preserved with Cholesterol Efflux Capacity and Antioxidative Actions in Dyslipidemic Patients. J Atheroscler Thromb. **2013;** 20 (9): 708–716.

52. Maruyama T, Takada M, Nishibori Y, Fujita K, Miki K, Masuda S, et al. Comparison of preventive effect on cardiovascular events with different statins: The circle study. Circ, **2011;** 75(8): 1951-1959. doi:10.1253/circj.CJ-10-1163

53. Toi T, Taguchi I, Yoneda S, Kageyama M, Kikuchi A, Tokura M, et al. Early effect of lipid-lowering therapy with pitavastatin on regression of coronary atherosclerotic plaque - Comparison with atorvastatin. Circ, **2009;** 73(8): 1466-1472. doi:10.1253/circj.CJ-08-1051.

54. Nishiguchi T, Kubo T, Tanimoto T, Ino Y, Matsuo Y, Yamano T, et al. Effect of Early Pitavastatin Therapy on Coronary Fibrous-Cap Thickness Assessed by Optical Coherence Tomography in Patients With Acute Coronary Syndrome: The ESCORT Study. JACC: Cardiovascular Imaging, **2018;** 11(6): 829-838. doi:10.1016/j.jcmg.2017.07.011

55. Malvestutto CD, Ma Q, Morse GD, Underberg JA, Aberg JA. Lack of pharmacokinetic interactions between pitavastatin and efavirenz or darunavir/ritonavir. J Acquir Immune Defic Synd, **2014;** 67(4): 390-396. doi:10.1097/QAI.0000000000000333

56. Gilbert JM, Fitch KV, Grinspoon SK. HIV-related cardiovascular disease, statins, and the REPRIEVE trial. Top antivir med, **2015;** 23(4): 146-149.

57. Kurihara Y, Douzono T, Kawakita K, Nagasaka Y. A Large–scale, Long–term, Prospective Post–marketing Surveillance of Pitavastatin. 薬理と治療, **2008;** 36(8): 709-731.