ACTA PHARMACEUTICA SCIENCIA

International Journal in Pharmaceutical Sciences, Published Quarterly

ISSN: 2636-8552
e-ISSN: 1307-2080,
Volume: 61, No: 1, 2023
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: 61, No: 1, 2023
Formerly: Eczacılık Bülteni/Acta Pharmaceutica Turcica
Founded in 1953 by Kasım Cemal Güven

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. Gülden Zehra OMURTAG
Editor

INSTRUCTIONS FOR AUTHORS

Manuscripts must be prepared using the manuscript template

Manuscripts should contain the following elements in the following order :

Title Page

Abstract

Keywords

Introduction (Without author names and affiliations)

Methodology

Results and Discussion

Statement of Ethics

Conflict of interest Statement

Author Contributions

Funding Sources (optional)

Acknowledgments (optional)

References

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

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

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

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

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

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

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

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

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

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

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

1. Scope and Editorial Policy

1.1. Scope of the Journal

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

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

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

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

1.2. Manuscript Categories

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

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

1.3. Prior Publication

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

1.4. Patents and Intellectual Property

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

1.5. Professional Ethics

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

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

1.5.1 Author Consent

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

1.5.2. Plagiarism

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

1.5.3. Use of Human or Animal Subjects

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

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

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

1.6 Issue Frequency

The Journal publishes 4 issues per year.

2. Preparing the Manuscript

2.1. General Considerations

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

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

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

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

2.1.1 Articles of all kind

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

2.1.2 Nomenclature

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

Authors may find the following sources useful for recommended nomenclature:

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

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

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

2.1.3 Compound Code Numbers

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

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

· Once in the abstract.

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

patent on first appearance.

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

2.1.4 Trademark Names

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

2.1.5 Interference Compounds

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

2.2 Manuscript Organization

2.2.1 Title Page. Title

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

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

2.2.2 Abstract and keywords

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

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

Keywords: instructions for authors, template, journal

2.2.3 Introduction

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

2.2.4. Methodology

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

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

2.2.5 Results and Discussion

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

2.2.6 Ancillary Information

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

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

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

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

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

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

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

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

2.2.7 References and Notes

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

References

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

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

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

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

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

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

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

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

Examples

For printed articles

• Article with 1-6 authors:

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

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

• Article with more than 6 authors:

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

Electronic journal article:

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

Electronic journal article with DOI:

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

Books and book chapters

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

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

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

2.2.8 Tables

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

2.2.9 Figures, Schemes/Structures, and Charts

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

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

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

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

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

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

- Black & White line art: 1200 dpi

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

- Color art (RGB color mode): 300 dpi

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

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

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

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

2.2.10 Image Manipulation

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

2.3 Specialized Data

2.3.1 Biological Data

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

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

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

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

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

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

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

2.3.2 Purity of Tested Compounds

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

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

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

2.3.3 Confirmation of Structure

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

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

3. Submitting the Manuscript

3.1. Communication and log in to Author's Module

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

3.2. Registration to System

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

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

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

3.3 Submitting A New Article

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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 resubmit (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 61:(1), 2023
DOI: 10.23893/1307-2080.APS6101

Development of Mefenamic Acid-Soluplus ® amorphous dispersions via hot melt extrusion and *in silico* prediction of oral absorption

Estrella CHAVERO[1], Aleksandra KUROWSKA[1], Shaila A LEWIS[1*]

[1] Department of Pharmaceutics, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal - 576104, Karnataka, India

ABSTRACT

The objective of this study was to increase the solubility of Mefenamic Acid (MA), a BCS class II drug by formulating amorphous solid dispersions via Holt-Melt Extrusion. The extrudates were prepared at different drug to polymer ratios and characterised by standard analytical techniques. Dissolution studies were performed in Phosphate buffer saline (PBS) pH 7.4 medium. Stability of the different ratios of MA: Soluplus (1:1, 1:4 and 4:1) was studied at room temperature for 12 months. Computer simulation using GastroPlus™ was run to depict the gastrointestinal absorption of MA in humans. DSC thermograms and the diffractograms of the solid dispersions confirmed amorphous nature. Dissolution studies showed enhanced dissolution rate of MA from the solid dispersions. Stability studies indicated 1:4 (MA: Soluplus®) dispersion as the most stable dispersion. GastroPlus™ simulation using *in vitro* data showed improvement in the PK parameters of the solid dispersión in comparison with pure MA.

Keywords: Hot-melt extrusion, Metanamic Acid, Soluplus, solubilisation, solid dispersion

INTRODUCTION

Mefenamic Acid (MA) [(2-(2,3-dimethylphenyl) aminobenzoic acid] is a widely prescribed non-steroidal anti-inflammatory drug (NSAID) for relief of pain primarily dysmenorrhoea and rheumatoid arthritis[1,2]. Poor solubility affects the

*Corresponding Author: E-mail: s.lewis@manipal.edu
ORCIDs:
Estrella Chavero: 0000-0003-3834-6608
Aleksandra Kurowska: 0000-0001-5950-0358
Shaila A Lewis: 0000-0002-2884-0015
(Received 05 Oct 2020, Accepted 23 Nov 2022)

rate of absorption of MA from the gastrointestinal tract [3,4]. Oral administration is associated with side effects in the gastrointestinal tract as perforation of the stomach, small and large intestine, ulceration and bleeding that may be fatal[4].

There are multiple delivery strategies for oral administration of poorly soluble drugs and some of them have been explored for the oral delivery of MA[5-7]. The use of amorphous form has the potential to increase oral absorption and is usually considered when drug cannot be suitably solubilised. Melt extrusion is one such means of obtaining amorphous forms. Specifically, amorphous solid dispersions (ASDs) are advantageous as they have high free energy and kinetic solubility due to structural changes that modify chemical and physical properties, such as endothermic end exothermic values, the lack of melting point observed in ASDs, and the lack of symmetry at conformational, translational and orientational structures.

Most of the published research data for solid dispersion of MA are based on carriers like PVP, polyoxyethylene, eudragit EPO, Pluronic F127® polyethylene glycol 4000 and Gelucire 50/13[8-15].

Polyvinyl caprolactam–polyvinyl acetate–polyethylene glycol graft copolymer (Soluplus®), is an attractive carrier for solid dispersion due to numerous advantages. As it has a low Tg, 70°C, it is suitable for poorly soluble drugs with high melting point and ensures that the API is thermally stable during the process. Being hydrophilic and non-ionic, solubility is unaltered along the GI tract. Apart from enhancing solubility, Soluplus® stabilizes the solid dispersion[16-19].

Hot melt extrusion (HME) is a documented process for the manufacturing of solid dispersions. Compared to other techniques for preparing solid dispersions, HME is a reliable and robust process, far less complex, cost efficient and avoids the use of organic solvents[20,21].

There are no reports on the use of Soluplus® with HME technology in preparation of solid dispersion of MA. MA is a challenging drug to process via HME owing to its high melting point indicating high crystallinity. Hence the present study was taken to explore HME with Soluplus® as a carrier, to generate solid dispersion of MA. To ensure that MA is indeed in its amorphous form, the performance of DSC, FTIR and XRD analysis were carried out and to confirm stability XRD was studied after 12 months. The novelty of this paper lies in the use of co rotating twin screw extruder for preparing solid dispersion of MA and *in silico* tool to predict the enhanced oral bioavailability with the obtained in vitro results. GastroPlus™ simulation software was used to predict the oral absorption of the solid dispersion. The *in vitro* data was used as the input function into a simulation software.

METHODOLOGY

Materials

Mefenamic acid was purchased from Yarrow Chem Products, Mumbai, India. Soluplus® (M_w = 118,000 g/mol, density = 1.08 g/cm^3) was a gift from BASF. All other chemicals and reagents were of analytical grade and used in the study without further purification.

Calculation of solubility parameter

To predict possibility of glass solution formation on melt extrusión, Hansen solubility parameter of MA and Soluplus® was calculated. In the present study prediction was based on comparison of solubility parameters of drug and excipient. The solubility parameters were obtained from literature.

Thermal analysis of the physical mixtures

The thermal properties of the physical mixtures were determined using a DSC (DSC-50, Shimadzu, Japan). Physical mixtures (100 mg) consisting of one (crystalline) drug and one polymer in concentrations ranging from 60 – 90% drug (w/w) were prepared by gentle mixing using a mortar and pestle. Samples of 4 – 6 mg were analyzed by DSC at a heating rate of 10 °C/min from ambient temperature to 20 °C above the Tm of the pure crystalline drug. The calibration of the baseline was done using empty aluminium pans as a reference, and temperature/ enthalpy using indium.

Preparation of hot-melt extrudates

HME operation was carried out by twin screw extruder (STEER-Omicron 10P, India). Soluplus® was blended with MA until it was mixed evenly and introduced manually into the extruder barrel. Three different extrudates were prepared, drug to polymer ratios, at 1:4, 1:1 and 4:1. The extrusion process was performed at various barrel temperatures 110°C, 130°C and 130°C while screw speed was fixed at 150 rpm. The external to internal screw diameter (Do/Di) ratio of the HME apparatus was 1.71. The samples were milled using mortar and pestle and meshed through a 60 mesh and stored in a desiccator until use.

Physical characterisation

Differential Scanning Calorimetry (DSC)

The DSC thermograms of the extrudates were recorded on a DSC (DSC-50, Shimadzu, Japan). The samples were weighed into aluminium pans, sealed and heated under nitrogen flow at a scanning rate of 10 °C min-1 to obtain a temperature range from 25 °C to 300 °C. The calibration of the baseline was done

using empty aluminium pans as a reference, and temperature/ enthalpy using indium.

Fourier Transform Infrared Spectroscopy (FTIR)

The FT-IR spectra (Shimadzu - FTIR 8300) were recorded based on the KBr pellet technique for the extrudates the drug and the polymer in the wavelength region of 400-4000 cm^{-2}.

X-Ray Diffraction (XRD)

The X-ray diffraction pattern of extrudates the drug and the polymer was recorded using benchtop X-ray Diffractometer (Rigaku MiniFlex 600, Japan) at an angle range of 10 to 80° at the rate of 2°/min. The experiment was carried out at room temperature.

Scanning electron microscope (SEM) analysis

The surface size, shape and structure of the pure drug and extrudates were evaluated using a JEOL JSM-7600 F, SEM. The samples to be examined were mounted on the SEM sample slab using a double-sided adhesive tape and were coated with gold (200 °A) under reduced pressure (0.001 torr) for 5 min to increase the conductivity using an ion sputtering device and viewed.

Solubility determination

MA and the extrudates were placed in a 2 ml Eppendorf tube with water and PBS until saturation and left for two days in the rotospin (Tarsons). The samples were filtered, and absorbance measured with the UV-Spectrophotometer[22].

Drug content

The drug content of the different extrudates were performed by weighing 10 mg of the different extrudates in 100ml methanol volumetric flask, sonication for 20 minutes and measurement of absorbance with UV-Spectrophotometer[23].

***In vitro* drug release studies**

Dissolution study was performed with TDT-08L Dissolution Tester USP Apparatus II (Electrolab) by placing 50 mg equivalent of drug content of the different extrudates in in 900ml of PBS pH 7.4 at 37°C and 75 rpm. Samples were taken at 15, 30, 45, 60, 90 and 120 minutes and media replaced immediately. Absorbance of samples was measured using the UV-Spectrophotometer[22].

***In silico* simulation of oral absorption**

An absorption model was built using the GastroPlus™ simulation software

(version 9.0, Simulations Plus Inc, Lancaster, CA, USA). Input parameters like drug solubility, pKa, effective permeability were determined *in silico* using the ADMET predictor module of GastroPlus™. The default human fasted physiological model in GastroPlus™ (Opt logD SA/v6.1) was used for simulations. Metabolism of MA is predominantly mediated via CYP enzymes particularly CYP2C9 in addition to CYP1A2. The GastroPlus™ default Km and Vmax values based on the Metabolism and Transporter module were utilised. Human PK parameters were estimated by fitting the 250mg capsule oral data from humans to a one compartment model in PKPlus[22]. The generated mean PK parameters were exported to the Pharmacokinetics tab to enable software prediction. Simulations were conducted using the dissolution data of solid dispersions.

Physical stability studies upon storage

Physical stability studies were conducted for 12 months at 30°C/75%RH. The prepared solid dispersions were stored in air tight containers. The stored samples were investigated for the recrystallisation tendency by XRPD after 12 months.

RESULTS AND DISCUSSION

Solubility parameter

The solubility parameter of MA and Soluplus® were obtained from literature. Based on the group contribution method, individual solubility parameter values (δ) for MA[25] and Soluplus®[17] are 21 MPa $^{1\backslash 2}$ and 23.77 MPa $^{1\backslash 2}$ respectively.

The use of solubility parameter in predicting miscibility between drugs and polymers is based on the standard solution theory "likes dissolves likes" whereby if two solvents have similar solubility parameters, they can be mixed to form a uniform solution with any ratios. Likewise, if drugs and polymers are predicted with close solubility parameters, a miscible drug-polymer solid dispersion could be prepared. It has been proposed empirically that compounds with a $\Delta\delta$ < 7.0 MPa$^{0.5}$ were likely to be miscible while compounds with a $\Delta\delta$ > 7.0 MPa$^{0.5}$ were expected to be immiscible[26,27]. The solubility parameter difference between Soluplus® and MA is 2.77, which is below 7.0 MPa$^{0.5}$

The small difference between the calculated solubility parameters of MA and Soluplus® indicates that MA is likely to be miscible with Soluplus®**.** The comparison of solubility parameters can be a rapid way to predict miscibility between drugs and polymers.

Thermal analysis of the physical mixtures

The DSC thermograms of the physical mixtures of MA: Soluplus® is displayed in figure1. The DSC thermogram of pure crystalline MA displayed a single endothermic melting event at 231 °C, with a melting enthalpy (ΔHm) of approximately 187.51 J/g, Physical and thermodynamic values measured for the physical mixtures are presented in Table 1. Drastic change in Cp value from pure drug to the polymer mixture could be due to slow transition of the phase of the drug which is significant.

Accordingly, it can be predicted that at 80%+20% (MA: Soluplus®) could be optimum as enthalpy change over at this composition. Based on these observations the ratios 1:4, 1:1 and 4:1 were selected for preparing solid dispersions.

Table 1. Thermodynamic values of the MA and physical mixtures measured by DSC

MEFENAMIC ACID %	PEAK Tm (°C)	ONSET (°C	ENDSET (°C)	ΔHm (J·g-1)	ΔCp (J·g-1·K-1)
100	231.92	230.17	236.09	-187.51J/g	0.37
90	230.47	226.08	233.37	-47.74J/g	0.09
80	229.64	224.15	232.75	-28.64J/g	0.05
70	230.28	225.31	232.31	-13.79J/g	0.027

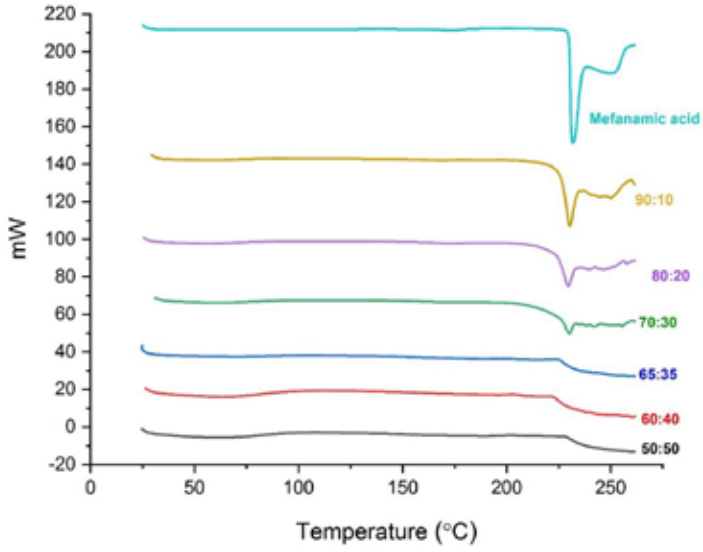


Figure 1. Thermograms of the MA-Soluplus physical mixtures (% w/w)

Preparation of Hot melt extrudates

Three different extrudates were prepared with drug to polymer ratios, at 1:4, 1:1 and 4:1. The melting point (Tm) of MA is 230°C The thermal decomposition kinetics of MA is reported in literature[28]. Positive value of the Gibbs free energy obtained from the study showed that the decomposition reaction of MA is nonspontaneous. Since the carrier Soluplus® has excellent plasticizing effect, the operation was possible at lower processing makes Soluplus® a good candidate for extrusión.

DSC

The DSC thermograms of pure MA and exrudates are displayed in figure 2. However, in the thermogram of solid dispersion (extrudates) the peaks of crystalline MA completely disappeared. This indicates crystalline MA was transformed into amorphous state by HME. Amorphisation was possible due to molecular interaction with carrier.

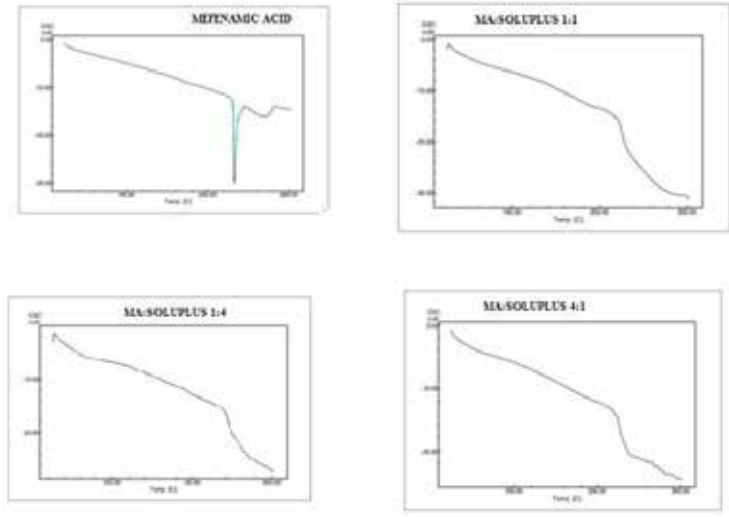


Figure 2. DSC thermograms of MA, MA: Soluplus (1:1,1:4 and 4:1)

FTIR

The FTIR spectra of pure MA shows N-H stretching, N-H bending and stretching vibrations of the COOH moiety. Characteristic peaks at 3309 and 1575 cm^{-1} indicate N-H stretching and N-H bending of secondary amine. The C=O stretch of carboxylic acid appears at 1649, C=C stretch at 1508. C-H symmetric bending vibrations of side chain, O-H stretching of C=O and C-OH appear at 2571 cm^{-1}. For the solid dispersions (extrudates) all assigned peaks showed no significant difference in comparison with pure drug except for some changes with

respect to H bond formation. The probability of H bonding is high in the amine and carbonyl groups of MA. Carboxyl group of MA strongly contributes to intermolecular interaction with Soluplus®.

4:1 indicated involvement of both C=O, C-H and N-H is not much affected. 1:1 only C=O not N-H but C-H bending is affected. 1:4 C=O, N-H, C-H all are affected. In conclusion both C=O, C-OH (of COOH) and N-H seem to have involved in H bonding within the MA. The H bond is cleaved and new H bond formed with the polymer-OH function at either end of the polymer chain.

XRD

The overlayed XRD spectra of MA and the extrudates is presented in figure 3. The characteristic XRD peaks of pure MA were observed at 2 θ equal to 6.5, 21.5 and 26.3 that coincided with those reported previously and thus confirming crystalline structure of pure MA[25,29]. XRD of solid dispersions showed disappearance of characteristic peaks of MA. The peaks of crystalline MA completely disappeared. This observation indicates amorphisation of MA due to molecular interaction with the carrier.

X-ray diffraction analysis can be useful to determine the amorphous or crystalline structure of the samples, as the beams go in different directions and layers. The straight peaks in the results indicate that the sample is in a crystalline form and when I t shows a halo it is much disorganised form, amorphous.

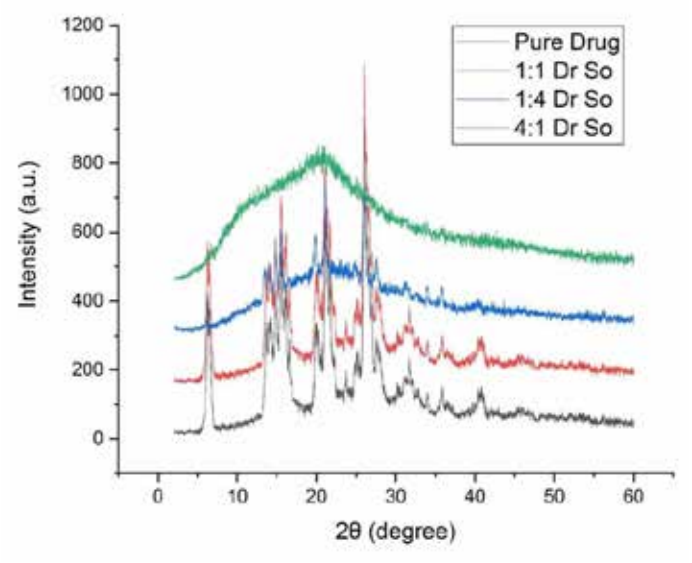


Figure 3. Xray diffractograms of MA, MA: Soluplus (1:1,1:4 and 4:1)

SEM analysis

SEM images of MA solid dipersions and pure MA captured at various magnifications is shown in figure 4. When compared to the extrudates the surface of pure MA was coarse in appearance.

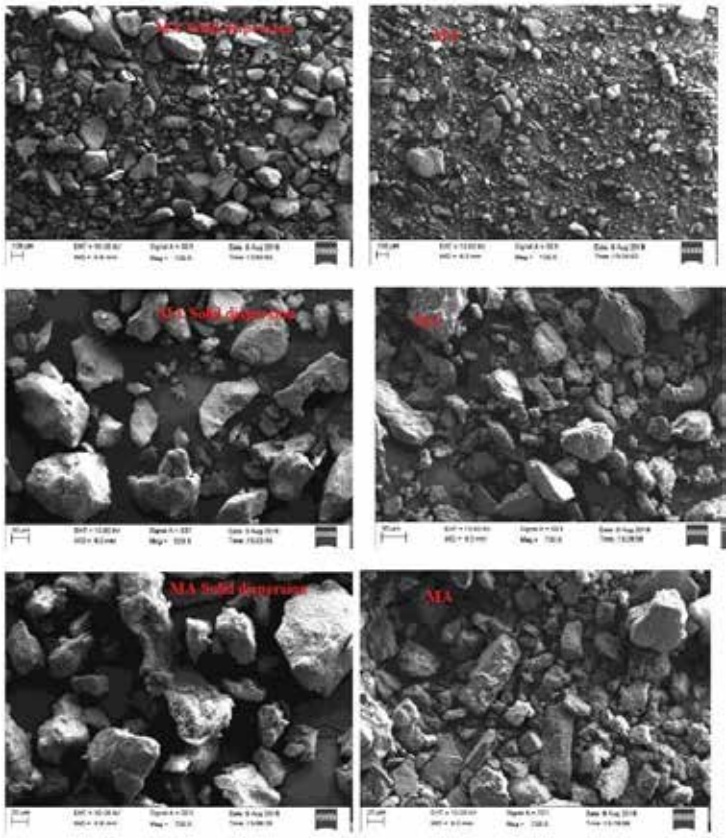


Figure 4. SEM images of MA solid dipersions and pure MA captured at various magnifications

In vitro dissolution

The dissolution data (Table 2) clearly indicates that the dissolution rate is controlled by the drug/carrier ratio of the formulation.

Table 2. In vitro Dissolution of MA and MA Solid Dispersions

Time (Min)	Cummulative Percent Released			
	MA	1:1	1:4	4:1
15	1.5	7.6	0.5	25.7
30	3.2	14.5	1.3	35.7
60	4.5	21.6	7.2	52.8
90	5.4	25.6	9.0	60.2
120	8.9	32.2	12.9	70.8
180	11.7	40.2	24.1	86.0
240	17.2	49.9	28.4	91.3

The dissolution rate increases with increasing the drug amount in the blend up to a drug load of 80% and decreases markedly from amounts with higher polymer load.

This observation leads to the assumption that the improvement of drug release is based on a chemical interaction between drug and carrier. The release of MA was less than 20% owing to hydrophobic nature of the drug. The release of MA from the ASDs was higher. Though solid dispersion transformed the drug into amorphous form, drug release was influenced by drug: polymer ratios. Slow release with higher polymer was hypothetised due to aggregation of the dispersion in the media. Initially, a polymer-rich diffusion layer is formed between the solid dispersion and the dissolution médium. When the amorphous molecules diffuse through the polymer-rich diffusion layer, crystallization may occur, which creates a high-energy interfacial boundary that slows down the dissolution rate. The viscosity of the polymer will also influence the dissolution rate of the drug from the amorphous solid dispersion.

In silico simulation of oral absorption

The built base absorption model was validated using the plasma data comparing the predicted with the observed values. The accuracy of prediction of the pharmacokinetic (PK) parameters was based on prediction fold error. The simulated model was considered to be of high prediction accuracy if prediction values were within two-fold of observed values[30]. Table 3 represents a summary of the input parameters used in the study. Figure 5 shows the predicted and observed plasma concentration-time profile of oral administration of 250 mg capsule of MA.

Table 3. Basic modeling parameters of MA fed into the Gastro-Plus™ software

Parameters	Value
Molecular weight ($gmol^{-1}$)	241.29[a]
PKa	3.95[a]
Solubility ($mgml^{-1}$)@pH4.5	0.0097[a]
Human permeability [$P_{eff}(cms^{-1}x10^{4})$]	6.5[b]
Particle density (gml^{-1})	1.2[b]
Diffusion coefficient (cm^2s^{-1})$x10^5$	0.6[b]
Log P	4.9[a]
Mean precipitation time (s)	900[b]
CL/F L/h	18.7[c]
Oral dose for Cp-time profile (mg)	250[d]

a. Predicted by ADMET predictor (Version 7.2.0.0, Simulations Plus, Inc., Lancaster, CA, USA)

b. Default GastroPlus™

c. Calculated by GastroPlus™

d. Literature value

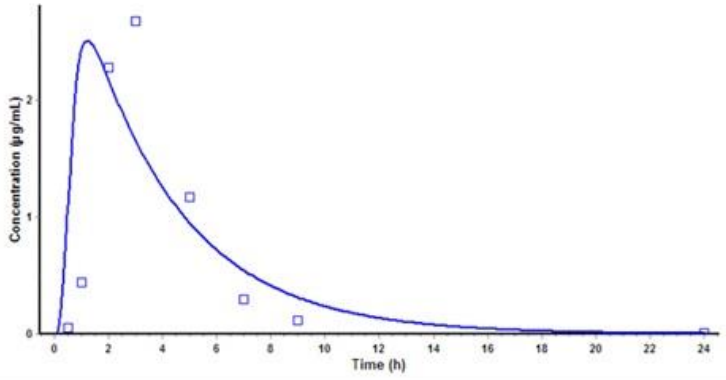


Figure 5. Observed (in square) and predicted (continuous line) plasma concentration–time profile following oral administration of 250 mg MA Capsule

The simulated profile fitted well with the observed (reported) curve. The observed and predicted PK parameters are displayed in Table 4. The fold error for the prediction accuracy of PK parameters was found to be < 2 indicating good prediction.

Table 4. Pharmacokinetic parameters of MA obtained from literature (observed) and Calculated by GastroPlusTM (predicted) for building absorption model

Pharmacokinetic Parameter	Observed	Calculated	Fold Error
Cmax(µg/ml)	2.68	2.5	0.932
Tmax (h)	3	1.2	0.4
$AUC_{(0-t)}$µg-h/ml	10.67	11.38	1.06

Among the solid dispersions the best disolution profile was seen with 4:1, so the dissolution data of 4:1 solid dispersión was used to simulate oral absorption profile in humans. The predicted PK parameters of the solid dispersión in comparison with pure MA is presented in Table 5. Solid dispersión of MA showed improvement in the PK parameters.

Table 5. Pharmacokinetic parameters generated by loading dissolution data into the developed absorption model of GastroPlusTM

Pharmacokinetic Parameter	MA	MA Solid dispersion (4:1)
Cmax(µg/ml)	0.39	1.24
Tmax (h)	10	3.04
AUC(0-t)µg-h/ml	6.44	8.73

Stability studies

The physical and chemical stability of the solid dispersions prepared by HME is of paramount importance as it can limit the commercial outcomes of solid dispersions. Recrystallization of the drug in the amorphous systems generally takes place with aging due to the high free energy of amorphous molecules compared to the crystalline form. The addition of a suitable polymer can delay this crystallization phenomenon according to many studies[31-34]. The viscosity of the polymer, as well as the intermolecular interactions (hydrogen bonds) that can occur between the API and the polymer, are most key factors in the stabilization of solid dispersion systems.

In the present study, we stored our samples of interest for a period of 12 months at normal conditions (30 °C/75% RH).

XRD analysis demonstrated the appearance of crystalline peaks in solid dispersions of 1:1 and 4:1 dispersions. However, they were not seen in 1:4 indicating stable dispersions (Figure 6).

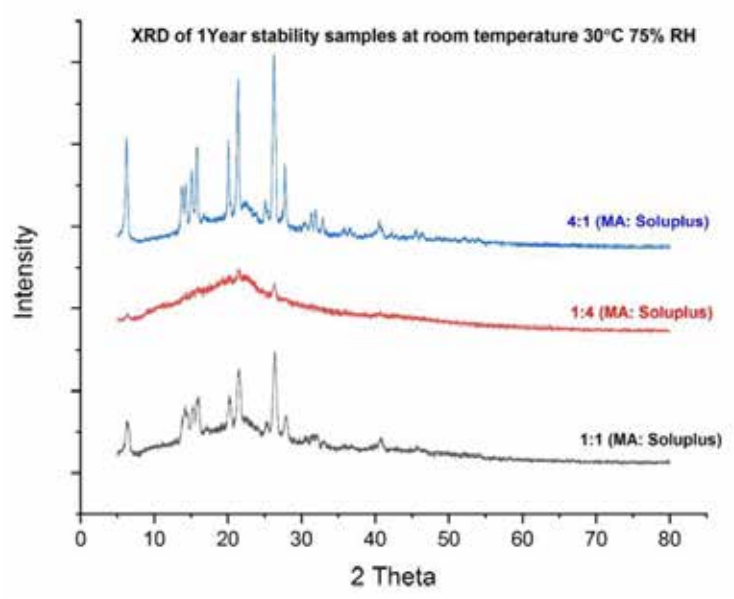


Figure 6. Diffractograms of the stability samples of MA solid dispersions

Steric hindrance by a polymer of different concentrations may slow crystallization mechanisms. Steric hindrance acts by preventing drug molecule aggregation and/or interaction that are the precursors for crystallization[35].

The appropriate use of polymer is very important to the physical stability of an ASD. The drug–polymer interactions are indicated by peak shifts or peak intensity changes corresponding to specific vibrational modes of the functional groups involved in intermolecular interactions in FTIR. From the FTIR results of the solid dispersions, 1:4 showed involvement of all N-H, C=O and C-H of COOH in the formation of hydrogen bonds which may also be the reason for better stability. Also in the 1:4 dispersion, the drug is well dispersed in the polymer and so the chances of self-contact interactions (drug-drug) which can be precursor for crystal growth is minimum. Moreover, hydrogen bonds between drug molecules and polymers not only increase the nucleation activation energy but also reduce crystal growth.

ACKNOWLEDGEMENTS

The authors are grateful to STEER Engineering, Bengaluru, India for providing Hot Melt Extruder (OMICRON 10), Simulations Plus, Lancaster USA for GastroPlus™ and Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education for providing the facilities for carrying out the research work.

CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

FUNDING SOURCES

This work did not receive any funding.

AUTHOR CONTRIBUTIONS

Estrella Chavero and Aleksandra Kurowska carried out the experiments and generated the data. Shaila Lewis supervised the project and wrote the manuscript with input from the co-authors.

REFERENCES

1. Reynolds JEF. In Martindale: The Extra Pharmacopeia. 31st ed., The Pharmaceutical Press, London 1998; 58-59.

2. Yazdanian M, Briggs K, Jankovsky C, Hawi A. The High Solubility Definition of the Current FDA Guidance on Biopharmaceutical Classification System May Be Too Strict for Acidic Drugs. Pharm. Res. 2004; 21: 293–99. doi: 10.1023/b:pham.0000016242.48642.71.

3. Graf GM, Jelen D, Jamnig H, Schabus W, Pipam RA. Comparison of the Efficacy and Rate of Side-Effects of Mefenamic Acid and Naproxen in Adult Patients Following Elective Tonsillectomy: A Randomized Double-Blind Study. Acute Pain. 2008; 10: 131–36. doi.org/10.1016/j.acpain.2008.07.003

4. Jelvehgari M, Hassanzadeh D, Kiafar F, Delf Loveym B, Amiri S. Preparation and Determination of Drug-Polymer Interaction and In-vitro Release of Mefenamic Acid Microspheres Made of CelluloseAcetate Phthalate and/or Ethylcellulose Polymers. Iran J Pharm Res. 2011; 10: 457–67. PMID: 24250377; PMCID: PMC3813045.

5. Sevgi F, Yurdasiper A, Kaynarsoy B, Turunç E, Güneri T, Yalçin A. Studies on Mefenamic Acid Microparticles: Formulation, In Vitro Release, and In Situ Studies in Rats. AAPS PharmSciTech. 2009; 10: 104–12. doi: 10.1208/s12249-008-9183-0.

6. Konnerth C, Braig V, Ito A, Schmidt J, Lee G, Peukert W. Formation of Mefenamic Acid Nanocrystals with Improved Dissolution Characteristics. Chemie Ing Tech. 2017; 89: 1060–1071. doi.org/10.1002/cite.201600190.

7. Nagabhushanam MV, Sudha Rani A. Dissolution Enhancement of Mefenamic Acid using Solid Dispersions in Crospovidone. Int J Pharm Sci. 2011; 3: 16–19. https://web.archive.org/web/20180413020232id_/http://ijppsjournal.com/Vol3Issue1/692.pdf.

8. Sambasiva Rao KRS, Chowdary KPR, Nagabhushanam M. In vitro Dissolution Studies on Solid Dispersions of Mefenamic Acid. Indian J Pharm Sci 2011; 73: 243. DOI: 10.4103/0250-474x.91575.

9. Fülöp I, Gyeresi A, Kiss L, Deli MA, Croitoru MD, Szabo-Revesz P,, Aigner Z. Preparation and Investigation of Mefenamic Acid - Polyethylene Gglycol - Sucrose Ester Solid Dispersions. Acta Pharm. 2015; 10: 457–67. DOI:10.1515/acph-2015-0035

10. Kang N, Lee J, Choi JN, Mao C, Lee EH. Cryomilling-Induced Solid Dispersion of Poor Glass Forming/Poorly Water-Soluble Mefenamic Acid with Polyvinylpyrrolidone K12. Drug Dev Ind Pharm. 2015; 41: 978–88. doi: 10.3109/03639045.2014.920024.

11. Andrews GP, Zhai H, Tipping S, Jones DS. Characterisation of the Thermal, Spectroscopic and Drug Dissolution Properties of Mefenamic Acid and Polyoxyethylene–Polyoxypropylene Solid Dispersions. J Pharm Sci. 2009; 98: 4545–4556. doi: 10.1002/jps.21752.

12. Kojima T, Higashi K. Suzuki T, Tomono K, Moribe K, Yamamoto K. Stabilization of a Supersaturated Solution of Mefenamic Acid from a Solid Dispersion with EUDRAGIT® EPO. Pharm Res. 2012; 29: 2777–2791. doi: 10.1007/s11095-011-0655-7

13. Alshehri S, Shakeel F, Ibrahim M, Elzayat E, Altamimi M, Shazly G, Mohsin K, Alkholief M, Alsulays B, Alshetaili A, Alshahrani A, Almalki B, Alanazi F. Influence of the Microwave Technology on Solid Dispersions of Mefenamic Acid and Flufenamic Acid. PLoS One. 2017; 12, e0182011. doi.org/10.1371/journal.pone.0182011.

14. Wong WS, Lee CS, Er HM, Lim WH. Preparation and Evaluation of Palm Oil-Based Polyesteramide Solid Dispersion for Obtaining Improved and Targeted Dissolution of Mefenamic Acid. J Pharm Innov. 2017; 12: 76–89. DOI:10.1007/s12247-017-9271-3

15. Linn M, Collnot EM, Djuric D. Soluplus® as an Effective Absorption Enhancer of Poorly Soluble Drugs In vitro and In vivo. Eur J Pharm Sci. 2012; 45: 336–343. DOI: 10.1016/j.ejps.2011.11.025

16. Zhang K, Yu H, Luo Q. Increased Dissolution and Oral Absorption of Itraconazole/Soluplus Extrudate Compared with Itraconazole Nanosuspension. Eur J Pharm Biopharm. 2013; 85: 1285–1292 . doi: 10.1016/j.ejpb.2013.03.002.

17. Zhang Y, Liu Y, Luo Y. Extruded Soluplus/SIM as an Oral Delivery System: Characterization, Interactions, In vitro and In vivo Evaluations. Drug Deliv. 2016; 23: 1902–1911. doi: 10.3109/10717544.2014.960982.

18. Shamma RN, Basha M. Soluplus®: A novel polymeric solubilizer for optimization of Carvedilol solid dispersions: Formulation design and effect of method of preparation Powder technology. 2013; 237: 406-414. DOI:10.1016/J.POWTEC.2012.12.038

19. Crowley MM., Zhang F, Repka MA. Pharmaceutical Applications of Hot-Melt Extrusion: Part I. Drug Dev Ind Pharm. 2007; 33: 909–926. DOI: 10.1080/03639040701498759

20. Shah S, Maddineni S, Lu J, Repka MA. Melt Extrusion with poorly soluble drugs. Int J Pharm. 2013; 453: 233–252. doi.org/10.1016/j.ijpharm.2012.11.001

21. Repka MA., Shah S, Lu J. Melt Extrusion: Process to Product. Expert Opin. Drug Deliv. 2012; 9: 105–125 . doi.org/10.1517/17425247.2012.642365

22. Lakshman D, Chegireddy M, Hanegave GK, Sree KN, Kumar N. Investigation of drug-polymer miscibility, biorelevant dissolution, and bioavailability improvement of Dolutegravir-polyvinyl caprolactam- polyvinyl acetate-polyethylene glycol graft copolymer solid dispersion. Eur J Pharm Sci. 2020; 142: 105137 doi: 10.1016/j.ejps.2019.105137

23. Sharma A, Jain CP. Preparation and characterization of solid dispersions of carvedilol with PVP K30. Res Pharm Sci. 2010; 5(1): 49-56. PMID: 21589768; PMCID: PMC3093098

24. Hamaguchi T, Shinkuma D, Yamanaka Y, Mizuno N. Bioavailability of Mefenamic Acid: Influence of Food and Water Intake. J Pharm Sci. 1986; 75: 891–893. doi: 10.1002/jps.2600750914

25. Romero S, Escalera B, Bustamante P. Solubility Behavior of Polymorphs I and II of Mefenamic Acid in Solvent Mixtures. Int J Pharm. 1999; 178: 193–202. doi: 10.1016/s0378-5173(98)00375-5

26. Greenhalgh D J, Williams A C, Timmins P, York P. Solubility Parameters as Predictors of Miscibility in Solid Dispersions. J Pharm Sci. 1999; 98: 1182–1190. doi.org/10.1021/js9900856

27 Hancock BC, York P, Rowe R C. The Use of Solubility Parameters in Pharmaceutical Dosage Form Design. Int J Pharm. 1997; 148: 1–21. doi.org/10.1016/S0378-5173(96)04828-4

28. Ramachandran E Ramukutty S. Thermal Decomposition Kinetics of Mefenamic Acid Crystals. Int J. Thermodynamics and Chemical Kinetics. 2016; 2: 21 - 24.

29. Cesur S, Gokbel S. Crystallization of Mefenamic Acid and Polymorphs. Cryst Res Technol 2008; 43: 720–728. doi.org/10.1002/crat.200711119.

30. De Buck SS, Sinha VK, Fenu LA, Nijsen MJ, Mackie CE, Gilissen RA. Prediction of Human Pharmacokinetics Using Physiologically Based Modeling: A Retrospective Analysis of 26 Clinically Tested Drugs. Drug Metab Dispos. 2007; 35: 1766- 1780. doi.org/10.1124/dmd.107.015644.

31. Qian F, Huang J,Hussain M A. Polymer Solubility and Miscibility: Stability Consideration

and Practical Challenges in Amorphous Solid Dispersion Development. J Pharm Sci. 2010; 99: 2941–2947. doi: 10.1002/jps.22074.

32. Breitenbach J. Melt Extrusion: From Process to Drug Delivery Technology. Eur J Pharm Biopharm. 2002; 54: 107–17. doi: 10.1016/s0939-6411(02)00061-9

33. Sarode AL, Sandhu H, Shah N, Malick W, Zia H. Hot melt extrusion (HME) for Amorphous Solid Dispersions: Predictive Tools for Processing and Impact of Drug–Polymer Interactions on Supersaturation. Eur J Pharm Sci. 2013; 48: 371–384. doi.org/10.1016/j.ejps.2012.12.012

34. Matsumoto T, Zografi G. Physical Properties of Solid Molecular Dispersions of Indomethacin with Poly(vinylpyrrolidone) and Poly(vinylpyrrolidone-co-vinyl-acetate) in Relation to Indomethacin Crystallization. Pharm Res. 1999; 16: 1722–1728. doi: 10.1023/a:1018906132279.

35. Huang Y, Dai WG. Fundamental Aspects of Solid Dispersion Technology for Poorly Soluble Drugs. Acta Pharm Sin B. 2014; 4: 18–25. doi.org/10.1016/j.apsb.2013.11.001.

Acta Pharm. Sci. Vol 61:(1), 2023
DOI: 10.23893/1307-2080.APS6102

Antibiotic therapy of adult inpatients with community-acquired pneumonia: a retrospective hospital-based study in Ukraine

Oksana HORODNYCHA[1*], Andriy ZIMENKOVSKY[1]

[1] Department of HealthCare Management, Pharmacotherapy and Clinical Pharmacy, Faculty of Pharmacy, Danylo Halytsky Lviv National Medical University, Lviv, Ukraine

ABSTRACT

This study aimed to assess the antibiotic therapy of community-acquired pneumonia (CAP) in adults. A single-center, retrospective study was conducted in one of Lviv city hospitals, Ukraine. Adults with CAP (n=181) were enrolled. Fluoroquinolones (45.3%), cephalosporins (27.8%), and macrolides (16.1%) were the most common antibiotics. Antibiotic-associated drug-related problems (DRPs) were found in 87.3% (95%CI 81.5%:91.8%) of the participants. 4 items of antibiotic-associated DRPs were identified: potential drug-drug interactions (76.6%), inappropriate dosing (14.0%), inappropriate length of therapy (7.5%), and contraindicated usage (1.9%). Spiramycin, metronidazole, levofloxacin, azithromycin, and cefoperazone were associated with the highest risk of DRPs. Age of patients ($p<0.001$), number of antibiotics ($p<0.001$), length of antibiotic therapy ($p=0.036$), and the total number of antibiotic-associated DRPs ($p=0.005$) were defined as factors that statistically contribute to the patient's health status on discharge. Antibiotics should be the drug class most commonly involved in the interventions to improve the safety and quality of CAP therapy.

Keywords: Community-acquired infection, bacterial pneumonia, anti-bacterial agents, inappropriate prescribing, risk factors

INTRODUCTION

Community-acquired pneumonia (CAP) is one of the most common infectious diagnoses and a frequent cause of hospital admissions among adults[1,2]. The estimated worldwide incidence of CAP varies between 1.5 to 14 cases per 1000 per-

*Corresponding author: E-mail: gor.oxana.27@gmail.com
ORCIDs:
Oksana Horodnycha: 0000-0002-6373-1447
Andriy Zimenkovsky: 0000-0002-9649-293X
(Received 19 Apr 2022, Accepted 12 Sep 2022)

son-years[3]. Bacterial pathogens are the most typical causative agents of CAP that require antimicrobial therapy[4]. Consequently, the CAP treatment can bring about numerous antibiotic-related adverse health and economic outcomes such as antimicrobial resistance, allergic reactions, *Clostridium difficile* infections, etc[5-7].

Problems associated with antibiotics treatment have a wide set of factors that can be considered as drug-related problems (DRPs)[8]. DRPs are known to be health and economic issues because they may affect treatment outcomes and incur considerable health costs[9-11]. For instance, in the United States the cost of negative outcomes of DRPs is estimating up to $672 billion US dollars per year[11].

According to the different studies, the incidence of antibiotic-associated DRPs is high[8,9,12,13]. Wrong dosage and frequency regimen, unnecessary treatment, inappropriate drug selection, duplicate therapy, drug interactions, contraindications are the most prevalent antibiotic-associated DRPs in hospitals[9,12-14]. The vast majority of DRPs can be attributed to the prescription of quinolones, macrolides and penicillins[8,9,12,14,15].

Different factors are associated with the occurrence of DRPs in inpatients with CAP[16]. Elder age, female gender, polypharmacy, comorbidity, and duration of hospitalization have been established as the significant determinants of DRPs[8,9,12]. Blix et al[12] developed a novel method with a drug risk ratio calculation for evaluation the risk of antibiotic-associated DRPs. This approach allows determining antibiotics that require heightened awareness and attention if they are going to be prescribed. Improving prescription of antibiotics has a positive influence the patients' health, treatment costs, and patients' quality of life[16].There are limited data in this area in Ukraine. Hence, this study aimed to (1) describe and characterize the prescription of antibiotics for inpatients with CAP, (2) identify the incidence and types of antibiotic-associated DRPs, (3) investigate the risk of occurrence of DRPs for the different antibiotics and (4) determine the factors (especially antibiotics-related) contributing to the patient's health condition on discharge.

METHODOLOGY

Definitions

A drug-related problem (DRP) is defined as "an event or circumstance involving drug therapy that actually or potentially interferes with desired health outcomes"[17]. In addition, DRP is known to be any problem involving drug therapy that can affect desired health outcomes[9]. Drug risk ratio – the number of times the antibiotic was associated with DRPs in relation to the number of times it was used[12]. Potential drug-drug interaction (DDI) is considered to be a concomitant prescription of two interacting drugs, regardless of whether an adverse patient outcome occurs[18].

Study design and data collection

This retrospective study was conducted in one of Lviv city hospitals (Ukraine) that admits inpatients over 18 years. We do not give the name of the hospital owing to the ethical reasons. During the 12 months (from January 2019 to December 2019), 188 adult patients with CAP were hospitalized to the therapeutic department and recruited to the study, but 181 patients were included in the final analysis (Figure 1). The reason for exclusion (n=7) was the absence of antibiotic treatment for inpatients with CAP.

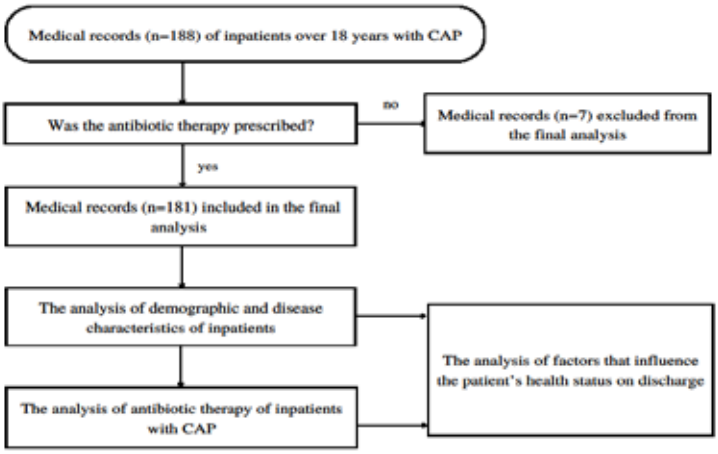


Figure 1. The Flowchart of the Study

Copies of medical records were acquired through an agreement with the hospital administration. Taking into consideration the retrospective design of this study, we did not (1) set a diagnosis, (2) evaluate the patient's health status on admission and discharge, (3) perform laboratory testing. All necessary data about patients (socio-demographic characteristics, medical history, comorbidities, health status on admission and discharge), diagnosis (type of CAP), and treatment (drugs, doses, frequency regimen, and length) was extracted from the medical records.

Antibiotic-related DRPs (inappropriate dosage and length of therapy, contraindications, potential DDIs) were identified by authors according to the Pharmaceutical Care Network Europe (PCNE) DRPs classification v5.01[17]. The appropriateness of antibiotic therapy was assessed by using Ukrainian guidelines for the management of CAP in adults[19], The State Register of Medicinal Products of Ukraine[20], and Medscape[21]. All types of potential DDIs according to the Med-

scape drug interaction checker (minor, significant, serious, and contraindicated)[21] were taken into account in this study.

Statistical analysis

Descriptive statistics were used to describe and summarize demographic and disease characteristics of patients. Qualitative variables were presented as frequencies and percentages. Quantitative variables were given as mean ± standard deviation (SD) and ranges.

The drug risk ratio was calculated for evaluation the risk of antibiotic-associated DRPs for antibiotics that had been prescribed at least 3 times.

Multiple logistic regression analysis was performed to figure out the factors that influence the patient's health status on discharge (patient fully recovered/ patient not fully recovered). Univariable analysis was conducted using simple logistic regression. Variables with a P value ≤0.25 were considered statistically significant and were included in the multiple logistic regression. A preliminary regression model was based on the results of backward and forward Wald methods. Multicollinearity and interaction among variables were checked. Hosmer-Lemeshow test, classification table and the area under the ROC-curve were applied to check the model fitness. Results were presented as adjusted odds ratio (OR), 95% confidence interval (CI) for OR, and P value for the variables included in the final model. A value of $P<0.05$ was considered statistically significant. The statistical analyses were performed with SPSS Trial.

RESULTS AND DISCUSSION

The distributions of demographic and clinical characteristics of the study population are summarized in Table 1.

Table 1. The Baseline Characteristics of the Patients

Characteristics	Frequency (%)	Mean (SD)
Gender		
male	94 (51.9)	
female	87 (48.1)	
Age, years		48.3 (18.1)
18-39	71 (39.2)	
40-64	67 (37.0)	
more than 65	43 (23.8)	
Co-morbidities		
yes	82 (45.3)	
no	99 (54.7)	
Previous drug allergy		
yes	16 (8.8)	
no	165 (91.2)	
Type of CAP		
right-sided	106 (58.6)	
left-sided	48 (26.5)	
bilateral	27 (14.9)	
Health status on admission		
moderate	143 (79.0)	
severe	38 (21.0)	
Number of prescribed medications		9.8 (3.5)
Length of stay at hospital, days		10.3 (2.7)
Health status on discharge		
patient fully recovered	54 (29.8)	
patient not fully recovered	127 (70.2)	

Note. CAP = community-acquired pneumonia; SD = standard deviation

The majority of inpatients were men (51.9%). The mean age was 48.3 ± 18.1 years (range 18-84 years). In 106 (58.6%) of medical records the right-sided CAP was noted, in 48 (26.5%) – left-sided and in 27 (14.9 %) – bilateral. The moderate health status on admission had 79.0% of patients, 21.0% – severe.

The average for a hospital stay was 10.3 ± 2.7 days (range 5-25). Each patient during hospitalization took at least 3 medicines (a mean of 9.8 ± 3.5 medicines per patient, range 3-22).

Antibiotics were prescribed in a total number of 353 items. The mean number of antibiotics received by the patients was 2.0 ± 0.7 ranging from 1 to 5 (Table 2).

Table 2. Characteristics of Antibiotic Therapy

Characteristics	Frequency (%)	Mean (SD)
Number of prescribed antibiotics in medical records, items 1 2 3 4 5	43 (23.7) 114 (63.0) 15 (8.3) 8 (4.4) 1 (0.6)	2.0 (0.7)
Groups of antibiotics Fluoroquinolones Cephalosporins Macrolides Penicillins with a beta-lactamase inhibitor Nitroimidazoles Others*	160 (45.3) 98 (27.8) 57 (16.1) 20 (5.7) 10 (2.8) 8 (2.3)	
The route of administration Injections Both injections and oral	108 (59.7) 73 (40.3)	
Length of antibiotic therapy, days 5-10 more then 10	124 (68.5) 57 (31.5)	10.0 (2.6)

The most frequent antibiotics prescribed were fluoroquinolones, followed by cephalosporins and macrolides, accounting for 45.3%, 27.8%, and 16.1% of prescriptions, respectively. Levofloxacin (n=157; 44.5%), cefepime (n=79; 22.4%) and azithromycin (n=42; 11.9%) were the most common antibiotics in this study.

Most of the patients received antibiotics in injections 5 to 10 days (a mean length of antibiotic therapy 10.0 ± 2.6 days; range 5-25).

Antibiotic therapy of CAP was associated with numerous DRPs, which were found in 158 (87.3%; 95%CI 81.5%:91.8%) medical records out of 181 enrolled. Totally 321 DRPs (on average 1.8 ± 1.4 DRPs, range 0-8 DRPs) of 4 items were identified in this study: (1) potential DDIs (76.6%), (2) inappropriate dosing (14.0%), (3) inappropriate length of antibiotic therapy (7.5%) and (4) contraindicated usage of antibiotics (1.9%) (Table 3).

Table 3. Identified Antibiotic-Associated DRPs (n=321)

Items of DRPs	Frequency	%
Potential DDIs	246	76.6
Dose-related DRPs	45*	14.0*
insufficient daily dose of antibiotics	32	10.0
exceeded daily dose of antibiotics	7	2.2
insufficient dose of solvent	1	0.3
exceeded dose of solvent	5	1.5
Length of antibiotic therapy	24*	7.5*
insufficient	20	6.2
exceeded	4	1.3
Contraindicated usage	6	1.9

The drug risk ratio was high for spiramycin (1.000), metronidazole (1.000), levofloxacin (0.828), azithromycin (0.714) and cefoperazone (0.600). Ceftriaxone, amoxicillin with a beta-lactamase inhibitor and cefepime were associated with a lower risk of DRPs, 0.533, 0.450 and 0.300 respectively.

Univariable analysis was done using simple logistic regression to identify the variables associated with the patient's health status on discharge (patient fully recovered/not fully recovered) (Table 4).

Table 4. Univariable Analysis of Factors Associated with the Patient's Health Status on Discharge

Variables	Crude OR (95%CI)	Wald Statistics (df)	P value[a]
Gender			
male	1.00		
female	0.74 (0.38-1.39)	0.921 (1)	0.337
Age, years	0.97 (0.94-0.98)	14.346 (1)	<0.001
Co-morbidities			
no	1.00		
yes	0.34 (0.17-0.69)	9.169 (1)	0.002
Previous drug allergy			
no	1.00		
yes	1.08 (0.36-3.26)	0.017 (1)	0.897
Type of CAP			
bilateral	1.00		
right-sided	1.67 (0.81-3.40)	1.922 (1)	0.166
left-sided	0.58 (0.20-1.66)	1.044 (1)	0.307
Health status on admission			
severe	1.00		
moderate	1.06 (0.48-2.32)	0.018 (1)	0.893
Length of stay at hospital, days	0.97 (0.85-1.09)	0.383 (1)	0.536
Number of prescribed medications	0.91 (0.82-1.00)	4.078 (1)	0.043
Number of prescribed antibiotics	1.38 (0.90-2.09)	2.120 (1)	0.145
Length of antibiotic therapy, days	0.91 (0.80-1.04)	1.954 (1)	0.162
The route of antibiotic administration			
both injections and oral	1.00		
injections	0.57 (0.30-1.08)	2.960 (1)	0.085
Number of antibiotic-related DRPs	0.79 (0.61-1.02)	3.224 (1)	0.073

Eight variables (age, co-morbidities, type of CAP, number of prescribed medications, number of prescribed antibiotics, length of antibiotic therapy, the route of antibiotic administration, and number of antibiotic-related DRPs) were found to have a P value ≤0.25 and included in the multiple regression model. Age of patients ($p<0.001$), number of antibiotics ($p<0.001$), length of antibiotic therapy ($p=0.036$), and total number of antibiotic-associated DRPs ($p=0.005$) were defined as factors that influence the patient's health status on discharge (Table 5).

Table 5. Factors Associated with the Patient's Health Status on Discharge, Final Model

Variables	Adjusted OR (95% CI)	Wald Statistic (df)	P value[a]
Age	0.96 (0.94-0.98)	13.237 (1)	<0.001
Number of prescribed antibiotics	3.10 (1.65-5.84)	12.333 (1)	<0.001
Length of antibiotic therapy	0.84 (0.71- 0.99)	4.379 (1)	0.036
Number of antibiotic-related DRPs	0.60 (0.42-0.86)	7.900 (1)	0.005

Note. OR = odds ratio; CI = confidence interval; DRPs = drug-related problems. Multicollinearity and interaction term were checked and were not found. Hosmer-Lemeshow test (P=0.686), classification table (70.2% of subjects are correctly classified by the model) and the area under the ROC-curve (74.7%; 95% CI 66.9%-82.5%) were applied to check the model fitness and reported to be fit. [a] – multiple logistic regression (Wald test), significant at P<0.05

This study aimed to characterize the antibiotic treatment for adult inpatients with CAP in Ukraine, describe the incidence and types of antibiotic-associated DRPs, investigate the risk of occurrence of DRPs for the different antibiotics and determine the factors contributing to the patient's health condition on discharge.

CAP is a common problem in adults[1,22,23]. The average age of hospitalized patients with CAP in the USA is 57 years[24], in France – 63 years[25], in Spain – 66 years[26], in Germany – 70 years[27]. Compared with these data, the mean age of inpatients in Ukraine is much less (48.3 years). In addition, we found that the incidence of CAP decreased with increasing age. This result is opposite to data in the other studies[1,2,24,28]. It could be, possibly, related to the high prevalence of self-treatment of respiratory tract infections among working-age adults in Ukraine, underestimating threats associated with ignoring the doctor's consultation, delaying medical care appointments, and, therefore, deterioration requiring hospitalization[29].

In our study, CAP was slightly more common among men (51.9%). This distribution confirms the results of previous studies in France[25], Spain[26], Germany[27], the Netherlands[28]. However, in numerous other studies the slight predominance of women has been established[1,30-32].

In 79.0% of medical records, the patient's health status on admission was noticed as moderate, in 21.0% as severe. Comorbidity had 45.3% of patients. The most common comorbidities were coronary heart disease, hypertension, cardio sclerosis and heart failure. As reported by Torres et al[33], the presence of comorbid conditions, especially chronic respiratory and cardiovascular diseases, increases the risk of CAP significantly. Furthermore, comorbidity and the severity of CAP influence the duration of hospitalization that varies considerably in

different countries (5 to 15 days)[23,25,28,32]. According to the results of this study, the mean length of stay at the hospital was 10.3 days.

The standard treatment of CAP includes antibiotics[19,34,35]. The vast majority of inpatients (63.0%) during the study period received two antibiotics, 13.3% – at least 3. As described in scientific literature, the administration of 3 or more antibiotics, increases the risk of prescribing medication errors[36]. In this study, fluoroquinolones (in particular levofloxacin) were the most common antibiotics (45.3%), followed by cephalosporins (27.8%) and macrolides (16.1%). Similar results have been found in Germany[27] and Denmark[30]. But in contrast to our study, moxifloxacin and ciprofloxacin were the most frequently administrated for inpatients with CAP[27,30]. Prescribing of other antibiotics (mainly nitroimidazoles, penicillins/cephalosporins with beta-lactamase inhibitors) was low. The choice of antibiotics influences the risk of CAP treatment failure with the following switching to another antimicrobial therapy[27]. Ott et al[27] established that moxifloxacin or a combination of β-lactam and macrolide are possible strategies to prevent the treatment failure.

The proper duration of antibiotic therapy is a serious controversial issue in the management of CAP[37-39]. The majority of guidelines do not have specific recommendations regarding the proper length of antibiotic treatment of inpatients with CAP[40]. It is generally accepted that the traditional antibiotic regimens are 7 to 14 days long[39]. Our findings are consistent with it because the mean length of antibiotic therapy was 10.0 days. However, in different countries, the duration of antibiotic treatment of CAP differs significantly[32,37]. The longer duration is associated with numerous antibiotics-related adverse health and economic outcomes such as antimicrobial resistance, allergic reactions, *Clostridium difficile* infections, etc[5,6,39,41]. Therefore, the efficacy of short versus traditional antibiotic courses has been investigated in recent studies. According to some of them, there is no difference in efficacy between antibiotic therapy of CAP throughout 3-5 days and 7-14 days, respectively[38,39,42]. Moreover, some guidelines have been updated and now suggest a 5-day course of antibiotic treatment[35].

For 59.7% of inpatients, antibiotics were prescribed in injections, for 40.3% in both injections and oral forms. Overall, the choice of the route of antibiotic administration depends on the severity of CAP, patient's health condition and risk factors for methicillin-resistant *Staphylococcus aureus* and *Pseudomonas* infections[35]. Sæterdal et al[43] found that there is little or no difference between the effectiveness of oral and intravenous antibiotic treatment in patients with CAP.

A lot of studies have described the inappropriateness of antibiotic prescription

so far[9,10,12,36]. The presence of antibiotic-related DRPs is associated with (1) the patient's duration of hospital stay, (2) polypharmacy, and (3) comorbidity[9]. In our study, antibiotic-related DRPs were found in 87.3% of medical records. This result is much higher compared to other studies (up to 72%)[9,12,14,15].

According to the scientific literature, beta-lactamase-resistant penicillins, quinolones, and macrolides provoke the highest risk of antibiotic-related DRPs[12]. Our results agree with these findings up to a point because the highest levels of drug risk ratio were defined for spiramycin, metronidazole, levofloxacin, azithromycin, and cefoperazone. Consequently, the abovementioned antibiotics are at the biggest risk of occurrence of DRPs. Ceftriaxone, amoxicillin with a beta-lactamase inhibitor, and cefepime were associated with a lower risk of DRPs.

In the present study, the most frequent antibiotic-associated DRPs were potential DDIs (76.6%). This is consistent with previous studies where DDIs were highly prevalent in patients with CAP[44]. DDIs can lead to (1) different adverse drug reactions, (2) decreasing clinical effectiveness, (3) toxicity etc[44]. For instance, we identified 7 contraindicated potential DDIs between ceftriaxone and calcium chloride/gluconate. This combination is associated with the risk of potentially fatal particulate precipitation in the lungs and kidneys[20,21].

Antibiotic dose-related DRPs were frequent (14.0%). This subset of DRPs involved both the wrong dosage of antibiotics and the wrong dosage of solvents. Amoxicillin with clavulanic acid, metronidazole and cefepime were the most frequently associated with DRPs «Insufficient daily dose of antibiotics». For example, amoxicillin/clavulanic acid 1000 mg/200 mg for injection/infusion should be used every 8 hours[20]. But it was prescribed every 12 or 24 hours. Metronidazole was administrated only once a day instead of 3 times per day[20]. The injection regimen of cefepime is every 8 to 12 hours[20]. However, our patients received it every 24 hours.

DRPs of item «Exceeded daily dosage of antibiotics» were less common and entirely related to azithromycin. According to the recommendations, the dose regimen of azithromycin for CAP treatment is 500 mg orally as a single dose on day 1, followed by 250 mg orally once a day on days 2 to 5[21]. Nevertheless, inpatients took 500 mg of azithromycin throughout therapy.

In addition, 6 DRPs regarding the wrong dosage of solvents were identified. The volume of sodium chloride was at least twice much or less as necessary. This subset of DRPs is rather significant because the inappropriate amount of solvent influences the rate of drug administration and, therefore, effectiveness and safety of therapy[45].

Although the duration of antibiotic therapy has recently become a controversial question, we identified 24 DRPs related to this issue. The necessary length of CAP pharmacotherapy should be at least 7 days for levofloxacin, cefepime and metronidazole, 6 days – for clarithromycin, 5 days – for amoxicillin and azithromycin[20]. However, the duration of treatment was insufficient in 20 medical records. At the same time, in 4 medical records, the length of antibiotic treatment was exceeded. All DRPs of this subset were associated with azithromycin prescription 7 to 11 days.

In this study, all cases of contraindicated usage of antibiotics have its origins from the allergy. Almost all DRPs of this item were related to cross allergy between cephalosporins and penicillins. Additionally, we identified one DRP of contraindicated levofloxacin prescribing due to the ofloxacin allergy.

The next step of this study was to identify the factors contributing to the patient's health condition on discharge. It is known that the awareness of the risk factors can improve the therapy and clinical outcomes[46,47]. Despite publishing numerous studies in scientific literature dedicated to determinants of DRPs[9,13], predictors of CAP antibiotic treatment failure, early deterioration or so[46-50], the information about factors that influence the patient's health condition on discharge is limited. According to our results, these factors include the age of patients ($p<0.001$), number of prescribed antibiotics ($p<0.001$), length of antibiotic therapy ($p=0.036$), and the total number of antibiotic-associated DRPs ($p=0.005$). Thus, interventions to reduce antibiotic-related factors may have a significant positive impact on CAP therapy.

Limitations

Limitations of this study include its retrospective design. Identifying of DPRs was based only on the information available from the medical records. Therefore, we were able to check potential DDIs, contraindications, appropriateness of doses, frequency and length of antibiotic therapy. We did not monitor the patient's condition throughout the therapy. Hence, DDIs could be considered only as potential.

Only 4 subsets of DRPs were included in the regression analysis. Adverse drug reactions, treatment failure, long-term clinical outcomes were not taken into consideration because the medical records did not include such kind of information or it was missed. Future studies are needed to explore the impact of other types of DRPs (not only antibiotic-associated) on a patient's health condition on discharge. Moreover, other factors that influence a patient's health condition on discharge cannot be excluded.

In this study, we had only 2 options of patient's health status on discharge: (1) patient fully recovered, (2) patient not fully recovered and was discharged under the care of primary care physicians. Further investigations are required to define factors contributing to the other options of patient's health status on discharge (died, discharged with deterioration, etc.).

In addition, the analysis of medical records only from one hospital limits the generalizability of our results.

In conclusion, CAP is a common problem in adults that requires antibiotic therapy. The prevalence of antibiotic-related DRPs in hospitalized patients with CAP was found to be high. Potential DDIs were defined as the most common antibiotic-associated DRPs. Spiramycin, metronidazole, levofloxacin, azithromycin and cefoperazone were identified as antibiotics with the highest drug risk ratio level and the biggest risk of occurrence of DRPs. Age of patients, number of prescribed antibiotics, length of antibiotic therapy, and the total number of antibiotic-associated DRPs were defined as the factors that significantly contribute to the patient's health status on discharge. Thus, antibiotics should be the drug class most commonly involved in the interventions to improve the safety and quality of CAP therapy.

STATEMENT OF ETHICS

The study received ethical approval from the Human Research Ethics Committee of Danylo Halytsky Lviv National Medical University (Protocol No.10 of 16.12.2019).

Due to the retrospective design of this study, we did not have written patient's consent for being included in the study. An agreement with the hospital administration was obtained.

CONFLICT OF INTEREST

Nothing to declare.

AUTHOR CONTRIBUTIONS

Concept – Oksana Horodnycha, Andriy Zimenkovsky (authors contributed equally); Design – Oksana Horodnycha, Andriy Zimenkovsky (authors contributed equally); Data Collection and Processing – Oksana Horodnycha; Statistical Analysis and Interpretation – Oksana Horodnycha; Literature Search – Oksana Horodnycha; Drafting of the manuscript – Oksana Horodnycha; Critical revision of the manuscript – Andriy Zimenkovsky.

FUNDING SOURCES

The authors received no financial support for the research, authorship and/or publication of this article.

ACKNOWLEDGEMENTS

Nothing to declare.

REFERENCES

1. Broulette J, Yu H, Pyenson B, Iwasaki K, Sato R. The incidence rate and economic burden of community-acquired pneumonia in a working-age population. Am Health Drug Benefits. 2013. Sep;6(8):494-503.

2. Kleinman NL, Yu H, Beren IA, Sato R. Work-related and health care cost burden of community-acquired pneumonia in an employed population. J Occup Environ Med. 2013 Oct;55(10):1149-56. doi: 10.1097/JOM.0b013e3182a7e6af

3. Regunath H, Oba Y. Community-Acquired Pneumonia [Internet]. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2021, https://www.ncbi.nlm.nih.gov/books/NBK430749/.

4. Pakhale S, Mulpuru S, Verheij TJ, Kochen MM, Rohde GG, Bjerre LM. Antibiotics for community-acquired pneumonia in adult outpatients. Cochrane Database Syst Rev. 2014 Oct 9;2014(10):CD002109. doi: 10.1002/14651858.CD002109.pub4

5. Davey P, Marwick CA, Scott CL, Charani E, McNeil K, Brown E, et al. Interventions to improve antibiotic prescribing practices for hospital inpatients. Cochrane Database Syst Rev. 2017 Feb 9;2(2):CD003543. doi: 10.1002/14651858.CD003543.pub4

6. Geller AI, Lovegrove MC, Shehab N, Hicks LA, Sapiano MRP, Budnitz DS. National estimates of emergency department visits for antibiotic adverse events among adults - United States, 2011-2015. J Gen Intern Med. 2018 Jul;33(7):1060-1068. doi: 10.1007/s11606-018-4430-x

7. Matsuura GT, Garrison MW. Antibiotic Collateral Damage: Resistance and Antibiotic-Associated Diarrhea. Hosp Pharm. 2011 Oct 1;46(10):758-768. doi: 10.1310/hpj4610-758

8. Nivya K, Sri Sai Kiran V, Ragoo N, Jayaprakash B, Sonal Sekhar M. Systemic review on drug related hospital admissions – a Pubmed based search. Saudi Pharm J. 2015 Jan;23(1):1-8. doi: 10.1016/j.jsps.2013.05.006

9. Bekele F, Fekadu G, Bekele K, Dugassa D, Sori J. Drug-related problems among patients with infectious disease admitted to medical wards of Wollega University Referral Hospital: Prospective observational study. SAGE Open Med. 2021 Jan 22;9:2050312121989625. doi: 10.1177/2050312121989625

10. Sekh M, Zimenkovsky A. Community-acquired pneumonia: quality of pharmacotherapy and expense of irrationality. Pharmacia. 2018;65(4):25-37. http://bsphs.org/wp-content/uploads/2019/03/Sekh.pdf.

11. Watanabe JH, McInnis T, Hirsch JD. Cost of Prescription Drug-Related Morbidity and Mortality. Ann Pharmacother. 2018 Sep;52(9):829-837. doi: 10.1177/1060028018765159

12. Blix HS, Viktil KK, Moger TA, Reikvam A. Risk of drug-related problems for various antibiotics in hospital: assessment by use of a novel method. Pharmacoepidemiol Drug Saf. 2008 Aug;17(8):834-41. doi: 10.1002/pds.1595

13. Mechessa DF, Dessalegn D, Melaku T. Drug-related problem and its predictors among pediatric patients with infectious diseases admitted to Jimma University Medical Center, Southwest Ethiopia: Prospective observational study. SAGE Open Med. 2020 Nov 12;8:2050312120970734. doi: 10.1177/2050312120970734

14. Kurniawati F, Munif Yasin N, Aulia F, Vinanda Krisha G. Drug-related problems of antibiotic use in gastroenteritis related to patient therapy outcomes at Universitas Gadjah Mada Hospital. J Basic Clin Physiol Pharmacol. 2021 Jun 25;32(4):761-766. doi: 10.1515/jbcpp-2020-0451

15. Tefera GM, Feyisa BB, Kebede TM. Antimicrobial use-related problems and their costs in surgery ward of Jimma University Medical Center: Prospective observational study. PLoS One. 2019 May 17;14(5):e0216770. doi: 10.1371/journal.pone.0216770

16. Bhagavathula SA, Meknonnen BG, Birarra KM, Tekle TM. Assessment of Drug Related Problems and its Associated Factors among Medical Ward Patients in University of Gondar Teaching Hospital, Northwest Ethiopia: A Prospective Cross-Sectional Study. J Basic Clin Pharma. 2017;8:S016-S021.

17. PCNE classification for drug related problems V5.01. Pharmaceutical Care Network Europe, https://studylib.net/doc/18191674/pcne-classification-scheme-for-drug-related-problems, Access date: 1.03.2021

18. Jazbar J, Locatelli I, Horvat N, Kos M. Clinically relevant potential drug-drug interactions among outpatients: A nationwide database study. Res Social Adm Pharm. 2018 Jun;14(6):572-580. doi: 10.1016/j.sapharm.2017.07.004

19. Unified clinical protocol of medical care for patients with community-acquired pneumonia, 2016. Adapted clinical evidence-based guidelines, http://www.ifp.kiev.ua/doc/staff/pneumonia_guidelines_2016.pdf, Access date: 1.09.2021

20. The State Register of Medicinal Products of Ukraine, http://www.drlz.com.ua/ibp/ddsite.nsf/all/shlist?opendocument, Access date: 21.06.2021

21. Medscape, https://reference.medscape.com/, Access date: 29.08.2021

22. Bonafede MM, Suaya JA, Wilson KL, Mannino DM, Polsky D. Incidence and cost of CAP in a large working-age population. Am J Manag Care. 2012 Jul;18(7):380-7.

23. File TM Jr, Marrie TJ. Burden of community-acquired pneumonia in North American adults. Postgrad Med. 2010 Mar;122(2):130-41. doi: 10.3810/pgm.2010.03.2130

24. Jain S, Self WH, Wunderink RG, Fakhran S, Balk R, Bramley AM, et al. Community-acquired pneumonia requiring hospitalization among U.S. adults. N Engl J Med. 2015 Jul 30;373(5):415-27. doi: 10.1056/NEJMoa1500245

25. Saba G, Andrade LF, Gaillat J, Bonnin P, Chidiac C, Illes HG, et al. Costs associated with community acquired pneumonia in France. Eur J Health Econ. 2018 May;19(4):533-544. doi: 10.1007/s10198-017-0900-z

26. Uranga A, España PP, Bilbao A, Quintana JM, Arriaga I, Intxausti M, et al. Duration of antibiotic treatment in community-acquired pneumonia: a multicenter randomized clinical trial. JAMA Intern Med. 2016 Sep 1;176(9):1257-65. doi: 10.1001/jamainternmed.2016.3633

27. Ott SR, Hauptmeier BM, Ernen C, Lepper PM, Nüesch E, Pletz MW, et al. Treatment failure in pneumonia: impact of antibiotic treatment and cost analysis. Eur Respir J. 2012 Mar;39(3):611-8. doi: 10.1183/09031936.00098411

28. Vissink CE, Huijts SM, de Wit GA, Bonten MJ, Mangen MJ. Hospitalization costs for community-acquired pneumonia in Dutch elderly: an observational study. BMC Infect Dis. 2016 Sep 2;16(1):466. doi: 10.1186/s12879-016-1783-9

29. Horodnycha OY, Makukh KI, Zimenkovsky AB. The study of permanent problems of pharmacist's protocol realization concerning wet cough. Farm Zh. 2021 Feb 17;1:80-90. doi: 10.32352/0367-3057.1.21.09

30. Aabenhus R, Hansen MP, Saust LT, Bjerrum L. Characterization of antibiotic prescriptions for acute respiratory tract infections in Danish general practice: a retrospective registry based cohort study. NPJ Prim Care Respir Med. 2017 May 19;27(1):37. doi: 10.1038/s41533-017-0037-7

31. Lopardo GD, Fridman D, Raimondo E, Albornoz H, Lopardo A, Bagnulo H, et al. Incidence rate of community-acquired pneumonia in adults: a population-based prospective active surveillance study in three cities in South America. BMJ Open. 2018 Apr 10;8(4):e019439. doi: 10.1136/bmjopen-2017-019439

32. Welker JA, Huston M, McCue JD. Antibiotic timing and errors in diagnosing pneumonia. Arch Intern Med. 2008 Feb 25;168(4):351-6. doi: 10.1001/archinternmed.2007.84

33. Torres A, Peetermans WE, Viegi G, Blasi F. Risk factors for community-acquired pneumonia in adults in Europe: a literature review. Thorax. 2013 Nov;68(11):1057-65. doi: 10.1136/thoraxjnl-2013-204282

34. Metlay JP, Waterer GW, Long AC, Anzueto A, Brozek J, Crothers K, et al. Diagnosis and Treatment of Adults with Community-acquired Pneumonia. An Official Clinical Practice Guideline of the American Thoracic Society and Infectious Diseases Society of America. Am J Respir Crit Care Med. 2019 Oct 1;200(7):e45-e67. doi: 10.1164/rccm.201908-1581ST

35. Pneumonia (community-acquired): antimicrobial prescribing. NICE guideline [NG138], 2019, https://www.nice.org.uk/guidance/ng138, Access date: 20.09.2021

36. Iftikhar S, Sarwar MR, Saqib A, Sarfraz M, Shoaib QU. Antibiotic prescribing practices and errors among hospitalized pediatric patients suffering from acute respiratory tract infections: a multicenter, cross-sectional study in Pakistan. Medicina (Kaunas). 2019 Feb 11;55(2):44. doi: 10.3390/medicina55020044

37. Aliberti S, Blasi F, Zanaboni AM, Peyrani P, Tarsia P, Gaito S, et al. Duration of antibiotic therapy in hospitalised patients with community-acquired pneumonia. Eur Respir J. 2010 Jul;36(1):128-34. doi: 10.1183/09031936.00130909

38. Palin V, Welfare W, Ashcroft DM, van Staa TP. Shorter and longer courses of antibiotics for common infections and the association with reductions of infection-related complications including hospital admissions. Clin Infect Dis. 2021 Nov 16;73(10):1805-1812. doi: 10.1093/cid/ciab159

39. Spellberg B, Rice LB. Duration of Antibiotic Therapy: Shorter Is Better. Ann Intern Med. 2019 Aug 6;171(3):210-211. doi: 10.7326/M19-1509

40. Mantero M, Tarsia P, Gramegna A, Henchi S, Vanoni N, Di Pasquale M. Antibiotic therapy, supportive treatment and management of immunomodulation-inflammation response in community acquired pneumonia: review of recommendations. Multidiscip Respir Med. 2017 Oct 5;12:26. doi: 10.1186/s40248-017-0106-3

41. Uranga A, Artaraz A, Bilbao A, Quintana JM, Arriaga I, Intxausti M, et al. Impact of reducing the duration of antibiotic treatment on the long-term prognosis of community acquired pneumonia. BMC Pulm Med. 2020;20(1):261. doi: 10.1186/s12890-020-01293-6

42. Wald-Dickler N, Spellberg B. Short-course antibiotic therapy-replacing constantine units with "shorter is better". Clin Infect Dis. 2019 Oct 15;69(9):1476-1479. doi: 10.1093/cid/ciy1134

43. Sæterdal I, Akselsen PE, Berild D, Harboe I, Odgaard-Jensen J, Reinertsen E, et al. Antibiotic Therapy in Hospital, Oral Versus Intravenous Treatment. Oslo, Norway: Knowledge Centre for the Health Services at The Norwegian Institute of Public Health (NIPH); 2010. Report from Norwegian Knowledge Centre for the Health Services (NOKC) No.02-2010.

44. Noor S, Ismail M, Ali Z. Potential drug-drug interactions among pneumonia patients: do these matter in clinical perspectives? BMC Pharmacol Toxicol. 2019 Jul 26;20(1):45. doi: 10.1186/s40360-019-0325-7

45. Savjani KT, Gajjar AK, Savjani JK. Drug solubility: importance and enhancement tech-

niques. ISRN Pharm. 2012 Jul 5;2012:195727. doi: 10.5402/2012/195727

46. Muro RP, Masoza TS, Kasanga G, Kayange N, Kidenya BR. Predictors and outcome of first line treatment failure among under-five children with community acquired severe pneumonia at Bugando Medical Centre, Mwanza, Tanzania: A prospective cohort study. PLoS One. 2020 Dec 11;15(12):e0243636. doi: 10.1371/journal.pone.0243636

47. Kolditz M, Ewig S, Klapdor B, Schütte H, Winning J, Rupp J, et al. Community-acquired pneumonia as medical emergency: predictors of early deterioration. Thorax. 2015 Jun;70(6):551-8. doi: 10.1136/thoraxjnl-2014-206744

48. Ito A, Ishida T, Tokumasu H, Washio Y, Yamazaki A, Ito Y, et al. Prognostic factors in hospitalized community-acquired pneumonia: a retrospective study of a prospective observational cohort. BMC Pulm Med. 2017 May 2;17(1):78. doi: 10.1186/s12890-017-0424-4

49. Kothe H, Bauer T, Marre R, Suttorp N, Welte T, Dalhoff K. Competence Network for Community-Acquired Pneumonia study group. Outcome of community-acquired pneumonia: influence of age, residence status and antimicrobial treatment. Eur Respir J. 2008 Jul;32(1):139-46. doi: 10.1183/09031936.00092507

50. Li M, Liu J, Tan R, Liu Z, Yin J, Qu H. Risk factors for slowly resolving pneumonia in the intensive care unit. J Microbiol Immunol Infect. 2016 Oct;49(5):654-662. doi: 10.1016/j.jmii.2014.11.005

Acta Pharm. Sci. Vol 61:(1), 2023
DOI: 10.23893/1307-2080.APS6103

Antioxidant and antibacterial activities of ethanolic extract of sintok lancang *(Cinnamomum javanicum* Blume*)* from Central Kalimantan

Susi NOVARYATIIN[1*], Syahrida D ARDHANY[1], Muhammad T RAHMAN[1], Muhammad D MUTTAWALI[1], Hikmah HIKMAH[1], Elin TRI[1], Fahruddin ARFIANTO[2], Nanang HANAFI[3]

[1] Department of Pharmacy, Faculty of Health Sciences, Muhammadiyah University of Palangkaraya, Palangka Raya,Indonesia

[2] Department of Agrotechnology, Faculty of Agriculture and Forestry, Muhammadiyah University of Palangkaraya, Palangka Raya, Indonesia

[3] Department of Forestry, Faculty of Agriculture and Forestry, Muhammadiyah University of Palangkaraya, Palangka Raya, Indonesia

ABSTRACT

The *C. javanicum* Blume is a typical plant that can be founded in Central Kalimantan. This research aimed to find out the total flavonoids, alkaloids, tannins, and compounds and to evaluate the antioxidant and antibacterial activity of ethanol extract of the stem of *C. javanicum*. The antioxidant activity was tested by two different assays: DPPH and FRAP. Total alkaloids, flavonoids and tannins content were 32.81±0.77 µg caffeine equivalent/mg, 126.96±3.17 µg quercetin equivalent/mg, and 42.89±0.77 µg catechin equivalent/mg, respectively. The IC_{50} value of DPPH was 20.63±0.82 ppm quercetin equivalent while the FRAP method was 968.38±22.25 µmol trolox/g. *C. javanicum* extract had antibacterial potential against the three bacteria tested, with the inhibition zones in the range of 3.17±0.90 - 8.90±1.50 mm. It can be stated that the ethanol extract of the stem of *C. javanicum* has high potential antioxidant activity, however, the antibacterial activity is classified as weak activity.

*Corresponding Author: E-mail: susi_novaryatiin@yahoo.com
ORCIDs:
Susi Novaryatiin: 0000-0003-0696-6546
Syahrida D Ardhany: 0000-0002-8606-8991
Muhammad T Rahman: 0000-0002-0293-0469
Muhammad D Muttawali: 0000-0002-0885-2959
Hikmah Hikmah: 0000-0002-5221-0419
Elin Tri: 0000-0002-1817-2279
Fahruddin Arfianto: 0000-0002-1405-5403
Nanang Hanafi: 0000-0003-2933-8788
(Received 30 May 2022, Accepted 26 Oct 2022)

Keywords: *Cinnamomum javanicum* Blume, DPPH, FRAP, disc-diffusion, medicinal plant

INTRODUCTION

Free radicals are known for their central role in various physiological conditions as well as their involvement in a variety of diseases. The accumulation of free radicals in the body produces oxidative stress, which has been reported to be associated with various diseases such as neurodegenerative disorders, diabetes mellitus, respiratory diseases, cardiovascular diseases, coronary heart diseases, rheumatoid arthritis, the development of cataracts, inflammatory diseases, autoimmune disorders, aging, and various cancers[1-3]. Antioxidants play an important role in neutralizing free radicals[3].

In addition, the resistance of bacterial species to many antibiotics is another major problem in antibiotic therapy that continuously encourages researchers to develop new antibiotics. Recently, most of the newly approved antibacterial agents are derived from natural products or their derivatives. Herbal remedies are one of the most important sources of natural antibacterial agents[1]. The antimicrobial activity was mainly related to the existence of secondary metabolites of phenols, terpenes, and alkaloids present in the plant extracts[2]. Therefore, there is a need to explore new, safe and inexpensive sources of natural antioxidant and antibacterial compounds.

Cinnamomum (Family Lauraceae) is a genus known for its fragrant leaves and bark. This genus has more than 300 species that are widespread in North America, Asia, Australasia, and Southeast Asia. Commercial species sold as spices are *C. verum*, *C. cassia*, *C. burmannii*, *C. zeylanicum*, and *C. loureiroi*[4].

Sintok lancang (*Cinnamomum javanicum* Blume) is a typical plant of Central Kalimantan, in this study found at Mungku Baru Forest (KHDTK), an educational forest managed by the collaboration of Muhammadiyah University of Palangkaraya with the Borneo Nature Foundation (BNF). Empirically, Sintok lancang was used for various diseases such as treating abdominal pain, wounds, and diabetes mellitus.

There has not been much research on *C. javanicum* from Indonesia regarding its potential use as an antioxidant and antibacterial agent. One of the studies done on *C. javanicum* from Malaysia showed that the leaf and bark extracts had antioxidant activity[5]. The antibacterial activity of the essential oil of *C. javanicum* found in Borneo was investigated using the broth dilution method, which showed that the inhibition against *S. aureus* with MIC and MBC values

was 250 μg/mL and 500 μg/mL, respectively[4]. Previous studies reported that the ethanolic extract of *C. javanicum* leaves from Central Kalimantan had a high potential for antioxidant activity as tested by DPPH and FRAP methods[6]. However, no studies on the antioxidant activity of these ethanolic extracts of *C. javanicum* stem from Central Kalimantan have been conducted. This study was advanced from the previous study, with the aim being to determine the total number of flavonoid, alkaloid, and tannin compounds and to evaluate the antioxidant and antibacterial activity of the ethanolic extract of stems of *C. javanicum*. This study will help to explore the potential of Indonesian medicinal plants as natural antioxidants and antibacterial agents.

METHODOLOGY

Plant material

C. javanicum was collected from Mungku Baru Forest (KHDTK), an educational forest managed by the cooperation of the Muhammadiyah University of Palangkaraya with the Borneo Nature Foundation (BNF). The collected plant material was identified by Dr. R. Hendrian, MSc (Indonesian Institute of Sciences, Research Center for Plant Conservation and Botanic Gardens, Bogor, Indonesia) with the document number B-833/IPH.3./KS/VII/2020.

Preparation of *Cinnamomum javanicum* extract

The stems of *C. javanicum* were dried in an oven at 45 °C. The dried stems were powdered by the grinder and then percolated using 96% ethanol at room temperature. Percolation is a continuous process in which the saturated solvent is continuously replaced by a new solvent[7]. The solvent is removed from the extract by using a rotary evaporator. Percentage yield (w/w) of the extracts is calculated by formula[8]: Weight of the extract ÷ Weight of the starting plant material) x 100%

Phytochemical qualitative screening

The ethanolic extract of *C. javanicum* was preliminarily screened for phytochemicals such as alkaloids, flavonoids, saponins, tannins, and steroids[9-12].

Total alkaloids content

Ten mg of *C. javanicum* extract were weighed and then dissolved in 10 mL of ethanol. The absorbance of one mL of the extract was measured with a spectrophotometer at 272 nm. The standard used for the calibration curve was caffeine[13]. Total alkaloid content is expressed as μg alkaloids per mg of extract.

Total flavonoids content

Five mg of *C. javanicum* extract were weighed and then dissolved in 10 mL of ethanol. One mL of the extract was placed in the volumetric flask, and one mL of $AlCl_3$ (2% b/v) and 8 mL of acetic acid (5% v/v) were added. After mixing, the solution was incubated for 20 min[14]. The solution absorbance was measured with a spectrophotometer at 412 nm. The standard used for the calibration curve was quercetin[15]. Total flavonoid content is expressed as µg of flavonoids per mg of extract.

Total tannins content

A total of 30 mg of sample was weighed and placed in a 10 mL volumetric flask. Add to the 0.5 mL catechin standard solution, 3.0 mL vanillin 4% and 1.5 mL concentrated HCl. The mixture was incubated for 10 min[15-16]. Absorbance was measured with a UV Vis spectrophotometer at 498 nm. The standard used for the calibration curve was catechin[17]. Total tannin content is expressed as µg tannins per mg extract.

Antioxidant activity by DPPH assay

A 0.4 mM DPPH solution was prepared and the absorbance was measured at 512 nm. The resulting absorbance of the DPPH solution is the absorbance control. The stem extract of *C. javanicum* (sample) was first dissolved in methanol with five variant concentrations of 10, 20, 30, 40, 50 ppm. One mL of 0.4 mM DPPH solution was added in a 5 mL volumetric flask, and then 4 mL of sample solutions of different concentrations were added. The prepared mixture was placed at 25 °C for 30 min, the absorbance was measured at 512 nm[18]. The calculation of the percent inhibition or DPPH scavenging effect was used according to the following formula:

$$DPPH\ Scavenging\ effect\ \% = \frac{(A-B)}{A} X\ 100\%$$

Where A was the absorbance of DPPH solution and B was the absorbance of sample solution[19-20].

Antioxidant activity by FRAP assay

Sample (0.2 g) dissolved with ethanol in a 10 mL volumetric flask. Two mL of sample solution were added to 3 mL of FRAP reagent in a test tube, followed by incubation for 16 minutes. Absorbance was measured with a UV Vis spectrophotometer at 595 nm. Antioxidant activity expressed in µmol trolox/g[19-21].

Antibacterial activity test

The antibacterial activity was determined by disc diffusion method with four variant concentrations 1%, 5%, 10%, and 15% against three bacterial strains: *Cutibacterium acnes/C. acnes* (ATCC 11827), *Staphylococcus epidermidis/S. epidermidis* (ATCC 12228) and *Staphylococcus aureus/S. aureus* (ATCC 25923). Ten mL of McFarland 0.5 standard were prepared in sterilized tubes. The suspension of bacteria was prepared by diluting the colonies of bacteria in a normally sterile saline solution and the turbidity was adjusted to 1-2x10^8 CFU/mL (based on McFarland 0.5 standard). A sterile cotton swab was dipped in a standardized suspension of bacteria and used to inoculate on Mueller-Hinton agar plates[22]. All discs were immersed in the ethanolic extract of *C. javanicum* and then placed on the plates. A disc immersed in 1% clindamycin gel (positive control) was also placed on the plate. These plates were incubated in the aerobic incubator for 24 h at 37 °C. A caliper was used to measure the diameter of the inhibition zone of each extract and positive controls.

RESULTS AND DISCUSSION

Extraction yield

One thousand two hundred grams of *C. javanicum* were extracted into 60.29 g of extract. Based on yield calculation, *C. javanicum* extraction yielded 5.03%. The yield value relates to the number of secondary metabolites that were captured during extraction[8].

Qualitative phytochemical screening

Phytochemical screening of *C. javanicum* stem by using the following standard methods[9-10]. The results of the phytochemical qualitative test of *C. javanicum* stem showed the presence of alkaloids using Mayer's reagents[11], flavonoids using the Shinoda test[12], tannins, saponins, and steroids (Table 1).

Table 1. The qualitative phytochemical of ethanolic extract of *C. javanicum* stem

Phytochemical compound	Result
Alkaloids	+
Flavonoids	+
Tannins	+
Saponins	+
Steroids	+

Total alkaloids, flavonoids, and tannins content

C. javanicum stem was extracted by percolation method. Total alkaloids, flavonoids, and tannins content were calculated by standard protocols, with the results of 32.81 ± 0.77 µg caffeine equivalent/mg, 126.96 ± 3.17 µg quercetin equivalent/mg, and 42.89 ± 0.77 µg catechin equivalent/mg, respectively (Table 2). The total flavonoid content is the largest compared to the total alkaloids and tannins content. In addition, the total alkaloids, flavonoids, and tannins of *C. javanicum* stem ethanol extract in this study were greater than the total content of the ethanolic extract of the leaves of *C. javanicum* obtained in the previous studies[6].

Phenolics and flavonoids are commonly found in all parts of the plant. This compound is a group of secondary metabolites made up of a large group of polyphenols that can scavenge free radicals and inhibit lipid oxidation[23]. Most antioxidant activities from plant sources correlate with phenolic and flavonoid contents[24]. Therefore, the measurement of phenol and flavonoid content can be used as a basis for rapid screening of antioxidant activities[1].

Table 2. Total alkaloids, flavonoids, and tannins content of ethanolic extract of *C. javanicum* stem

Assay \ Sample	Ethanolic extract of *C. javanicum*
Total Alkaloid (µg caffeine equivalent/mg)	32.81 ± 0.77
Total Flavonoid (µg quercetin equivalent/mg)	126.96 ± 3.17
Total Tannin (µg catechin equivalent/mg)	42.89 ± 0.77

Antioxidant activity

DPPH and FRAP methods were used to evaluate the antioxidant activity of *C. javanicum* extract. The antioxidant activity results showed that the IC_{50} value of DPPH was 20.63 ± 0.82 ppm quercetin equivalent while the FRAP method was 968.38 ± 22.25 µmol trolox/g (Table 3).

Table 3. Antioxidant activity of ethanolic extract of *C. javanicum* stem

Assay \ Sample	Ethanolic extract of *C. javanicum*	Quercetin
DPPH (IC_{50} ppm)	20.63 ± 0.82	6.98
FRAP (µmol trolox/g)	968.38 ± 22.25	-

According to some publications, the antioxidant activity with the DPPH method was classified by IC_{50} as very strong (< 50 ppm), strong (50-100 ppm), moderate (101-150 ppm), and low (> 150 ppm)[19,25], while the antioxidant activity with the FRAP method was classified as very low FRAP (< 10 µmol/g), low FRAP (10-50 µmol/g), good FRAP (50-100 µmol/g), high FRAP (100-400 µmol/g) and very high FRAP (> 400 µmol/g)[26]. A low IC50 value (the extract concentration, required to scavenge 50% of DPPH free radicals) means strong antioxidant activity[27]. The ethanolic extract of *C. javanicum* stem is included in the very strong antioxidant activity (20.63 ± 0.82 ppm) and very high FRAP (968.38 ± 22.25 µmol trolox/g). The antioxidant activity in this study was better than in the previous study, where the ethanolic extract of *C. javanicum* leaves included very strong antioxidant activity (26.99 ± 0.27 ppm) and very high FRAP (779.73 ± 19.66 µmol trolox/g)[6]. When compared with the antioxidant activity of *C. javanicum* leaves and bark study conducted in Malaysia with the DPPH method (223.5 ppm and 197.4 ppm)[5], *C. javanicum* in Indonesia, especially Central Kalimantan gives better antioxidant activity.

DPPH is a stable free radical with unpaired electrons distributed throughout the molecule and is widely used to test the free radical scavenging capacity of a variety of samples[28]. The DPPH assay is based on both electron transfer and hydrogen atom transfer reactions. The reduction in absorbance of DPPH caused by antioxidants is due to a reaction between the antioxidant molecules and the radical, which results in radical scavenging by hydrogen donation. This is visualized as purple-to-yellow discoloration. The advantage of the DPPH test is that it is simple, fast, and economical. Although the DPPH test is simple, its sensitivity can be affected by several factors, such as solvent type, reaction time, temperature, and freshness of DPPH reagent[1,29], while the FRAP test is a non-specific, redox-linked, colorimetric assay related to the molar concentration of the antioxidant present. The FRAP assay is a typical method based on electron transfer, which measures the reduction of ferric ion (Fe^{3+})-ligand complex to the ferrous (Fe^{2+}); complexed by antioxidants in acidic media. One limitation of the FRAP assay is its tendency to precipitate, form suspensions, and color

the cuvette. Therefore, the timing of $FeCl_3$ addition is essential to prevent error interpretation. However, the FRAP or DPPH assay is simple, economical, fast, and requires no specialized equipment[29].

Antibacterial activity

In this study, an antibacterial activity test was performed for *C. javanicum* extract and clindamycin gel. Clindamycin is used as a positive control because it is a lincosamide antibiotic used to treat anaerobic, streptococcal, and staphylococcal infections, with *in vitro* bactericidal activity against a wide variety of anaerobic bacteria, including *Bacteroides fragilis* as well as some *Staphylococcus* sp.[30]. It is also known to be one of the antibiotics used to treat acne[31]. The diameters of the zones of inhibition produced by the clindamycin gel against *C.acnes*, *S. epidermidis,* and *S. aureus* were 34.17 ± 2.48 mm, 28.87 ± 0.75 mm, and 29.53 ± 1.06 mm, respectively (Table 4).

Table 4. Antibacterial activity of ethanolic extract of *C. javanicum* stem

Materials	Concentration (%)	Inhibition zone diameter (mm) (mean±SD; n=3)		
		C. acnes	S. epidermidis	S. aureus
Clindamycin gel	1	34.17 ± 2.48	28.87 ± 0.75	29.53 ± 1.06
Ethanolic extract of C. javanicum stem	1	4.20 ± 1.58	7.47 ± 3.87	4.33 ± 0.38
	5	5.10 ± 1.02	7.53 ± 1.80	4.80 ± 0.28
	10	4.00 ± 1.80	7.33 ± 0.38	4.03 ± 1.03
	15	3.17 ± 0.90	8.90 ± 1.50	5.83 ± 0.76

This study showed that *C. javanicum* extract was effective against the three bacteria tested and its inhibition zone was in the range of 3.17 ± 0.90 - 8.90 ± 1.50 mm (Figure 1). The highest antibacterial activity was found for *C. javanicum* extract against *S. epidermidis,* with inhibition zone diameters of 7.47 ± 3.87 mm, 7.53 ± 1.80 mm, 7.33 ± 0.38 mm and 8.90 ± 1.50 mm at concentrations of 1%, 5%, 10% and 15% (Table 4). The antibacterial activity of the extract can be divided into three levels: weak activity (inhibition zone less than 12 mm), moderate activity (inhibition zone 12-20 mm) and strong activity (inhibition zone greater than 20 mm)[32].

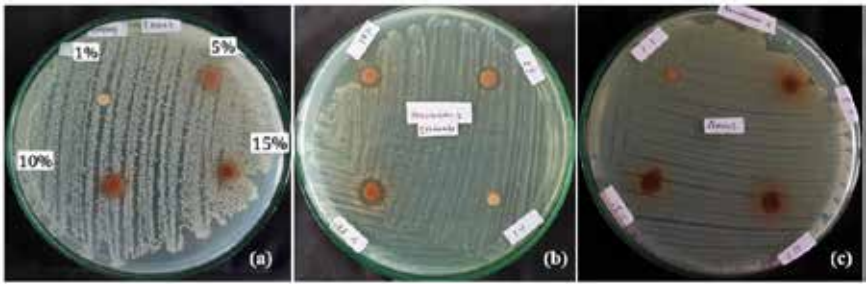


Figure 1. Antibacterial activity of ethanolic extract of *C. javanicum* stem against *C. acnes* (a), *S. epidermidis* (b), and *S. aureus* (c)

It can be concluded that the ethanol extract of the stem of *C. javanicum* has high potential antioxidant activity, but the antibacterial activity is classified as weak activity. The inhibition zones produced in this study fall on the concentration of extract of 10%. Further studies are needed to identify the factor that affects the rise and fall of the inhibition zone. In addition, negative control must be used in further study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING SOURCES

This research was funded by an internal grant from Muhammadiyah University of Palangkaraya and supported by a RisetMu grant batch 5 from Majelis Diktilitbang PP Muhammadiyah for publication.

REFERENCES

1. Safari M, Ahmady-Asbchin S. Evaluation of antioxidant and antibacterial activities of methanolic extract of medlar (*Mespilus germania* L.) leaves. Biotechnol Biotechnol Equip. 2019; 33(1): 372-378. https://doi.org/10.1080/13102818.2019.1577701.

2. Paliwal SK, Sati B, Faudjar S, Sharma S. Antioxidants and antibacterial activities of various extracts of Inula cuspidate C.B. Clarke stem. Beni-Suef Univ J Basic Appl Sci. 2017; 6(20): 97-2015. http://dx.doi.org/10.1016/j.bjbas.2016.10.003.

3. Baba SA, Malik SA. Evaluation of antioxidant and antibacterial activity of methanolic extracts of Gentiana kurroo royle. Saudi J Biol Sci. 2014; 21: 493-498. http://dx.doi.org/10.1016/j.sjbs.2014.06.004.

4. Vairappan CS, Nagappan T, Julip J. The essential oil profiles and antibacterial activity of six wild Cinnamomum species. Nat Prod Commun. 2014; 9(9): 1387-1389. https://doi.org/10.1177/1934578X1400900941.

5. Salleh WMNHW, Ahmad F, Yen KH. Evaluation of antioxidant, anticholinesterase and antityrosinase activities of malaysian cinnamomum species. Dhaka Univ J Pharm Sci. 2015; 14(2): 125-132. https://doi.org/10.3329/dujps.v14i2.28500.

6. Ardhany SD, Novaryatiin S, Hanafi N. The phytochemical screening and antioxidant potential of *Cinnamomum javanicum* Blume leaves from Central Kalimantan. Bull Pharm Sci. 2021; 44(1): 81-88. https://doi.org/10.21608/BFSA.2021.174133.

7. Zhang QW, Lin LG, Ye WC. Techniques for extraction and isolation of natural products: a comprehensive review. Chin Med. 2018; 13(20): 1-26. https://doi.org/10.1186/s13020-018-0177-x.

8. Kusuma SAF, Mita SR, Firdayani I, Mustarichie R. Study on the antibacterial activity of fruit extracts of klutuk banana (*Musa balbisiana* Colla) against *Shigella dysenteriae* ATCC 13313. Asian J Pharm Clin Res. 2017; 10(7): 220-223. https://doi.org/10.22159/ajpcr.2017.v10i7.18561.

9. Vijayan K, Gopinathan M, Ambikapathy V. Phytochemical screening and antioxidant activity of *Diospyros ebenum* J. koening ex retz, leaves extract. Int J Pharm Sci Res. 2020; 11(10): 5163-5169. https://doi.org/10.13040/IJPSR.0975-8232.11(10).5163-69.

10. Ahmed Z, Aziz S, Hanif M, Mohiuddin SG, Khan SHA, Ahmed R, et al. Phytochemical screening and enzymatic and antioxidant activities of *Erythrina suberosa* (Roxb) Bark. J Pharm Bioallied Sci. 2020; 12(2): 192-200. https://doi.org/10.4103/jpbs.JPBS_222_19.

11. Senhaji S, Lamchouri F, Toufik H. Phytochemical content, antibacterial and antioxidant potential of endemic plant *Anabasis aretioides* Coss. & Moq. (Chenopodiaceae). Biomed Res Int. 2020; 2020(6): 1-16. https://doi.org/10.1155/2020/6152932.

12. Mondal S, Rahaman ST. Flavonoids: a vital resource in healthcare and medicine. Pharm Pharmacol Int J. 2020; 8(2): 91-104. https://doi.org/10.15406/ppij.2020.08.00285.

13. Sathishkumar T, Baskar R. Screening and quantification of phytochemicals in the leaves and flowers of *Tabernaemontana heyneana* Wall. A near threatened medicinal plant. Indian J Nat Prod Res. 2014; 5(3): 237-243.

14. Hasan AEZ, Nashrianto H, Juhaeni RN, Artika IM. Optimization of conditions for flavonoids extraction from mangosteen (*Garcinia mangostana* L.). Der Pharm Lett. 2016; 8(18): 114-120.

15. Beyene BB, Alem FA, Ayana MT. Determination of antioxidant and antibacterial activities of leaf extracts of *Plumbago zeylanica* (amira). Cogent Chem. 2020; 6(1831715): 1-16. https://doi.org/10.1080/23312009.2020.1831715.

16. Hayat J, Akodad M, Moumen A, Baghour M, Skalli A, Ezrari S, et al. Phytochemical screening, polyphenols, flavonoids and tannin content, antioxidant activities and FTIR characterization of *Marrubium vulgare* L. from 2 different localities of Northeast of Morocco. Heliyon. 2020; 6(11): e05609. https://doi.org/10.1016/j.heliyon.2020.e05609.

17. Formagio ASN, Volobuff CRF, Santiago M, Cardoso CAL, Vieira MDC, Pereira ZV. Evaluation of antioxidant activity, total flavonoids, tanins and phenolic compounds in Psychotria leaf extracts. Antioxidants. 2014; 3: 745-757. https://doi.org/10.3390/antiox3040745.

18. Manssouri M, Znini M, Majidi L. Studies on the antioxidant activity of essential oil and various extracts of *Ammodaucus leucotrichus* Coss. & Dur. fruits from Morocco. J Taibah Univ Sci. 2020; 14(1): 124-130. https://doi.org/10.1080/16583655.2019.1710394.

19. Sukweenadhi J, Yunita O, Setiawan F, Kartini, Siagian MT, Danduru AP, et al. Antioxidant activity screening of seven Indonesian herbal extract. Biodiversitas. 2020; 21(5): 2062-2067. https://doi.org/10.13057/biodiv/d210532.

20. Andres AI, Petron MJ, Lopez AM, Timon ML. Optimization of extraction conditions to improve phenolic content and in vitro antioxidant activity in craft brewers' spent grain using response surface methodology (RSM). Foods. 2020; 9(1398): 1-13. https://doi.org/10.3390/foods9101398

21. Mitrevska K, Grigorakis S, Loupassaki S, Calokerinos AC. Antioxidant activity and polyphenolic content of North Macedonian wines. Appl Sci. 2020; 10(6): 1-10. https://doi.org/10.3390/app10062010.

22. Novaryatiin S, Ardhany SD. The antibacterial activity of bawang Dayak (*Eleutherine bulbosa* (Mill.) Urb.) from Central Kalimantan against acne-causing bacteria. Int J Appl Pharm. 2019; 11(Special issue 5): 22-25. https://doi.org/10.22159/ijap.2019.v11s5.T0032.

23. Kamtekar S, Keer V, Patil V. Estimation of phenolic content, flavonoid content, antioxidant and alpha-amylase inhibitory activity of marketed polyherbal formulation. J App Pharm Sci. 2014; 4 (9): 61-65. https://doi.org/10.7324/JAPS.2014.40911.

24. Azam M, Eden WT, Fibriana AI, Rahayu SR. Antioxidant properties of capsule dosage form from mixed extracts of *Garcinia mangostana* Rind and *Solanum lycopersicum* fruit. Acta Pharm Sci. 2021; 59(1): 69-80. https://doi.org/10.23893/1307-2080.APS.05904.

25. Najafabadi SF, Safaeian L, Zolfaghari B. In vitro antioxidant effects of different extracts obtained from the leaves and seeds of *Allium ampeloprasum subsp. Persicum*. J HerbMed Pharmacol. 2019; 8(3): 256-260. https://doi.org/10.15171/jhp.2019.37.

26. Fernandes RPP, Trindade MA, Tonin FG, Lima CG, Pugine SMP, Munekata PES, et al. Evaluation of antioxidant capacity of 13 plant extracts by three different methods: cluster analyses applied for selection of the natural extracts with higher antioxidant capacity to replace synthetic antioxidant in lamb burgers. J Food Sci Technol. 2016; 53(1): 451-460. https://doi.org/10.1007/s13197-015-1994-x.

27. Iduğ T, Hızlı H, Şen A, Koç F. In vitro antimicrobial and antioxidant activity of some berry species. Acta Pharm Sci. 2018; 56(3): 51-59. https://doi.org/10.23893/1307-2080.APS.05617.

28. Kusuma IW, Arung ET, Kim YU. Antimicrobial and antioxidant properties of medicinal plants used by the Bentian tribe from Indonesia. Food Sci Hum Wellness. 2014; 3(3-4): 191–196.

29. Shahidi F, Zhong Y. Measurement of antioxidant activity. J Func Foods. 2015; 18(Part B): 757-781. https://doi.org/10.1016/j.jff.2015.01.047.

30. Markey L, Pugliese A, Tian T, Roy F, Lee K, Kumamoto CA. Decreased ecological resistance of the gut microbiota in response to clindamycin challenge in mice colonized with the fungus *Candida albicans*. mSphere. 2021; 6(1): 1-16. https://doi.org/10.1128/mSphere.00982-20.

31. Novaryatiin S, Ardhany SD. Potential anti-acne: bawang Dayak (*Eleutherine bulbosa* (Mill.) Urb.) from Central Kalimantan-Indonesia. Pharmacog J. 2020; 12(1): 52-57. https://doi.org/10.5530/pj.2020.12.9.

32. Shahbazi Y. Antibacterial and antioxidant properties of methanolic extracts of apple (*Malus pumila*), grape (*Vitis vinifera*), pomegranate (*Punica granatum* L.), and common fig (*Ficus carica* L.) fruits. Pharm Sci. 2017; 23: 308-315. https://doi.org/10.15171/PS.2017.45.

Acta Pharm. Sci. Vol 61:(1), 2023
DOI: 10.23893/1307-2080.APS6104

JNK and p53 inhibition in regeneration-competent cells of nerve tissue: a novel approach for treatment of ethanol-induced neurodegeneration

Gleb Nikolaevich ZYUZ'KOV[1*], Larisa Arkad`Evna MİROSHNİCHENKO[1], Tatyana Yur`Evna POLYKOVA[1], Larisa Alexandrovna STAVROVA[1], Elena Vladislavovna SİMANİNA[1]

[1] Russian Academy of Sciences, Tomsk National Research Medical Center, Goldberg Research Institute of Pharmacology and Regenerative Medicine, Tomsk, Russia

ABSTRACT

This study aimed to explore the involvement of JNK and p53 in the implementation of nervous tissue regeneration-competent cell functions in ethanol-induced neurodegeneration. The studies were conducted on the C57B1/6 mice. Ethanol-induced neurodegeneration was modeled *in vitro* and *in vivo*. The effects of the JNK and p53 inhibitors on the colony-forming capacity of NSC and neuronal-committed progenitors, their proliferative activity and intensity of specialization, as well as the neurotrophin secretion by astrocytes, oligodendrocytes, and microglial cells, were studied. The stimulating role of JNK and p53 in the mitotic activity and specialization of intact NSCs was shown. Inversion of the role of these signaling molecules in the regulation of NSC proliferation in ethanol-induced neurodegeneration has been revealed. It has been found that JNK and p53 are not involved in regulating the NCP functions. The ambiguous role of JNK and p53 in the production of neurotrophic growth factors by neuroglia. Increased secretion of neurotrophins by oligodendrocytes and microglia during the JNK and p53 blockage in the conditions of alcohol exposure was found. These results show the potential for using JNK or p53 inhibitors as novel effective drugs for alcohol encephalopathy therapy.

Keywords: Neural stem cells, neuroglia, JNK, p53, neurodegenerative diseases

*Corresponding author: E-mail: zgn@pharmso.ru
ORCIDs:
Gleb Nikolaevich Zyuz'kov: 0000-0003-0384-333X
Larisa Arkad`Evna Miroshnichenko: 0000-0002-3684-5510
Tatyana Yur`Evna Polykova: 0000-0003-0971-7844
Larisa Alexandrovna Stavrova: 0000-0002-4729-6406
Elena Vladislavovna Simanina: 0000-0003-1890-6946
(Received 01 Dec 2021, Accepted 19 Sep 2022)

INTRODUCTION

In recent decades, the possibility of using individual intracellular signaling pathways as pharmacological targets has been actively studied. In oncopharmacology, this direction is one of the main modern trends in the creation of antitumor drugs[1]. The biggest pharmaceutical companies have developed several dozen antiblastic drugs based on intracellular signaling molecule inhibitors responsible for the growth and development of transformed cells. One of the advantages of these drugs is their selectivity, including not only to the tissue affected by the pathological process but also in some cases - to the type of tumor[2, 3].

A promising direction to solving the problems of regenerative medicine is the "Strategy of pharmacological regulation of intracellular signal transduction in regeneration-competent cells"[4-7]. This approach involves the use as targets of individual signaling molecules of progenitors and microenvironment cells of tissues (regulators of reparation) to create drugs with selective regenerative activity[8,9]. However, the development of this concept of pharmacotherapy requires a detailed understanding of the peculiarities of intracellular signaling in cells of different tissues to determine the potential targets for selective tissue-specific effects.

The implementation of this direction is particularly relevant for neurological practice to improve the effectiveness of the treatment of neurodegenerative diseases[10, 11]. Promising is the development of approaches to stimulation of neurogenesis by activation of the functions of progenitor cells of nerve tissue - multipotent neural stem cells (NSC) and neuronal-committed progenitors (NCP)[6, 12]. At the same time, it is known the importance of JNK- and p53-pathways (interacting with each other (have some "crossroads")), in regulating the processes of progenitor proliferation and differentiation[13, 14], as well as in the implementation of the functions of cytokine-producing cells[15]. However, there is no detailed understanding of their role in the functioning of nerve tissue regeneration-competent cells.

The work aimed to study the participation of JNK and p53 in the realization of the growth potential of nerve tissue precursors (NSC, NCP) and the production of neurotrophic growth factors by different neuroglial cells.

METHODOLOGY

Chemicals and Drugs

MACS Neuro Medium; anti-PSA-NCAM MicroBeads; anti-ACSA-2 MicroBead Kit; Anti-O4 MicroBeads; Anti-CD11b (Microglia) MicroBeads (all manufactured by Miltenyi Biotec, Germany); JNK inhibitor «SP600125» (InvivoGen, USA); p53 inhibitor «Pifithrin-α, Cyclic» (Santa Cruz Biotechnology, Inc. USA); hydroxyurea (Calbiochem, USA); plastic plates for cultural studies (Costar, USA).

Animals and Experimental Design

All animal experiments were carried out following the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. The study was approved by the Institute's local Ethics Committee (protocol GRIPh&RM-2020-09). Experiments were carried out on C57B1/6 mice (n=84) at the age of 2-2.5 months, weighing 20-22 g. Animals of the 1st category (conventional mice) were obtained from the Experimental Biological Models Department of Goldberg Research Institute of Pharmacology and Regenerative Medicine (Tomsk, Russia) (certificate available). Before the beginning of experiments (during 10 days) and over the study period, animals were contained in a vivarium (air temperature 20–22°C, humidity 50-60 %) in plastic cages (10-15 mice) on a normal diet (solid diet pellets (Limited Liability Company «Assortiment Firm», Sergiev Posad city, Russia), water ad libitum. To exclude seasonal fluctuations of studied parameters, all the experiments were performed in the autumn-winter period. The animals were removed from the experiment (sacrificed) using CO_2 cameras.

Using the cultural methods, we studied the direct effect of the JNK inhibitor and p53 inhibitor (at a concentration of 10 μM and 5 μM, respectively) on the realization of the growth potential of neural tissue precursor cells (NSC, NCP) and the secretion of neurotrophic growth factors (neurotrophins) by glial cells (astrocytes, oligodendrocytes, microglia) in the conditions of modeling ethanol-induced neurodegeneration *in vitro* and *in vivo*[6, 9]. The working concentrations of inhibitors of signaling molecules were determined following the instructions of the companies-developers of these reagents.

In vitro ethanol-induced neurodegeneration was obtained by adding ethanol to the culture medium at a concentration (65 mM). *In vivo* modeling of the pathological state was carried out by oral administration of a 30% C_2H_5OH solution (through a probe daily at a dose of 3 g/kg/day for 8 weeks)[6, 12]. In this case, instead of drinking water of free access, a 5% ethyl alcohol solution was used. Cellular materials for the study were taken 10 days after the end of the intro-

duction of ethanol *in vivo*. The control group in the same model was injected with distilled water in an equivalent volume (mice had free access to drinking water).

Determination of Progenitors Functional Activity

NSC were studied during the cultivation of unfractionated cells of the subventricular zone (SVZ) of the cerebral hemispheres. To study NPCs, PSA-NCAM+ cells were isolated from the SVZ cells. For this, an immunomagnetic separator MIniMACS Cell Separator (Miltenyi Biotec, Germany) was used[6, 8, 9]. PSA-NCAM+ (CD56+) cells were obtained by positive selection [16] (using appropriate antibody kits according to the methodological manufacturer's instructions). The obtained unfractionated and PSA-NCAM$^+$ cells at a concentration of 10^5 / ml were incubated in MACS Neuro Medium (Miltenyi Biotec, Germany) for 5 days in a CO_2 incubator at 37°C, 5% CO_2, and 100% air humidity. After incubation in both cases (during the cultivation of unfractionated cells and PSA-NCAM$^+$ cells), the content of clonogenic cells, their mitotic activity, and intensity of specialization were calculated. The number of NSC and NCP was determined by the yield in the respective cultures of colony-forming units (CFU, neurospheres containing more than 100 cells). The proliferative activity of the progenitors was assessed by the method of cell suicide technic using hydroxyurea (1 μM)[6]. The pool of CFU in the S-phase of the cell cycle was determined according to the formula: $N = [(a-b)/a] \times 100\%$, where a is the average for the group the number of CFU from cells not treated with hydroxyurea; b - the average for the group the number of CFU from cells treated with hydroxyurea. The intensity of the processes of specialization (differentiation/maturation) of progenitors was determined by calculating the ratio of the corresponding cluster-forming (ClFU, neurospheres of 30 - 100 cells) to CFU – the differentiation index[6, 9].

Study of Neurotrophic Growth Factors Secretion by Neuroglial Cells

Individual fractions of glial cells (astrocytes - ACSA-2$^+$ cells, [17] oligodendrocytes - O4$^+$ cells, [18] microglia - CD11b$^+$ cells[19]) were also obtained from the SVZ using immunomagnetic positive selection (using appropriate antibody kits according to the guidelines of Miltenyi Biotec, Germany) The isolated cells at a concentration of 2×10^6 / ml were incubated in MACS Neuro Medium (Miltenyi Biotec, Germany) for 2 days in a CO_2 incubator at 37 ° C, 5% CO_2 and 100% air humidity to obtain supernatants. To determine their secretory activity (neurotrophic growth factors production), the effect of conditioned media from cells on the level of neurospheres formation (CFU) in the test system (culture of the SVZ unfractionated cells) was studied[5, 6].

Statistical Analysis

The results were analyzed with one-way ANOVA followed by Dunnett's test, Wilcoxon's test for dependent samples, and Mann–Whitney test for independent samples. The data are expressed as arithmetic means. The significance level was $p < 0.05$[20].

RESULTS AND DISCUSSION

Effect of Ethanol on the Functioning of Various Types of Regeneration-Competent Cells of Nervous Tissue

The addition of a neurotoxic dose of ethanol to the culture medium did not cause a change in the level of the colony- and cluster-forming capacity of the unfractionated cells (Figure 1, A, B) and PSA-NCAM$^+$ cells (Figure 2, A, B) from the SVZ. However, there was a decrease in the NSC (CFU-N_{NSC}) and NCP (CFU-$N_{PSA\text{-}NCAM+}$) mitotic activity (up to 77.3% and 80.8% of background values, respectively) against the background of no changes in the speed of their specialization (Figure 1, C, D; 2, C, D).

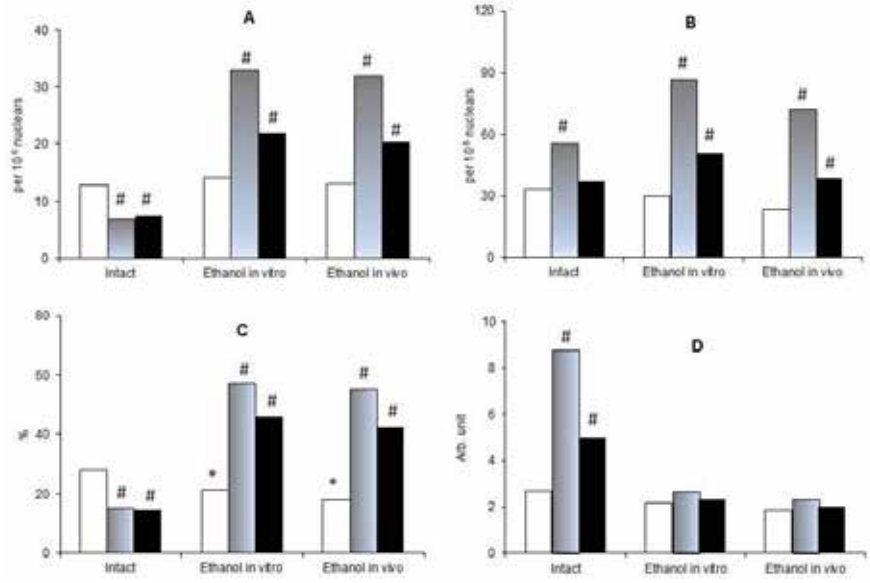


Figure 1. Number of CFU-N_{NSC} (A), CIFU-N_{NSC} (B), NSC proliferative activity (CFU-N_{NSC} in the S-phase of the cell cycle) (C), and NSC differentiation index (CIFU-N_{NSC} / CFU-N_{NSC}) (D). Here and in figures 2 and 3: cell culture without alcohol (intact); with alcohol (ethanol *in vitro*); and mice after prolonged administration of ethanol per os (ethanol *in vivo*). White bars - without signaling molecule inhibitors (white bars); gray bars - with the JNK inhibitor; black bars - with the p53 inhibitor; * - the significance of differences in indicators with intact was noted at $p < 0.05$; # - the significance of differences with the group without signaling molecule inhibitors was noted at $p < 0.05$.

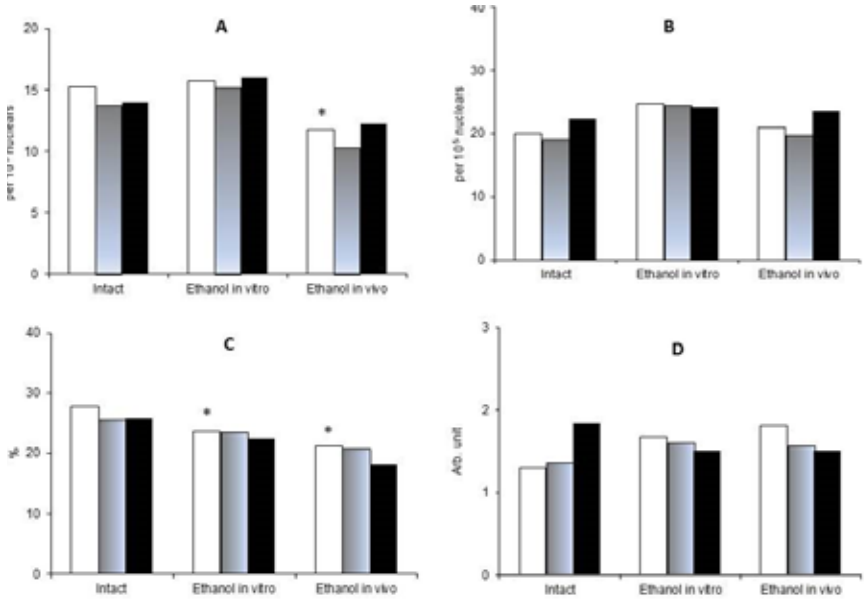


Figure 2. Number of CFU-$N_{PSA\text{-}NCAM+}$ (A), CIFU-$N_{PSA\text{-}NCAM+}$ (B), NCP proliferative activity (CFU-$N_{PSA\text{-}NCAM+}$ in the S-phase of the cell cycle) (C), and NCP differentiation index (CIFU-$N_{PSA\text{-}NCAM+}$ / CFU-$N_{PSA\text{-}NCAM+}$) (D).

The long-term introduction of ethyl alcohol *per os* was accompanied by the development of similar changes in the functioning of progenitors. However, in this case, there was a further decrease in the number of CFU-$N_{PSA\text{-}NCAM+}$ in the culture of clonogenic PSA-NCAM$^+$ cells (Figure 2, A) and the intensity of differentiation/maturation of NSC (Figure 1, D).

The ethanol addition *in vitro* was not accompanied by a change in the neurotrophin secretion by astrocytes and oligodendrocytes (Figure 3, A, B). However, there was a decrease in the value of this parameter in the conditioned media from microglial cells (up to 81.8% of the background value - a similar indicator in culture without ethanol) (Figure 3, C).

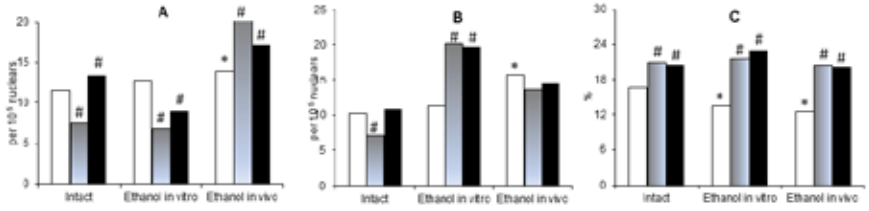


Figure 3. Effect of conditioned media of astrocytes (A), oligodendrocytes (B), and microglial cells (C) on the level of neurosphere formation in the test system.

Other changes in the production of humoral regulators were reported in cells of mice subjected to chronic alcohol intoxication. In this case, there was an increase in the neurotrophic growth factors secretions by astrocytes and oligodendrocytes (up to 121.7% and 153.7% of the initial control, respectively) against the background of the significant drop in the level of neurospheres formation in the test system containing the conditioned media from microglia (up to 75.8% of control).

Astrocytes and oligodendrocytes (primarily polydendrocytes, also called NG-2 cells and oligodendrocyte progenitor cells (OPC)) are known to produce neurotrophin family growth factors such as nerve growth factor (NGF), neurotrophic brain factor (BDNF).), neurotrophins 3 and 4 (NT3 and NT4), glial cell neurotrophic factor (GDNF), etc[21, 22]. Microglia secretes a wide range of cytokines, having both a stimulating effect on the NSC proliferation (leukemia inhibition factor (LIF), ciliary neurotrophic factor (CNTF), and others) and causing inhibition of their growth potential (interleukin-1, 6, 15 (IL-1, 6, 15), tumor necrosis factor-alpha (TNF-α)[23, 24]. Therefore, it is obvious that the detected dynamics of changes on the part of microglia reflect the parameter, which is also dependent on the products of inhibitors to realize the growth potential of progenitors - pro-inflammatory cytokines[25].

These findings were largely in line with the previously obtained data[6, 12] on the violation of implementation, primarily the proliferation of the nerve tissue progenitors under the influence of neurotoxic doses of ethanol. Therefore, it should be considered that the most vulnerable, especially with prolonged exposure to alcohol on the body, are precursors determined in the neuronal direction of development. Thus, the results indicate the development of ethanol-induced neurodegeneration of compensatory response from of astroglia and oligodendroglia, aimed at stimulation of neurogenesis in the conditions of violation of the NSC proliferation[12, 23]. At the same time, the detected decrease of the neurospheres formation under the influence of the microglial cells conditioned media is not so unambiguous. This circumstance was due, first of all, to the increase in

the secretion of the pro-inflammatory cytokines[25], rather than to a decrease in the neurotrophins production. It is known that the intensity of the inflammatory reaction in the nervous tissue in chronic alcohol consumption, in many respects, determines the development further of the pathological process. Expressed inflammation may not only not contribute to tissue repair, but also damage it[26, 27].

Role of JNK and p53 in Implementation Functions of Nervous Tissue Progenitors Under Conditions of Their Optimal Vital Activity

The addition of the signaling molecule inhibitors to the culture with the SVZ unfractionated cells was accompanied by a significant decrease in the level of colony formation. The number of CFU-N_{NSC} was 54.6% and 58.5% of the background value under the blockade of JNK and p53, respectively (Figure 1, A). The state of the ClFU-N_{NSC} (which are precursors with a lower self-renewal capacity and proliferating potential[6, 12]) was characterized by less unambiguous changes. The violation of JNK phosphorylation led to an increase in the number of ClFU-N_{NSC} in culture (up to 166.7% of the background), while the inactivation of p53 did not affect the value of this parameter (Figure 1, B). In both cases, however, there was a significant increase in the intensity of progenitor specialization processes. The differentiation index was 327.5% and 185.5% of control with the JNK and p53 inhibitors respectively (Figure 1, D).

Other phenomena were observed in the study of the role of JNK and p53 in the functioning of the neuronal-committed progenitors. The blockade of signal transduction via JNK and p53 did not change the values of the studied indicators in the culture of PSA-$NCAM^+$ cells (Figure 2).

Thus, JNK- and p53-pathways are essential in regulating the cell cycle of only NSC. They are responsible for maintaining their multipotency and self-renewal capacity. That is, JNK and p53 play one of the key roles in the maintaining the "deep reserve" of the CNS regeneration – resident stem cells[5, 28].

Role of JNK and p53 in Growth Factors Secretion by Neuroglial Cells Under Conditions of Their Optimal Vital Activity

It is known that the functioning of progenitor cells depends on the state of the microenvironment of tissue cells. They can exert their influence by secretion of humoral factors and direct intercellular communications that provide, among other things, the provision of cytokines in a biologically active form[4, 23]. In this regard, when choosing the targets among the intracellular signaling molecules in the progenitors should take into account their role in regulating the functions of individual populations of neuroglia[12].

Studies of JNK and p53 participation in the growth factors productions by different fractions of glial cells have revealed ambiguous changes. Thus, the JNK and p53 inhibitors caused a significant reduction in the neurotrophins secretion by astrocytes (ACSA-2$^+$ cell) (Figure 3, A), reaching 54.3% and 76.1% of the background, respectively. The capacity of microglia (CD11b$^+$ cells) to influence the realization of the growth potential of precursors (due to the production of humoral factors) during the blockade of JNK and p53, on the contrary, increased (up to 116.7% and 122.7% of the baseline, respectively) (Figure 3, C). At the same time, there were no changes in the functioning of oligodendrocytes (O4$^+$ cells) in any of the cases (Figure 3, B).

Role of JNK and p53 in Implementation Functions of Nervous Tissue Progenitors in Ethanol-Induced Neurodegeneration

The study of the effect of alcohol on the participation of JNK- and p53-pathways in realizing the growth potential of neural tissue precursors revealed some interesting phenomena. The addition into the culture medium with ethanol the JNK and p53 inhibitors led to a significant increase in the NSC proliferative activity and neurosphere formation (Figure 1, A, C). At the same time, the JNK blockade accelerated the NSC specialization to 123.6% of control (medium with ethanol without the signaling molecules inhibitors) (Figure 1, D). The inactivation of JNK and p53 in the neuronal-committed progenitors in the simulation of ethanol-induced neurodegeneration *in vitro* did not cause changes in their functioning (Figure 2).

Similar patterns were revealed in the cultivation of nerve tissue cells in alcoholized mice. The inactivation of JNK and p53 led to an increase in the content of CFU-N_{NSC} and their pro-proliferative activity in the culture of unfractionated cells of the SVZ (Figure 1, A, C). Besides, the JNK inhibitor also saw an increase in the NSC specialization index (Figure 1, D). However, there was no change in JNK and p53 involvement in regulating the NCP functions. The blockage of these signaling molecules in neuronal-committed progenitors of mice, which had long been treated with ethanol, did not affect the realization of their growth potential (Figure 2).

The results indicate an inversion of the role of JNK and p53 in the regulation of the NSC proliferation in ethanol-induced neurodegeneration.

Role of JNK and p53 in Growth Factors Secretion by Neuroglial Cells in Ethanol-Induced Neurodegeneration

Changes in the functioning of different types of neuroglial cells depending on their living conditions under the JNK and p53 blockade were ambiguous. The

inactivation of JNK and p53 in the incubation of astrocytes in the presence of C_2H_5OH *in vitro* was accompanied by a drop in the neurotrophic growth factors production (up to 45.1% and 78.4% of control values, respectively - in a medium with ethanol without the signaling molecules inhibitors). However, in mice that received ethyl alcohol *per os*, JNK and p53 blockade in astrocytes did not affect the neurotrophin secretion (Figure 1, A).

Another phenomenology was observed in the study of the functioning of oligodendrocytes. Disruption of signal transduction via JNK and p53 in oligodendrocytes in exposure to ethanol *in vitro*, on the contrary, led to an increase in their growth factors production, especially expressed in the use of the JNK inhibitor (Figure 3, B). At the same time, the blockade of JNK in the oligodendrocytes of animals, which were treated with alcohol for a long time, caused, on the contrary, a significant reduction in the neurotrophin secretion (up to 84.1% of similar parameters in cells without signaling modification). The p53 inhibitor, in this case, did not affect the production of growth factors by oligodendrocytes.

One-way changes were observed on the part of the secretory activity of microglial cells. In all cases (the addition of C_2H_5OH *in vitro* and the introduction of ethanol to mice *per os*), inactivation of signaling molecules led to an increase in the secretion of growth factors by microglial cells. A particularly pronounced increase in this indicator was with the use of the JNK inhibitor (up to 150.0% and 156.2% of the control values respectively) (Figure 3, C).

Thus, JNK and p53 played an ambiguous role in regulating the secretory function of different types of neuroglial cells when modeling ethanol-induced neurodegeneration *in vitro* and *in vivo*.

The findings confirm the evidence that there are significant peculiarities of the role of JNK and p53 in the regulation of the cell cycle of different types of regeneration-competent cells[5, 8, 13, 14]. It was found that JNK- and p53-pathways are responsible for maintaining the "deep reserve" of the plasticity of the CNS - for maintaining the self-renewal, rapid proliferation, and multiple differentiation of NSC. In the functioning of the neuronal-committed progenitors (the most mobile department of tissue-specific regeneration[5, 9]) these signaling molecules do not take significant participation.

However, it is known that balanced neurogenesis can occur through the implementation of the functions of exclusively multipotent NSC (without the participation of NCP)[5, 28, 29]. Therefore, the important role of JNK and p53 in the progression of the NSC cell cycle indicates the potential for their use as pharmacological targets (Figure 4).

At the same time, it has been found that ethanol-induced neurodegeneration leads to the inversion of the role of JNK and p53 in the regulation of the proliferation of multipotent NSC. The blockage of these signaling molecules in the conditions of ethanol intoxication can significantly increase the degree of realization of the growth potential of NSC and thus, obviously, stimulate neurogenesis. But it should be taken into account that such therapy can lead to the transformation of the ethanol-induced pattern of intracellular signaling into the «original» (normal) state in the *de novo* formed progenitors[6, 9, 12]. That is, to cause a "reverse" inversion of the role of JNK and p53 in the regulation of the NSC functions. In such conditions, the JNK and p53 inhibitors will, on the contrary, inhibit the implementation of the functions of these intact progenitor cells.

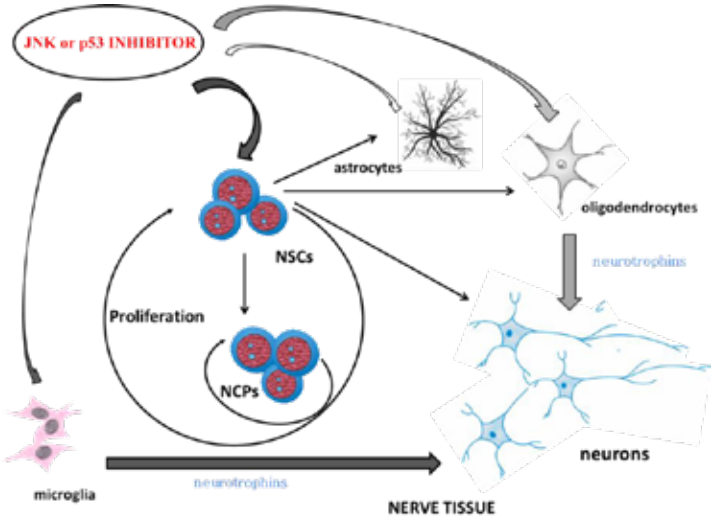


Figure 4. Effect of JNK or p53 inhibitor on the regeneration-competent cells of the nerve tissue in ethanol-induced neurodegeneration. NSCs - neural stem cells; NCPs - neuronal-committed progenitor cells; grey arrows are a stimulating influence; white arrows are an inhibitory effect.

Therefore, the use of JNK and p53 inhibitors in alcoholic encephalopathy may be promising only during the manifestation of the disease (and the cancellation of their use when there are clinical signs of reconvalescence)[30].

The detected increase in the secretion of neurotrophic growth factors by oligodendrocytes and microglia when blockaded in them JNK and p53 in the conditions of exposure to ethanol is an additional reason in favor of the possibility of accelerating the reparation of the CNS by inactivating these signal molecules.

But it should be taken into account that JNK and p53 inhibitors have an ambiguous effect on the functioning of astrocytes in the modeling of ethanol-induced neurodegeneration *in vitro* and *in vivo*. Further research should determine how much this factor can be neglected.

Besides, the anti-mutagenic properties of the p53 protein (p53 - "guardian of the genome"[31]) are known. This determines the need for further consideration of the carcinogenic safety of the potential use of its potency modifiers as drugs. The p53 can also be targeted via JNK-pathway[13]. However, it is only one of the directions of signal transduction via JNK[32, 33], which in some cases is not implemented. Besides, even the anti-blastic properties of JNK inhibitors are known[4, 34]. Therefore, the potential for drug (carcinogenic) safety in JNK activity/expression inhibitors is higher.

The results reveal the feasibility of further study of the possibility of creating novel effective drugs for the therapy of alcoholic encephalopathy based on JNK or p53 inhibitors[34]. The development of pharmacotherapy approaches using JNK activity/expression inhibitors is especially promising.

AUTHOR CONTRIBUTION

Conceptualization: Gleb N. Zyuz`kov; Methodology: Gleb N. Zyuz`kov, Larisa A. Miroshnichenko; Animal caring: Tatyana Yu. Polyakova, Larisa A. Stavrova and Elena V. Simanina; Writing: Gleb N. Zyuz`kov.

ETHICS APPROVAL

The study was approved by the Institute`s local Ethics Committee (Goldberg Research Institute of Pharmacology and Regenerative Medicine, Tomsk National Research Medical Center, Russian Academy of Sciences) (protocol GRIPh&RM-2020-09). No humans were used in this research. All animal experiments were carried out following the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

ACKNOWLEDGEMENT

The studies were carried out as part of the execution of the State task of the Ministry of Science and Higher Education of Russia on the topic No. FGWM-2022-0018. This work was supported by the Russian Foundation for Basic Research (RFBR) in the framework of scientific project No. 18-015-00013.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

REFERENCES

1. Lee YT, Tan YJ, Oon CE. Molecular targeted therapy: Treating cancer with specificity. Eur J Pharmacol. 2018; 834: 188-196. https://doi.org/10.1016/j.ejphar.2018.07.034.

2. Li B, Rampal RK, Xiao Z. Targeted therapies for myeloproliferative neoplasms. Biomark Res. 2019; 7(1): 15. https://doi.org/10.1186/s40364-019-0166-y.

3. Li GZ, Raut CP. Targeted therapy and personalized medicine in gastrointestinal stromal tumors: drug resistance, mechanisms, and treatment strategies. Onco Targets Ther. 2019; 12: 5123. https://doi.org/10.2147/OTT.S180763.

4. Zyuz'kov GN. Targeted Regulation of Intracellular Signal Transduction in Regeneration-Competent Cells: A new Direction for Therapy in Regenerative Medicine. Biointerface Res Appl Chem. 2021; 11(4): 12238-12251. https://doi.org/10.33263/BRIAC114.1223812251.

5. Zyuz'kov GN, Suslov NI, Miroshnichenko LA, Simanina EV, Polyakova TY, Stavrova LA, et al. Halogenated (Cl-ion) songorine is a new original agonist of fibroblast growth factor receptors of neuronal-committed progenitors possessing neuroregenerative effect after cerebral ischemia and hypoxia in experimental animals. Biointerface Res Appl Chem. 2019; 9(5): 4317-4326. https://doi.org/10.33263/BRIAC95.317326.

6. Zyuz'kov GN, Miroshnichenko LA, Simanina EV, Stavrova LA, Polyakova TY. Intracellular signaling molecules of nerve tissue progenitors as pharmacological targets for treatment of ethanol-induced neurodegeneration. J Basic Clin Physiol Pharmacol. 2021; 32(6). https://doi.org/10.1515/JBCPP-2020-0317.

7. Zyuz'kov GN, Miroshnichenko LA, Polyakova TY, Stavrova LA, Simanina EV. Wound healing properties of the protein kinase A inhibitor and the mechanisms of their development. Bangladesh J Pharmacol. 2021; 16(2): 19-26. https://doi.org/10.3329/bjp.v16i1.50575.

8. Zyuz'kov GN, Zhdanov VV, Udut EV, Miroshnichenko LA, Polyakova TY, Stavrova LA, et al. Peculiarities of Intracellular Signal Transduction in the Regulation of Functions of Mesenchymal, Neural, and Hematopoietic Progenitor Cells. Bull Exp Biol Med. 2019; 167(2): 201-206. https://doi.org/10.1007/s10517-019-04491-3.

9. Zyuz'kov GN, Miroshnichenko LA, Polyakova TY, Stavrova LA, Simanina EV, Zhdanov VV, et al.. Peculiarities of the Involvement of MAPKS ERK1/2 and p38 in the Implementation of the Functions of Neural Stem Cells and Neuronal Committed Precursors in Ethanol-Induced Neurodegeneration. Bull Exp Biol Med. 2020; 169(5): 609-613. https://doi.org/10.1007/s10517-020-04938-y.

10. Brick RM, Sun AX, Tuan RS. Neurotrophically Induced Mesenchymal Progenitor Cells Derived from Induced Pluripotent Stem Cells Enhance Neuritogenesis via Neurotrophin and Cytokine Production. Stem Cells Transl Med. 2018; 7(1): 45-58. https://doi.org/10.1002/sctm.17-0108.

11. Koeppen AH. The neuropathology of the adult cerebellum. Handb Clin Neurol. 2018; 154: 129-149. https://doi.org/10.1016/B978-0-444-63956-1.00008-4.

12. Zyuz'kov GN, Stavrova LA, Miroshnichenko LA, Polyakova TY, Simanina EV. Prospects for the Use of NF-κb Inhibitors to Stimulate the Functions of Regeneration-Competent Cells of Nerve Tissue and Neuroregeneration in Ethanol-Induced Neurodegeneration. Biointerface Res Appl Chem. 2021; 11(1): 8065-8074. https://doi.org/10.33263/BRIAC111.80658074.

13. Saha MN, Jiang H, Yang Y, Zhu X, Wang X, Schimmer AD, et al. Targeting p53 via JNK pathway: a novel role of RITA for apoptotic signaling in multiple myeloma. PLoS One. 2012; 7(1): e30215. https://doi.org/10.1371/journal.pone.0030215.

14. Yang Q, Qian L, Zhang S. Ginsenoside Rh1 Alleviates HK-2 Apoptosis by Inhibiting ROS and the JNK/p53 Pathways. Evid Based Complement Alternat Med. 2020; 2020: 3401067. https://doi.org/10.1155/2020/3401067.

15. Zhang T, Li H, Shi J, Li S, Li M, Zhang L, et al. p53 predominantly regulates IL-6 production and suppresses synovial inflammation in fibroblast-like synoviocytes and adjuvant-induced arthritis. Arthritis Res Ther. 2016; 18(1): 271. https://doi.org/10.1186/s13075-016-1161-4.

16. Seki T. Expression patterns of immature neuronal markers PSA-NCAM, CRMP-4 and NeuroD in the hippocampus of young adult and aged rodents. J Neurosci Res. 2002; 70(3): 327-34. https://doi.org/10.1002/jnr.10387.

17. Kantzer CG, Boutin C, Herzig ID, Wittwer C, Reiß S, Tiveron MC, et al. Anti-ACSA-2 defines a novel monoclonal antibody for prospective isolation of living neonatal and adult astrocytes. Glia. 2017; 65(6): 990-1004. https://doi.org/10.1002/glia.23140.

18. Werneburg S, Fuchs HLS, Albers I, Burkhardt H, Gudi V, Skripuletz T, et al. Polysialylation at Early Stages of Oligodendrocyte Differentiation Promotes Myelin Repair. J Neurosci. 2017; 37(34): 8131-8141. https://doi.org/10.1523/JNEUROSCI.1147-17.2017.

19. Martin E, El-Behi M, Fontaine B, Delarasse C. Analysis of Microglia and Monocyte-derived Macrophages from the Central Nervous System by Flow Cytometry. J Vis Exp. 2017; Jun 22(124): 55781. https://doi.org/10.3791/55781.

20. Curtis MJ, Bond RA, Spina D, Ahluwalia A, Alexander SP, Giembycz MA. Experimental design and analysis and theirreporting: new guidance for publication in BJP. Br J Pharmacol. 2015; 172(14): 3461–3471. https://doi.org/10.1111/bph.12856.

21. Alghamdi B, Fern R. Phenotype overlap in glial cell populations: astroglia, oligodendroglia and NG-2(+) cells. Front Neuroanat. 2015; 9: 49. https://doi.org/10.3389/fnana.2015.00049

22. Chang HM, Wu HC, Sun ZG, Lian F, Leung PCK. Neurotrophins and glial cell line-derived neurotrophic factor in the ovary: physiological and pathophysiological implications. Hum Reprod Update. 2019; 25(2): 224-242. https://doi.org/10.1093/humupd/dmy047.

23. Verkhratsky A, Ho MS, Zorec R, Parpura V. The Concept of Neuroglia. Adv Exp Med Biol. 2019; 1175: 1-13. https://doi.org/10.1007/978-981-13-9913-8_1.

24. Hu Z, Deng N, Liu K, Zhou N, Sun Y, Zeng W. CNTF-STAT3-IL-6 Axis Mediates Neuroinflammatory Cascade across Schwann Cell-Neuron-Microglia. Cell Rep. 2020; 31(7): 107657. https://doi.org/10.1016/j.celrep.2020.107657.

25. Matsumoto J, Dohgu S, Takata F, Machida T, Bölükbaşi Hatip FF, Hatip-Al-Khatib I, et al. TNF-alpha-sensitive brain pericytes activate microglia by releasing IL-6 through cooperation between IkappaB-NFkappaB and JAK-STAT3 pathways. Brain Res. 2018; 1692: 34-44. https://doi.org/10.1016/j.brainres.2018.04.023.

26. Macht V, Crews FT, Vetreno RP. Neuroimmune and epigenetic mechanisms underlying persistent loss of hippocampal neurogenesis following adolescent intermittent ethanol exposure. Curr Opin Pharmacol. 2020; 50: 9-16. https://doi.org/10.1016/j.coph.2019.10.007.

27. Zhang K, Wang H, Xu M, Frank JA, Luo J. Role of MCP-1 and CCR2 in ethanol-induced neuroinflammation and neurodegeneration in the developing brain. J Neuroinflammation. 2018; 15(1): 197. https://doi.org/10.1186/s12974-018-1241-2.

28. Katsimpardi L, Lledo PM. Regulation of neurogenesis in the adult and aging brain. Curr Opin Neurobiol. 2018; 53: 131-138. https://doi.org/10.1016/j.conb.2018.07.006.

29. Zyuz'kov GN, Miroshnichenko LA, Polyakova TYu, Simanina EV, Stavrova LA. Targeting cAMP-pathway in Regeneration-Competent Cells of Nervous Tissue: Potential to Create

a Novel Drug for Treatment of Ethanol-Induced Neurodegeneration. Cent Nerv Syst Agents Med Chem. 2021; 21(2): 1-9. https://doi.org/10.2174/1871524921666210907102847.

30. Udut VV, Naumov SA, Evtushenko DN, Udut EV, Naumov SS, Zyuz'kov GN. A case of xenon inhalation therapy for respiratory failure and neuropsychiatric disorders associated with COVID-19. EXCLI Journal. 2021; 20: 1517-1525. https://doi.org/10.17179/excli2021-4316.

31. Mantovani F, Collavin L, Del Sal G. Mutant p53 as a guardian of the cancer cell. Cell Death Differ. 2019; 26: 199-212. https://doi.org/10.1038/s41418-018-0246-9.

32. Kumar A, Singh UK, Kini SG, Garg V, Agrawal S, Tomar PK, et al. JNK pathway signaling: a novel and smarter therapeutic targets for various biological diseases. Future Med Chem. 2015; 7(15): 2065-86. https://doi.org/10.4155/fmc.15.132.

33. Li GQ, Chen M. Cardioprotective effect of erythropoietin in rats with acute myocardial infarction through JNK pathway. Eur Rev Med Pharmacol Sci. 2019; 23(3 Suppl): 153-160. https://doi.org/10.26355/eurrev_201901_18642.

34. Zyuz'kov GN, Zhdanov VV, Miroshnichenko LA, Polyakova TY, Simanina EV, Stavrova LA, et al. Role of JNK and p53 in Implementation of Functions of Various Types of Regeneration-Competent Cells of the Nervous Tissue. Bull Exp Biol Med. 2021; 171: 333–35 https://doi.org/10.1007/s10517-021-05222-3.

Acta Pharm. Sci. Vol 61:(1), 2023
DOI: 10.23893/1307-2080.APS6105

Synthesis, structure elucidation and *in vitro* microsomal metabolism of adamantane hydrazide-hydrazone derivatives

Göknil Pelin COŞKUN[1*], Basak TÜRK ERBUL[1], Jülide Cansu KARAKOÇ[1], Mert ÜLGEN[1]

[1] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Acibadem Mehmet Ali Aydınlar University, 34684, İçerenköy, Istanbul, Türkiye.

ABSTRACT

The discovery of adamantane has led to the researchers to find different drug molecules which exhibit diverse biological activities. On the other hand, hydrazide-hydrazones possess strong anticancer, antibacterial, antienflamatory and antiseptic activites. However, we have limited knowledge on the metabolic profiles of adamantane hydrazide-hydrazones. The metabolic pathway of adamantane hydrazide-hydrazones emerged to be an important step for pre-clinical drug discovery studies. In this study, the metabolic profile of adamantane hydrazones was the main motivation for our research group. The synthesis of hydrazide-hydrazone derivatives as substrates of this study was performed via condensation of corresponding hydrazide with aldehyde and ketone and their authentic metabolites were also synthesized. Following their structures were elucidated with spectroscopic methods, their *in-vitro* microsomal metabolic study was performed. The results indicated that the aldehyde derivative is susceptible to metabolic hydrolysis, whereas ketone derivative is stable for metabolic changes. LC-MS results proved the metabolic hydrolysis.

Keywords: Adamantane, *in vitro* metabolism, hydrazide-hydrazone, hydrolysis.

INTRODUCTION

Adamantane was first discovered in Hodonin, Czechoslovakia in 1933 from crude oil. The discovery of this interesting ring opened new fields in chemistry including drug development. Adamantane ring were then derivatized with dif-

*Corresponding author: E-mail: pelin.coskun@acibadem.edu.tr
ORCIDs:
Göknil Pelin Coşkun: 0000-0001-5168-3866
Başak Türk Erbul: 0000-0001-8263-8283
Jülide Cansu Karakoç: 0000-0002-7804-341X
Mert Ülgen: 0000-0003-4913-4950
(Received 19 Sep 2022, Accepted 03 Oct 2022)

ferent class of substituents leading a diverse biological activities [1]. Drugs having adamantane ring exhibit surprising clinical efficacy. The most common and well known drugs that have adamantane ring are amantadine, rimantadine, memantine, tromantadine and saxagliptin. These drugs are known to have different pharmacological activities ranging from antiviral to antidiabetic [2].

Hydrazide-hydrazone derivatives have an azomethine structure linked to an amide (-CH=N-NH-CO-) which plays a significant role in terms of pharmacological activites. Recent studies have showed their promising antimicrobial, anticancer, antituberculosis, antiviral and anticonvulsant activities [3-18].

The literature search indicated that there was limited number of studies related with adamantane hydrazide-hydrazones. Wassel and co-workers recently reported the synthesis and the carbonic anhydrase activity of adamantane hydrazide-hydrazones [19]. A hydrazone-adamantane structure with pentyl moiety showed excellent inhibitory activity and presented good pharmacokinetic properties. Both carbonic anhydrase and antimicrobial activity studies on adamantane-hydrazone derivatives were conducted by Pham and co-workers [3]. Although these compounds exhibited good biological activites, no metabolic study was performed showing their metabolic profile. There are very few studies on the metabolism of hydrazone compounds in the literature and these studies have been done on certain prototypes. Kömürcü et al. synthesized a number of hydrazide-hydrazones to investigate their antibacterial activity. They also investigated *in vitro* microsomal metabolites of 4-fluorobenzene derivative as a prototype following *in vitro* microsomal metabolism [20-21]. In another study by Ulgen et al, *in vitro* metabolism of benzoic acid benzylidenehydrazide was studied and it was observed that the compound produced the corresponding hydrolytic metabolites, namely benzoic acid hydrazide and benzaldehyde together with a p-hydroxylation metabolite which occured by oxidation from the para position of the benzylidene due to the strong electron withdrawing effect of the benzoyl ring [22]. Motiur Rahman et al. studied *in vitro* micromosal metabolism of 2-fluorescein hydrazones which are topoisomerase inhibitors. The study was performed on rat liver microsomes and metabolites were analyzed in the LC-MS system. They found out that the compounds undergone biotransformation to a hydroxyl derivative and tetra hydroxyl derivative. The metabolic products was recorded as hydroxylation and reduction. It was concluded that the hydrazone compounds needed co-factors for *in vitro* metabolism [23]. The literature data revealed limited knowledge on the microsomal metabolism of hydrazone derivatives. The hydrazone compounds were promising derivatives in medicinal chemistry field, therefore, it is vital to investigate their biological

profile before clinical studies. On the light of foregoing, we have designed and synthesized adamantane hydrazide-hydrazone derivatives from aldehyde and ketone. The ketone derivative compound is original molecule. The *in vitro* metabolism studies for both compounds were performed to establish the potantial stability of ketone substrate over the aldehyde hydrazone.

METHODOLOGY

All the chemicals were purchased from Sigma Aldrich and Merck. All the substrates and potential metabolites were synthesized according to the reported procedures [3, 24].

Melting point was recorded on a Stuart SMP50 Automatic Melting Point apparatus and uncorrected. The structures of adamantane derivatives were confirmed by FT-IR, LC-MS spectra. FT-IR analysis were performed with Thermo Scientific Nicolet IS10 device. LC-MS separation of adamantane derivatives were performed by an Agilent 1260 Infinity II LC-MS chromatographic system comprised of a G7115A 1260DAD WR detector, a G7311B 1260 Quad Pump system, a G1328C 1260 Manual Injection unit and a G6125B LC/MSD detector. An ACE C18 column was used as a stationary phase. NMR spectra were recorded on Bruker 400 MHz (Billerica, MA) for ^{1}H-NMR. Data are reported as follows: chemical shift, multiplicity (b.s.: broad singlet, d: doublet; m: multiplet, s: singlet, and t: triplet), coupling constants (Hz), integration. Adult male Suffolk white pigs were used in this study. ß-Nicotinamidedinucleotidephosphate (disodium salt, NADP) and glucose-6-phosphate (disodium salt, G-6-P) were purchased from Sigma. Glucose-6-phosphate dehydrogenase suspension (Reinheit grade II, 10 mg per 2 ml; G-6-PD) was obtained from Sigma Aldrich. Dichloromethane was obtained from Merck.

Experimental

Synthesis

Methyl adamantane-1-carboxylate: This compound was synthesized according to method presented in the literature [3]. Yield: 97% mp: 35-36°C. FT-IR υmax (cm^{-1}): 2927, 2850 (C-H), 1732 (C=O), 1450, 1425 (C-H), 1238 (C-O) [8].

Adamantane-1-carbohydrazide (M1): This compound was synthesized according to method presented in the literature [3]. Yield: 95% mp: 147-148°C. FT-IR υmax (cm^{-1}): 3332, 3275 (N-H), 2908, 2848 (C-H), 1616 (C=O), 1521 (N-H), 1456, 1367 (C-H) [8].

N'-[(1*E*)-1,1-Diphenylmethylidene]adamantane-1-carbohydrazide: (S1) Adamantane-1-carbohydrazide (M1) (0.01 mol) was dissolved in ethanol and equimolar amount of benzophenone was added. The reaction mixture was refluxed

for 2 hours and monitored by TLC. After the reaction completed, the mixture was evaporated under atmospheric pressure and solid product was crystallized with methanol. M.p 204-205°C. Yield: 88% FT-IR υmax (cm^{-1}): 3367 (N-H), 2903, 2874 (C-H), 1689 (C=O), 1521 (N-H), 1489 (C=N). ^{1}H-NMR (300 MHz, DMSO-d_6):1.4-2.1 (m, 17H, Adamantane and DMSO), 7.4-7.8 (m, 10H, Ar-H), 8.8 (s, 1H, CH=N). ^{13}C-NMR (75 MHz, DMSO-d6): 27.78, 36.26, 38.56, 39.52, 39.53, 39.74, 39.95, 40.15, 40.36, 40.57, 127.76, 128.67, 128.97, 130.21, 130.30, 130.47, 137.28 (CH=N), *the C=O peak was not detected due to noise*. MS-APCI (m/z): [M+1] 359. LC-MS: [M+1] 359.

N'-[(4-Chlorophenyl)methylidene]adamantane-1-carbohydrazide (S2): This compound was synthesized according to method presented in the literature [3]. M.p 205-207°C. Yield: 82% FT-IR υmax (cm^{-1}): 3289 (N-H), 2903, 2850 (C-H), 1647 (C=O), 1602 (C=N),), 1521 (N-H), 1489, 803 (C-Cl). MS-APCI (m/z): [M+1] 317; [M+2] 319. LC-MS: [M+1] 317.

[(4-Chlorophenyl)methylidene]hydrazine (M6): 4-chlorobenzaldehyde (M5) (0.001 mol) was refluxed with hydrazine hydrate (5ml) in the presence of ethanol. After the reaction completed, the mixture was evaporated under atmospheric pressure and solid product was crystallized with ethanol. M.p 59-61°C. Yield: FT-IR υmax (cm^{-1}): 3065 (C-H), 1605 (C=N). MS-APCI (m/z): [M+1] 155. LC-MS: [M+1] 155.

(Diphenylmethylidene)hydrazine (M4): Benzophenone (M2) (0.001 mol) was refluxed with hydrazine hydrate (5ml) in the presence of ethanol. After the reaction completed, the mixture was evaporated under atmospheric pressure and solid product was crystallized with ethanol. M.p 96-97°C. Yield: 74% FT-IR υmax (cm^{-1}): 3422, 3262 (N-H), 3187, 3054 (=C-H), 1580 (C=C), 1491 (C=N). MS-APCI (m/z): [M+1] 197. LC-MS: [M+1] 197.

LC-MS analysis

A methanol/water mixture was used with a gradient elution as a mobile phase. The substrates and their potential metabolites were separated according to their mass/charge ratio and their molecular ion peaks were determined. The retention times (Rt) of the substrate and possible metabolites were recorded. A DAD detector was also used to compare UV spectra of standard and metabolic products.

Biological studies

The animals were deprived of food overnight prior to sacrifice, but were allowed water ad libitum. They were previously fed on a balanced diet. Hepatic washed microsomes were prepared as described by Schenkman and Cinti [25] and Ulgen [26].

Incubation and extraction procedures

Incubations were carried out in a shaking water-bath at 37°C using a standard co-factor solution consisting of NADP (2 μmole), G-6-P (10 μmole), G-6-PD suspension (1 unit) and aqueous $MgCl_2$ (50% w/w) (20 μmole) in phosphate buffer (0.2M, pH 7.4, 2 ml) at pH 7.4. Co-factors were pre-incubated for 5 min to generate NADPH, before the addition of microsomes (1 ml equivalent to 0.5 g original liver) and substrate (5 μmole) in methanol (5 μl). Incubation was continued for 30 min, terminated and extracted with dichloromethane (3x5 ml). Organic extracts were evaporated to dryness under a steream of nitrogen [10]. The residues were reconstituted in 200 μl of methanol for LC-MS. The reconstituted extracts were analysed using the reverse-phase LC-MS system described in the text.

RESULTS AND DISCUSSION

Chemistry

The ester and hydrazide derivatives of adamantane were synthesized according to the reported method [3]. Both IR and LC-MS results were in accordance with the expected data. Briefly, adamantane carboxylic acid was reacted with methanol in the presence of concentrated sulphuric acid to give methyl adamantane-1-carboxylate. IR and LC-MS studies proved to formation of ester derivative. The carbonyl peak was shifted from 1701 to 1732 cm^{-1} confirming the ester carbonyl in IR studies (Table 1).

Table 1. Chromatographic and spectroscopic properties of the substrates and their potential metabolites

Compound (abbreviation)	M.w. (g/mol)	Molecular ion peak [M+1] (m/z)	IR peak (C=O) (cm^{-1})	IR peak (C=N) (cm^{-1})	LC-MS retention time (min)
S1	358.49	359	1689	-	
S2	316.83	317	1647	-	4.56
M3	150.24	151	1732	-	NT
M1	194.27	195	1616	-	3.39
M4	196.25	197	-	1516	3.95
M6	154.60	155	-	1501	6.61

LC-MS results indicated the molecular weight of the compound. To synthesize hydrazide derivative, methyl adamantane-1-carboxylate was heated with hydrazine-hydrate in the presence of methanol. The formation of the hydrazide (adamantane-1-carbohydrazide) was also proved with IR and LC-MS studies. The carbonyl peak resulting from hydrazide formation shifted from 1732

cm^{-1} to 1616 cm^{-1} in IR studies. LC-MS and ^{1}H-NMR spectra also proved to formation of hydrazide (Figure S1 and Figure S2). The substrate synthesis was performed using simple addition reaction steps. Both N'-[1,1-diphenylmethylidene]adamantane-1-carbohydrazide and N'-[(4-chlorophenyl)methylidene] adamantane-1-carbohydrazide were synthesized in the presence of ethanol and addition of benzophenone and 4-chlorobenzaldehyde respectively. The formation of hydrazone structure were proved with IR and LC-MS studies; following the presence of hydrazide C=O and azomethine –CH=N- peaks in IR and moleculer weight in LC-MS. Finally, we performed the synthesis of possible metabolites; (diphenylmethylidene)hydrazine and [(4-chlorophenyl)methylidene] hydrazine. The synthetic procedures for compounds were already reported in literature; therefore we simply performed the synthesis and proved the formation of the compounds by comparison with previous chromatographic and spectroscopic data.

LC-MS

Adamantane 1-carboxylic acid and 1-adamantanecarbohydrazide have no detectable UV absorbtion. Therefore, we were only able to determine the compounds by their MS spectrum. In order to determine the suitable mobile phase for the seperation of both substrate and their potential metabolites, few experimental procedures were performed. Hydrazone functional groups generally do not need any buffer medium in liquid chromatography methods. However, a buffer was necessary for aldehyde/ketone hydrazones (M4 and M6) and other possible metabolites. First attempts were made by using buffer medium as mobile phase. Acetonitrile/phosphate buffer and methanol/phosphate buffer systems were used. The seperation of the substrate and possible metabolites were achieved. Unfortunately, we were unable to identify the molecular ions in MS detector due to the strong shielding effect of triethylamine in the buffer solutions. Therefore, we immediately developed a buffer free mobile phase system. After several attempts, a gradient elution with a methanol/water mixture was found to be the best system for the separation of the substrates and their potential metabolites.

In vitro metabolic experiments were performed for two different substrates. Substrate 1 (S1) was synthesized from ketone, benzophenone derivative. Substrate 2 (S2) was synthesized from an aldehyde derivative. Both of the substrates have hydrazone functionality. Literature data mostly suggested the hydrolytic degredation of hydrazone compounds in *in vitro* metabolism studies [5]. Here in this study, we found a stable hydrazone compound, consisting of two large hydrophylic phenly ring. Following the metabolic experiments, no

hydrolytic change was observed for S1 (Figure 1). However, S2 resulted with the hydrolysis of hydrazone structure (Figure 2). The control experiments with denaturated microsomes or in the absence of co-factors were also carried out to establish whether hydrolysis was enzymatic and co-factor dependent. For S2, enzymatic hydrolysis was observed both in the presence of enzyme and co-factors and in the absence of co-factors, indicating that the reaction was enzymatic and co-factor independent (Figure 3). In the control experiment with denaturated enzyme but with co-factors, no hydrolytic metabolite was observed. The authentic and metabolically formed hydrolytic products were compared with their MS spectra (Figure 4).

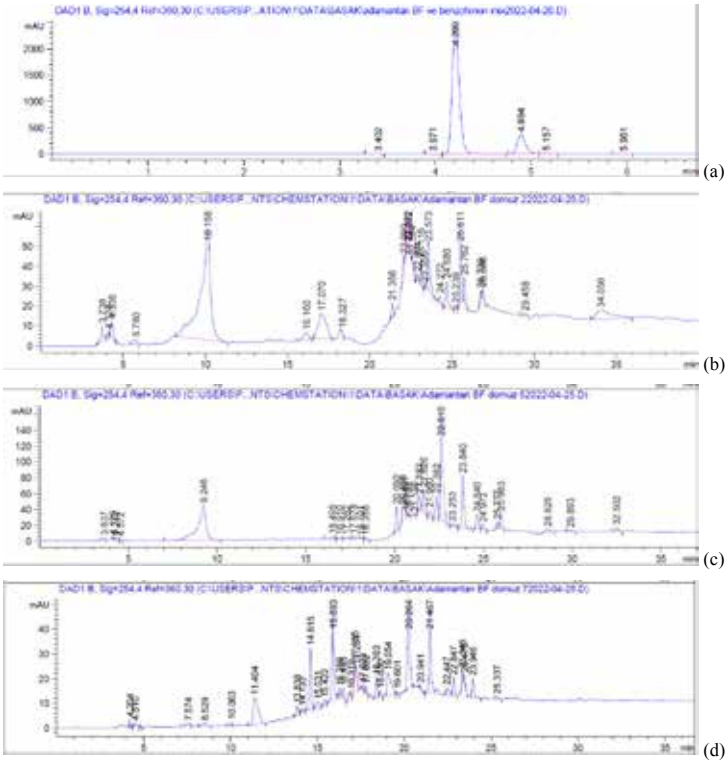


Figure 1. HPLC chromatogram of (a) S1 and its potential metabolites; S1 (4.209 min) and M2 (4.894 min); (b) HPLC chromatogram from test incubation mixture; HPLC chromatogram of S1 test: S1 (10.15 min), the other peaks resulted from microsomal mixture, no hydrolytic product was observed (c) HPLC chromatogram from control experiment with denaturated microsomes; HPLC chromatogram of S1 control (no microsome): S1 (9.24 min), the other peaks resulted from microsomal mixture, no hydrolytic product was observed (d) HPLC chromatogram from control experiment without co-factors; HPLC chromatogram of S1 control (no co-factor): S1 (11.404 min), the other peaks resulted from microsomal mixture, no hydrolytic product was observed.

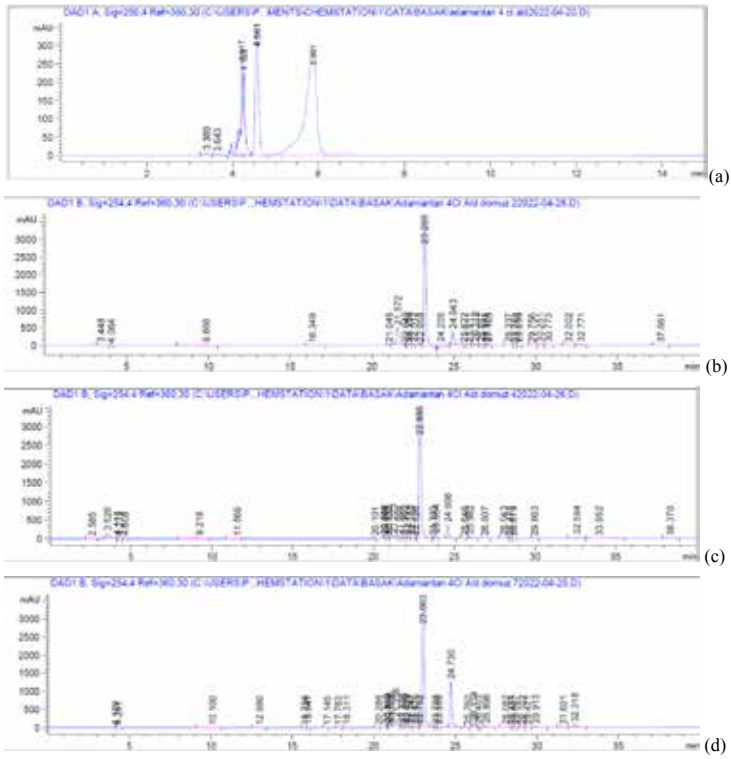


Figure 2. HPC chromatogram of (a) substrate 2 and its potential metabolites; HPLC chromatogram of S2 (4.561 min), M5 (5.901 min), M1 (3.917 min) (b) HPLC chromatogram from test incubation mixture; HPLC chromatogram of S2 test: S2 (23.206 min), M5 (24.943 min), M1 (NT), the other peaks resulted from microsomal mixture (c) HPLC chromatogram from control experiment with denaturated microsomes; HPLC chromatogram of S2 control (no microsome): S2 (22.856 min), M5 (NT), M1 (NT), the other peaks resulted from microsomal mixture (d) HPLC chromatogram from control experiment without co-factors; HPLC chromatogram of S2 control (no-cofactor): S2 (23.003 min), M5 (24.730), M1 (NT), the other peaks resulted from microsomal mixture.

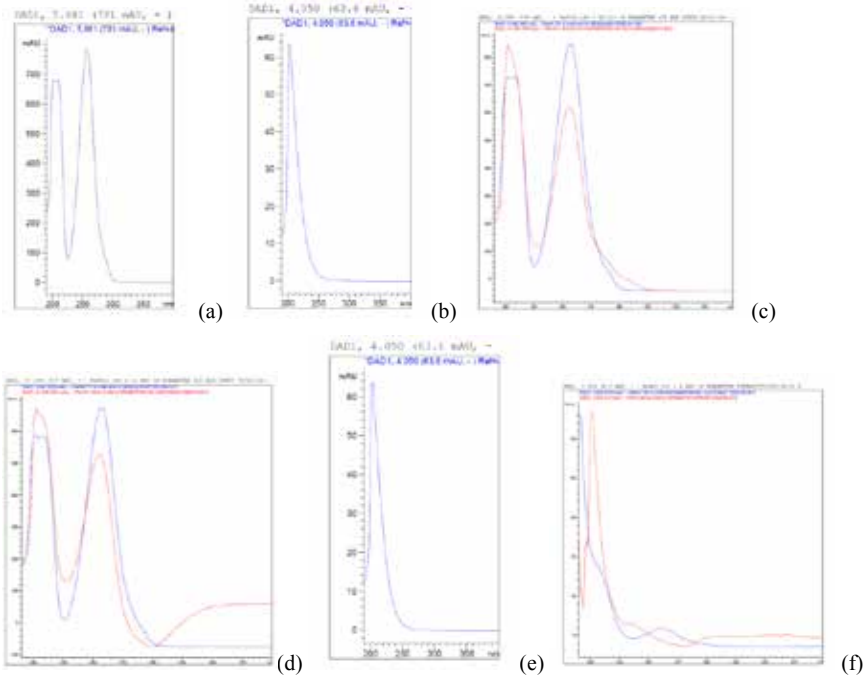


Figure 3. (a) UV spectra of M5 standard; (b) UV spectra of M1 standard; (c) Comparison of UV spectra from standard M5 and metabolically formed S1 test (22.010 min); (d) Comparison of UV spectra from standard M5 and metabolically formed M5 and S2 control with no co-factor (21.652 min); (e) M1 standard UV; (f) Comparison of UV spectra from standard M1 and metabolically formed S2 test (3.463 min); (d), (e), and (f) UV spectrum of S2 with pig microsomes fortified with NADPH

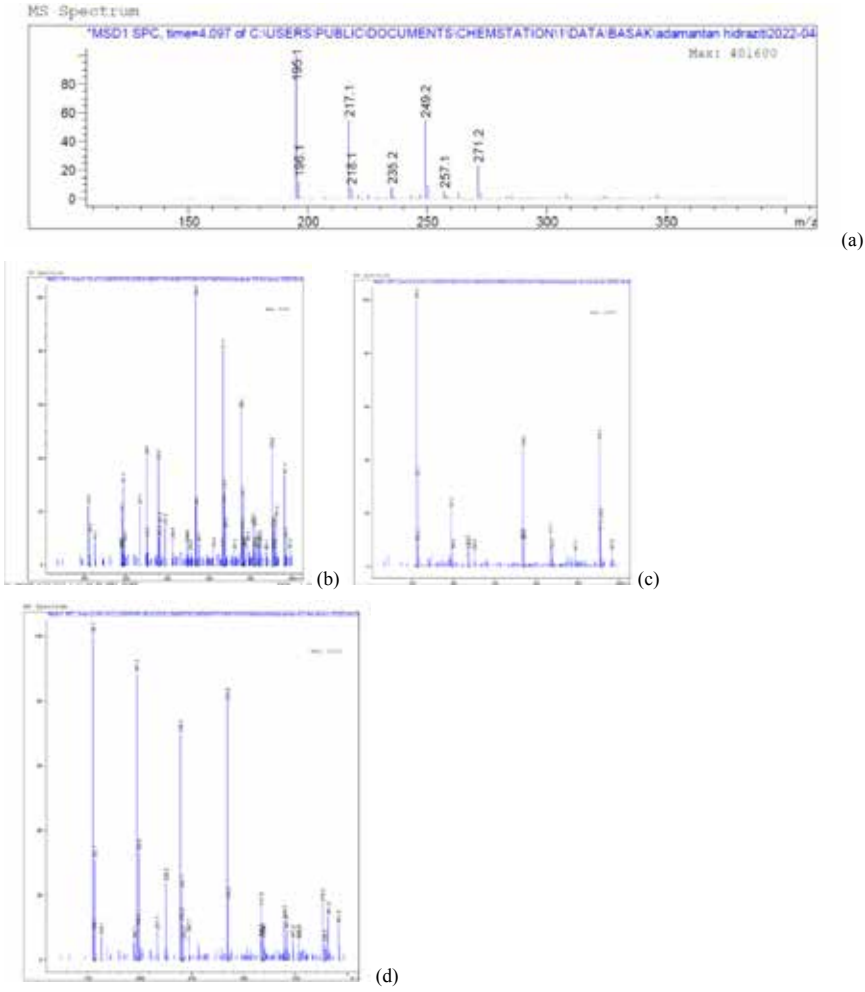


Figure 4. (a) MS spectrum of M1 (m/z=195); (b) MS spectrum of S2 test (2.13 min); (c) MS spectrum of S2 control (with denaturated microsome) (3.41 min); (d) MS spectrum of S2 control (with nocofactor) (2.45 min); (b), (c) and (d) Mass spectrum of S2 with pig microsomes fortified with NADPH

The UV visibility and a certain UV detection was not applicable for adamantane hydrazide as the compound have no UV absorbance. The formation of metabolites was determined with an MS detector. Another challenge within this study was the ionization of aldehyde standard was not observed. Even if we tried different mobile phases and also a gradient elution, aldehyde was not observed in MS detector. Finally a UV detector was used for aldehyde (M5) and an MS detector was used for hydrazide (M1) seperately. On both detectors, we successfully showed the formation of hydrolysis only on S2 but not with S1 (Figure 5).

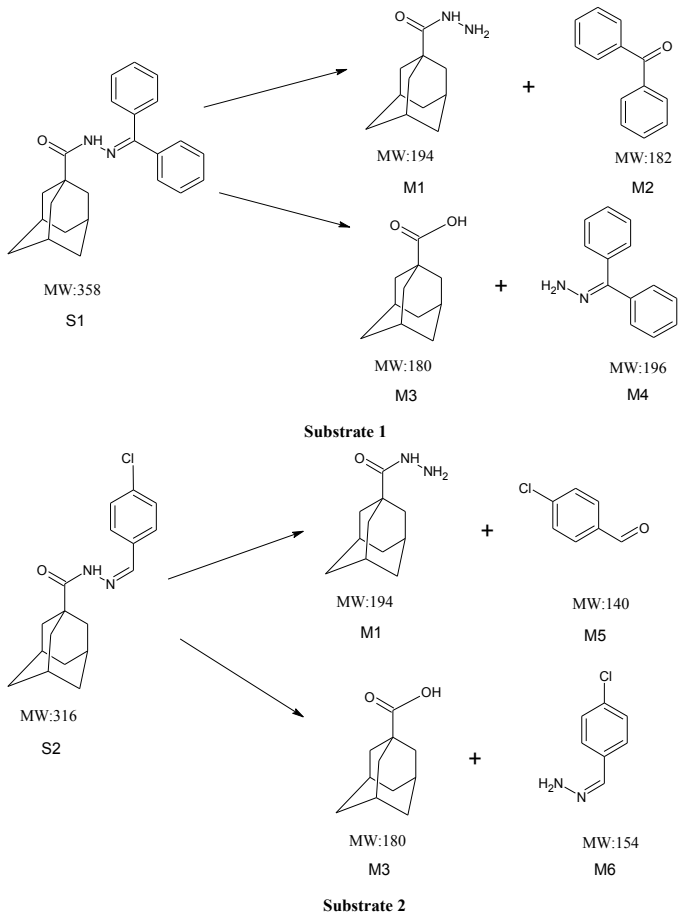


Figure 5. Potential metabolic profile of S1 and S2 and molecular weights of metabolites (see text for abbreviations)

The metabolic stability of ketone-derived hydrazone and aldehyde-derived hydrazone covers the novelty of this study. To the best of our knowledge, there are no comparation metabolic study was performed before. However, some studies revealed the metabolic stability of some different hydrazone compounds. Kovarikova [27] and co-workers investigated the the stability of aromatic hydrazones in plasma and in some different biological enviroment. Three different aroylhydrazones (pyridoxal isonicotinoyl hydrazone, salicylaldehyde isonicotinoyl hydrazone and pyridoxal 2-chlorobenzoyl hydrazone) were synthesized in this study and metabolic stability of those compounds were analysed. The results reveiled that the compounds are prone to degredation to their corresponding aldehyde and hydrazide intermediates in the presence of plasma and therefore, their half-life is short.

Another metabolic stability test for hydrazone molecules was presented by Kalia and co-workers; indicating that the hydrazone structures (the ones with aldehyde derivatives) are susceptible to hydrolysis in biological conditions. The synthesized compounds are derivatives of alkyl or acyl hydrazones. This study also its first representative of the comparition of oxime and hydrazone stability in biological conditions. The authors reveiled that the oximes are more stable than hydrazones; which also correletes with our current results [28].

Currently, no metabolic stability test were presented in the literature containing ketone-derived hydrazone compounds. It can be clearly understood that, even in the phyological contidions, aldehyde-derived hydrazones are hydrolized to their corresponding hydrazide and aldehyde. The existance of one proton in hydrazone, most propably favors the hydrolysis. Within this study, the metabolic enzymes clearly facilitates the hydrolysis on aldehyde-derived hydrazone compound. However, when hydrogen is exchanged with a larger group like phenly, the compound kept its stability both in physological conditions and in enzymatic enviroment.

In conclusion, the present study indicates that the hydrolytic profile of the hydrazone structures mostly depends on their steric hindrance for metabolic resistance. S1 was observed to be more stable to metabolic hydrolysis compared to S2, its aldehyde derivative hydrazone. It was also observed that the hydrazone formation depends on enzymes and do not require the co-factors for hydrolysis. These results indicated the metabolic stability of S1 for future studies.

ETHICAL STATEMENT

The pig livers were donated by Acibadem University, Animal Laboratory Centre from the Project by Dr. Mehmet Emin Aksoy; laparoscopic and robotic surgery, with the 2021-01 ethical approval number. At the end of the training, liver tissue was obtained from the euthanized pig.

Human And Animal Rights

No humans were used in this study. All animal research procedures were followed in accordance with the standards set forth in the eighth edition of Guide for the Care and Use of Laboratory Animals (published by the National Academy of Sciences, The National Academies Press, Washington, D.C.).

CONFLICT OF INTEREST

The authors declare no conflicts of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The pig livers were donated by Acibadem University, Animal Laboratory Centre from the Project by Dr. Mehmet Emin Aksoy; laparoscopic and robotic surgery course, with the 2021-01 ethical approval number. The liver tissue was obtained from the euthanized pig at the end of course. Authors are gratefull to Associate Prof. Füsun Göktaş from Istanbul University, Department of Pharmaceutical chemistry, Faculty of Pharmacy, for providing us 1-carboxyadamantan.

REFERENCES

1. Dembitsky VM, Gloriozova TA, Poroikov VV. Pharmacological profile of natural and synthetic compounds with rigid adamantane-based scaffolds as potential agents for the treatment of neurodegenerative diseases. Biochem Biophys Res Commun. 2020; 529(4): 1225-1241. https://doi.org/10.1016/j.bbrc.2020.06.123

2. Spilovska K, Zemek F, Korabecny J, Nepovimova E, Soukup O, Windisch M, Kuca K. Adamantane - A Lead structure for drugs in clinical practice. Curr Med Chem. 2016; 23: 3245-3266. https://doi.org/10.2174/0929867323666160525114026

3. Pham VH, Phan TPD, Phan DC, Vu BD. Synthesis and Bioactivity of Hydrazide-Hydrazones with the 1-Adamantyl-Carbonyl Moiety. Molecules. 2019; 24(21): 4000. https://doi.org/10.3390/molecules24214000

4. Han MI, Gürol G, Yıldırım T, Kalaycı S, Şahin F, Küçükgüzel ŞG. Synthesis and antibacterial activity of new hydrazidehydrazones derived from benzocaine. Marmara Pharm J. 2017; 21(4): 961-966. https://doi.org/10.12991/mpj.2017.34

5. Küçükgüzel ŞG, Mazı A, Şahin F, Öztürk S, Stables J. Synthesis and biological activities of diflunisal hydrazide-hydrazones. Eur J Med Chem. 2003; 38: 1005-1013. https://doi.org/10.1016/j.ejmech.2003.08.004

6. Rollas S, Küçükgüzel ŞG. Biological activities of hydrazone derivatives. Molecules. 2007; 12: 1910-1939. https://doi.org/10.3390/12081910

7. Aydın S, Basu NK, Arora P, Basu A, Nichols DB, Talele TT, Akkurt M, Çelik İ, Büyükgüngör O, Küçükgüzel ŞG. Microwave assisted synthesis of some novel flurbiprofen hydrazide-hydrazones as anti-HCV NS5B and anticancer agents. Marmara Pharm J. 2013; 17: 26-34. https://doi.org/10.12991/201317389

8. Han Mİ, Yeşil Baysal ÖD, Başaran GŞ, Sezer G, Telci D, Küçükgüzel ŞG. Design, synthesis and anticancer activity studies of novel 4-butylaminophenyl hydrazide-hydrazones as apoptotic inducers. Tetrahedron. 2022; 115: 132797. https://doi.org/10.1016/j.tet.2022.132797

9. Şenkardeş S, Kaushik-Basu N, Durmaz İ, Manvar D, Basu A, Atalay R, Küçükgüzel ŞG. Synthesis of novel diflunisal hydrazideehydrazones as anti-hepatitis C virus agents and hepatocellular carcinoma inhibitors. Eur J Med Chem. 2016; 108: 301-308 https://doi.org/10.1016/j.ejmech.2015.10.041

10. İnce U, Han Mİ. Investigation of the Antimicrobial Activity of Some Ethyl Paraben Hydrazide-Hydrazone Derivatives. Turk J Pharm Sci. https://doi.org/10.4274/tjps.galenos.2022.57699

11. Çıkla-Süzgün P, Küçükgüzel ŞG. Recent Advances in Apoptosis: The Role of Hydrazones. Mini Rev Med Chem. 2019; 19(17): 1427-1442.

https://doi.org/10.2174/1389557519666190410125910

12. Sarıözkan S, Türk G, Çıkla-Süzgün P, Güvenç M, Yüce A, Yay AH, Cantürk F, Küçükgüzel ŞG. Effect of etodolac hydrazone, a new compound synthesised from etodolac, on spermatozoon quality, testicular lipid peroxidation, apoptosis and spermatozoon DNA integrity. Andrologia. 2016; 48(2): 177-188. https://doi.org/10.1111/and.12429

13. Han Mİ, Atalay P, Tunç CÜ, Ünal G, Dayan S, Aydın Ö, Küçükgüzel ŞG. Design and synthesis of novel (S)-Naproxen hydrazide-hydrazones as potent VEGFR-2 inhibitors and their evaluation in vitro/in vivo breast cancer models. Bioorg Med Chem. 2021; 37: 116097. https://doi.org/10.1016/j.bmc.2021.116097

14. Şenkardeş S, Han Mİ, Kulabaş N, Abbak M, Çevik Ö, Küçükgüzel İ, Küçükgüzel ŞG. Synthesis, molecular docking and evaluation of novel sulfonyl hydrazones as anticancer agents and COX-2 inhibitors. Mol Divers. 2020; 24(3): 673-689. https://doi.org/10.1007/s11030-019-09974-z

15. Han MI, Bekçi H, Uba AI, Yıldırım Y, Karasulu E, Cumaoğlu A, Karasulu HY, Yelekçi K, Yılmaz Ö, Küçükgüzel ŞG. Synthesis, molecular modeling, in vivo study, and anticancer activity of 1,2,4-triazole containing hydrazide-hydrazones derived from (S)-naproxen. Arch Pharm (Weinheim). 2019; 352(6): 1800365. https://doi.org/10.1002/ardp.201800365

16. Küçükgüzel ŞG, Koç D, Çıkla-Süzgün P, Özsavcı D, Bingöl-Özakpınar Ö, Mega-Tiber P, Orun O, Erzincan P, Sağ-Erdem S, Şahin F. Synthesis of Tolmetin Hydrazide-Hydrazones and Discovery of a Potent Apoptosis Inducer in Colon Cancer Cells. Arch Pharm (Weinheim). 2015; 348(10): 730-742. https://doi.org/10.1002/ardp.201500178

17. Yapar G, Esra Duran H, Lolak N, Akocak S, Türkeş C, Durgun M, Işık M, Beydemir Ş. Biological effects of bis-hydrazone compounds bearing isovanillin moiety on the aldose reductase. Bioorg Chem. 2021; 117: 105473. https://doi.org/10.1016/j.bioorg.2021.105473

18. Sellitepe HE, Oh JM, Doğan İS, Yildirim S, Aksel AB, Jeong GS, Khames A, Abdelgawad MA, Gambacorta N, Nicolotti O, Mathew B, Kim H. Synthesis of N′-(4-/3-/2-/Non-substituted benzylidene)-4-[(4-methylphenyl)sulfonyloxy] Benzohydrazides and Evaluation of Their Inhibitory Activities against Monoamine Oxidases and β-Secretase. Applied Sciences. 2021; 11(13): 5830. https://doi.org/10.3390/app11135830

19. Wassel MMS, Ragab A, Elhag Ali GAM, Mehany ABM, Ammar YA. Novel adamantane-pyrazole and hydrazone hybridized: Design, synthesis, cytotoxic evaluation, SAR study and molecular docking simulation as carbonic anhydrase inhibitors. J Mol Struct. 2021; 1223: 128966. https://doi.org/10.1016/j.molstruc.2020.128966

20. Komurcu SG, Rollas S, Ulgen M, Gorrod JW, Cevikbas A. Evaluation of some arylhydrazones of p-aminobenzoic acid hydrazide as antimicrobial agents and their in vitro hepatic microsomal metabolism. Boll Chim Farm. 1995; 134 (7): 375-379. PMID: 7546542

21. Küçükgüzel SG, Küçükgüzel I, Ulgen M. Metabolic and chemical studies on N-(4-chlorobenzyl)-N'-benzoylhydrazine. Farmaco. 2000; 55(9-10): 624-630. https://doi.org/10.1016/s0014-827x(00)00077-x

22. Eldirdiri N, Ulgen M, Jacob P, Gorrod J. The in vitro metabolism of norcotinine and related biotransformation products by microsomal preparations. Eur J Drug Metab Pharmacokinet. 1997; 22: 385-390. https://doi.org/10.1007/BF03190974

23. Rahman AFMM, Al-Shakliah NS, Yin W, Kadi AA. In vitro Investigation of Metabolic Profiling of a Potent Topoisomerase Inhibitors Fluorescein Hydrazones (FLHs) in RLMs by LC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci. 2017; 1054: 27-35. https://doi.org/10.1016/j.jchromb.2017.03.041

24. Turk-Erbul B, Karaman EF, Duran GN,Ozbil M, Ozden S, Goktas F. Synthesis, in vitro cytotoxic andapoptotic effects, and molecular docking study of noveladamantane derivatives. Arch Pharm. 2021; 354: e2000256. https://doi.org/10.1002/ardp.202000256

25. Schenkman JB, Cinti DL. Preparation of microsomes with calcium. Methods Enzymol 1978; 52: 83-89. https://doi.org/10.1016/s0076-6879(78)52008-9

26. Ulgen M. Techniques in in vitro oxidative microsomal phase I drug metabolism. J Pharm Univ Mar. 1993; 9(2): 165-185.

27. Kovaříková P, Mrkvičková Z, Klimeš J. Investigation of the stability of aromatic hydrazones in plasma and related biological material. J Pharm Biomed Anal. 2008; 47(2): 360-370. https://doi.org/10.1016/j.jpba.2008.01.011

28. Kalia J, Raines RT. Hydrolytic stability of hydrazones and oximes. Angew Chem Int Ed Engl. 2008; 47(39): 7523-7526. https://doi.org/10.1002/anie.200802651.

Acta Pharm. Sci. Vol 61:(1), 2023
DOI: 10.23893/1307-2080.APS6106

Biological activity and chemical composition of the essential oil from the fruits of *Ferula halophila* Peşmen

Mine KÜRKÇÜOĞLU[1*], Gökalp İŞCAN[1], Fatma TOSUN[2], Mahmut MİSKİ[3]

[1] Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, Eskisehir, Türkiye

[2] Department of Pharmacognosy, School of Pharmacy, Istanbul Medipol University, Istanbul, Türkiye

[3] Department of Pharmacognosy, Faculty of Pharmacy, Istanbul University, Istanbul, Türkiye

ABSTRACT

The hydrodistilled essential oils of the dried fruits of *F. halophila* Peşmen, an endemic species growing near Salt Lake in Central Anatolia, were analyzed by GC and GC-MS systems; 27 and 24 compounds representing 92.1% and 79.8% of the essential oils were characterized, respectively. The main component of the essential oils was identified as β-phellandrene. The antibacterial and anticandidal effects of the essential oils were determined by using partly modified CLSI methods M7-A7 and M27-A2, respectively. The essential oils from two specimens showed weak to moderate inhibitory effects on the tested pathogenic bacteria (MIC, 125-2000 μg/mL) and *Candida* panels (MIC, 156-1250 μg/mL).

Keywords: Antibacterial, anticandidal, essential oil, *Ferula halophila*

INTRODUCTION

The genus *Ferula* (Apiaceae) comprises more than 220 species[1] and is widespread throughout the Mediterranean area and Central Asia. It is represented by 24 species, 13 of which are endemic in the Flora of Turkey[2-4].Several species, such as *Ferula assa-foetida*, *Ferula gummosa* and *Ferula latisecta* have been used in folk medicine to treat stomachache, hysteria, infant colitis, and asthma[5]. The extracts of *Ferula persica*, *Ferula mongolica*, *Ferula ferulago* and *Ferula sinaica* have been used in traditional medicine as antidiabetic[6], abortive[7], an-

*Corresponding author: E-mail: mkurkcuo@anadolu.edu.tr,
ORCIDs:
Mine Kürkçüoğlu: 0000-0002-9375-0294
Gökalp İşcan: 0000-0003-1210-0490
Fatma Tosun: 0000-0003-2533-5141
Mahmut Miski: 0000-0003-2653-0563
(Received 01 Dec 2022, Accepted 17 Jan 2023)

tispasmodic[8] and muscle relaxant[9] respectively. Different parts of *Ferula* have been used in treating various diseases such as neurological disorders, inflammations, dysentery, digestive disorders, rheumatism, headache, arthritis, and dizziness[10]. They also have been reported as antipyretic[11], contraceptive[12,13], and smooth muscle relaxant[14]. *F. assa-foetida* is used traditionally in the treatment of diabetes, asthma, epilepsy, stomachache, flatulence, intestinal parasites, weak digestion, bronchitis, and influenza. It was also believed that this plant has aphrodisiac, sedative and diuretic properties[15].

Recent studies have shown *Ferula assa-foetida* gum extract improved the morphological changes of the diabetic pancreas and stimulated the regeneration of the β cells[16]. Several studies showed hypotensive, neuroprotective, memory-enhancing, anti-oxidant, hepatoprotective, antimicrobial, anticarcinogenic, anti-obesity and anthelmintic effects for various species of *Ferula* and their constituents[17,18]. Pharmacological and biological studies indicate that the extracts and compounds of the genus *Ferula* have various biological activities, such as antibacterial[18], anti-inflammatory[19], antihypertensive[20] and cytotoxic[21]. The main phytochemical components present in the genus *Ferula* are coumarins, coumarin esters, sesquiterpenes, sesquiterpene lactones, monoterpene, monoterpene coumarins, prenylated coumarins, sulfur-containing compounds, phytoestrogen, flavonoids and carbohydrates[19].

Previous studies reported that the aerial parts extracts of *Ferula halophila* exhibited antiviral[22], antioxidant, α-amylase, α-glucosidase, tyrosinase and cholinesterase inhibitory activity and contain phenolic compounds[23,24]. Different bioactivities and uses of the *Ferula* species essential oils have been reported[25]. There are no reports in the literature dealing with the essential oil of *F. halophila*. This work is the first report concerning the chemical composition and antibacterial and antifungal activity of the essential oil obtained from the fruits of *F. halophila*. The hydrodistilled essential oils of the dried fruits of endemic species *Ferula halophila* were analyzed by GC and GC-MS and tested for their antibacterial and antifungal activity using micro-broth dilution methods.

METHODOLOGY

Plant material

The fruit specimens of *Ferula halophila* were collected near Salt Lake in Central Anatolia in June and July 2012. A voucher specimen identified by Prof. Dr. H. Duman (Gazi University, Ankara) was deposited in the Herbarium of Gazi University (GAZI Nr. 9898000001582).

Isolation of the essential oils

Dried and crushed fruits of the plant were subjected to hydro-distillation for 3 h using a Clevenger-type apparatus. The oil yields of the fruits collected in June and July were 1.3 and 0.6 %, respectively on a moisture-free basis. The oil was dried over anhydrous sodium sulphate and stored in sealed vials in the dark, at 4°C, ready for GC and GC/MS analyses and biological activity.

GC and GC/MS conditions

GC/MS: The GC/MS analysis was carried out with an Agilent 5975 GC-MSD system. Innowax FSC column (60m x 0.25mm, 0.25μm film thickness) was used with helium as carrier gas (0.8 mL/min.). GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, and kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/min. Split ratio was adjusted 40:1. The injector temperature was at 250°C. MS were taken at 70 eV. Mass range was from m/z 35 to 450.

GC: The GC analysis were done with Agilent 6890N GC system fitted with a FID detector set at a temperature of 300 °C. To obtain the same elution order with GC-MS, simultaneous auto-injection was done on a duplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

Identification of compounds

The components of essential oils were identified by comparison of their mass spectra with those in the Baser Library of Essential Oil Constituents, Wiley GC/MS Library, Adams Library, Mass Finder Library and confirmed by comparison of their retention indices. Alkanes were used as reference points in calculating relative retention indices (RRI). Relative percentage amounts of the separated compounds were calculated from FID chromatograms. The results of the analysis are shown in Table 1.

Table 1. The Composition of the essential oils of *Ferula halophila*

RRI	Compounds	EO1	EO2	IM
1032	α-Pinene	1.0	0.3	t_R, MS
1174	Myrcene	3.3	1.8	t_R, MS
1176	α-Phellandrene	1.7	0.6	t_R, MS
1203	Limonene	1.4	1.1	t_R, MS
1210	β-Phellandrene	71.8	37.8	t_R, MS
1280	*p*-Cymene	0.5	0.5	t_R, MS
1290	Terpinolene	tr	-	t_R, MS
1481	Longipinene	0.3	1.1	MS
1493	α-Ylangene	0.3	2.0	MS
1504	Daucene	0.1	-	MS
1513	Longicyclene	0.1	0.6	t_R, MS
1549	β-Cubebene	0.4	0.9	MS
1550	cis-α-Bergamotene	-	tr	MS
1568	trans-α-Bergamotene	-	0.6	MS
1590	Bornyl acetate	0.3	-	t_R, MS
1612	β-Caryophyllene	-	0.9	t_R, MS
1661	α-Himachalene	0.8	4.5	MS
1687	α-Humulene	0.3	-	t_R, MS
1711	g-Himachalene	1.0	3.8	MS
1729	γ-Himachalene	0.6	2.9	MS
1743	Eremophilene	4.9	7.0	MS
1755	Dauca-8,11-diene	0.2	1.0	MS
1783	β-Sesquiphellandrene	0.1	-	MS
1786	ar-Curcumene	0.2	-	MS
2008	Caryophyllene oxide	0.4	1.2	t_R, MS
2045	β-Himachalene oxide	tr	0.5	MS
2045	Carotol	tr	-	MS
2179	6-Epi-cubenol	-	0.8	MS
2232	α-Bisabolol	0.5	1.9	t_R, MS
2232	2-Himachalen -7-ol	0.8	7.3	MS
2296	Myristicine	1.1	0.7	MS
	Grouped compounds (%)			
	Monoterpene hydrocarbons	79.7	42.1	
	Sesquiterpene hydrocarbons	9.3	25.3	
	Oxygenated sesquiterpenes	1.7	11.7	
	Others	1.4	0.7	
	Total %	92.1	79.8	

EO1 and EO2: Dried fruits essential oils of *F. halophila* collected in June and July. RRI: Relative retention indices calculated against *n*-alkanes; %: calculated from the FID chromatograms; tr: Trace (<0.1 %). Identification method (IM): t_R, identification based on the retention times of genuine compounds on the HP Innowax column; MS, identified on the basis of computer matching of the mass spectra with those of the in-house Baser Library of Essential Oil Constituents, Adams, MassFinder and Wiley libraries and comparison with literature data.

Antimicrobial assay

Antibacterial and anticandidal effects of the samples were evaluated by using partly modified CLSI (formerly NCCLS) micro dilution broth methods M7-A7 and M27-A2 respectively[26,27].

Escherichia coli NRRL B-3008, *P. aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 13311, *Bacillus cereus* NRRL B-3711, *B. subtilis* NRRL B-4378, *Serratia marcescens* NRRL B-2544, *Staphylococcus epidermidis* ATCC 12228, *E. coli* O157:H7 RSSK 234 (RSSK; RSHM National Type Culture Collection Strains of Bacteria), two different strains of *Candida albicans* (clinically isolated, Osmangazi University, Faculty of Medicine, Department of Microbiology and ATCC 90028), *C. utilis* NRRL Y-12968, *C. krusei* NRRL Y-7179, *C. glabrata* (clinically isolated, Osmangazi University, Faculty of Medicine, Department of Microbiology and ATCC 90028) were used as the test microorganisms. Chloramphenicol (Merck), Ampicillin (Merck), Amphotericin-B (Sigma-Aldrich) and Ketoconazol (Sigma-Aldrich) were used as standard antimicrobial agents.

RESULTS AND DISCUSSION

The essential oils of the dried fruits of *F. halophila* collected in June and July were analysed by GC and GC-MS systems; 27 and 24 compounds representing 92.1% and 79.8% of the essential oils were characterized, respectively. The main component of the essential oils was identified as β-phellandrene (72%) collected in June. The other EO distilled from July samples were consist of several sesquiterpenes and decreased β-phellandrene content (38%). Monoterpene hydrocarbons (June and July; 79.7%, 42.1%), sesquiterpene hydrocarbons (9.3%, 25.3%) and oxygenated sesquiterpenes (1.7%, 11.7%) were the main groups present in the oils respectively. Monoterpene hydrocarbons were the most abundant among these groups representing 79.7% collected in June harvest while 42.1% in the sample of collected in July, followed by sesquiterpenes and oxygenated sesquiterpenes 11.0% and 37.0 % respectively. While monoterpenes were high in the essential oil obtained from the plant material collected in June, monoterpenes were decreased and sesquiterpenes increased in the samples collected in July.

The antibacterial and anticandidal effects of the essential oils were determined by using partly modified CLSI methods M7-A7 and M27-A2, respectively. Tables 2 and 3, show that the essential oils from two specimens exhibited weak to moderate inhibitory effects on the tested pathogenic bacteria (MIC, 125-2000 µg/mL) and *Candida* panels (MIC, 156-1250 µg/mL). Interestingly, essential oils obtained from June and July plant samples were demonstrated different bioactivity.

Table 2. Antibacterial effects of F. halophila essential oils (MIC, µg/mL)

Microorganisms	EO1	EO2	S1	S2
Escherichia coli	2000	2000	3.9	1
Pseudomonas aeruginosa	2000	2000	62.5	15.6
Salmonella typhimurium	500	500	3.9	1
Bacillus cereus	1000	1000	7.8	1
Bacillus subtilis	500	125	1.9	1
Serratia marcescens	1000	500	15.6	15.6
Staphylococcus epidermidis	1000	1000	3.9	1
E. coli O157:H7	1000	2000	3.9	1

EO1 and EO2: Dried fruits essential oils of *F. halophila* collected in June and July, **S1:** Chloramphenicol, **S2:** Ampicillin

Table 3. Anticandidal effects of F. halophila essential oils (MIC, µg/mL)

Microorganisms	EO1	EO2	S1	S2
*Candida albicans**	625	625	0.05	0.1
Candida utilis	625	156	1.6	0.05
Candida tropicalis	625	1250	0.2	0.2
Candida krusei	625	312	1.6	0.2
Candida albicans	1250	625	0.1	0.2
Candida glabrata	1250	1250	3.2	0.2

EO1 and EO2: Dried fruits essential oils of *F. halophila* collected in June and July, **S1:** Ketoconazole, **S2:** Amphotericin-B, *: Clinically isolated strain

Except from *E. coli* O157:H7 and *C. tropicalis*, essential oil of the *F. halophila* collected in July were more active against all test microorganisms having MIC values between 125-2000 µg/mL. July sample was also rich in himachalenes. A previous study was reported that the himachalanes were demonstrated antibacterial activity at various doses 46 to 3000 µg/mL (MIC) [28]. In other study a correlation have been found between the antibacterial activity against MRSA and sesquiterpene compounds in *Ferula akitsckensis* essential oil obtained from leaves at budding stage [29]. β-phellandrene-rich June essential oil were showed weaker effects against all test panel between the concentration of 625 to 2000 µg/mL.

To our knowledge, no previous study has examined the antimicrobial effects of *Ferula halophila* essential oils. Furthermore, it was also showed in this study that the plants collected in different months have different inhibitory effects.

In a previous study, dichloromethane extract of the roots of *Ferula halophila* was evaluated for its antimicrobial activity against 36 different pathogenic bacteria and *Candida* strains. *Stenotrophomonas maltophilia* and *Candida albicans* were inhibited by the extract at lowest concentration (0.3 mg/ml). MIC values have been determined for other test strains ranking from 10 mg/ml to 0.3 mg/ml [30].

In other study, antimicrobial activities of water, methanol extracts and their several fractions of the aerial and underground parts of *F. halophila* were screened by using disc diffusion method. *Staphylococcus aureus, Bacillus cereus* and *B. subtilis* were determined as the most susceptible test strains with 7-10 mm inhibition zones. Chloroform fraction were demonstrated moderate antibacterial effects [31].

REFERENCES

1. Plants of the World Online- Royal Botanical Gardens, Kew. https://powo.science.kew.org/taxon/30105171-2#publications

2. Peşmen H. *Ferula* L. In Flora of Turkey and the East Aegean Islands. Davis, PH Ed., Edinburgh University Press, Edinburgh, 1972; Vol.4, pp 440-453.

3. Pimenov MG, Leonov MV. The Asian Umbelliferae biodiversity database (ASIUM) with particular reference to South-West Asian taxa. Turk J Bot. 2004; 28**:** 139–145.

4. Akalın E, Tuncay HO, Olcay B, Miski M. A new *Ferula* (Apiaceae) species from Southwest Anatolia: *Ferula pisidica* Akalın & Miski. Plants. 2020; 9: 740. https://doi.org/10.3390/plants9060740

5. Eigner D, Scholz D. *Ferula asa-foetida* and *Curcuma longa* in traditional medical treatment and diet in Nepal. J Ethnopharmacol. 1999; 67: 1–6.

6. Afifi FU, Abu-Irmaileh B. Herbal medicine in Jordan with special emphasis on less commonly used medicinal herbs. J Ethnopharmacol. 2000; 72: 101–110.

7. Choudhary MI, Baig I, Nur-e-Alam M, Shahzad-ul-Hussan S, Bunderya M, Oyun Z, et al. New *a*-glucosidase inhibitors from the Mongolian medicinal plant *Ferula mongolica*. Helv Chim Acta. 2001; 84: 2409–2416.

8. Abd-el-Razek M, Ohta S, Ahmed AA, Hirata T. Monoterpene coumarins from *Ferula ferulago*. Phytochemistry. 2001; 57:1201-1203.

9. Aqel MB, Al-Khalil S, Afifi F, Al-Eisawai D. Relaxant effects of *Ferula sinaica* root extract on rabbit and guinea pig smooth muscle. J Ethnopharmacol, 1991; 31: 373-381.

10. Tamemoto K, Takaishi Y, Chen B, Kawazoe K, Shibata H, Higuti T, et al. Sesquiterpenoids from the fruits of *Ferula kuhistanica* and antibacterial activity of the constituents of *F. kuhistanica*. Phytochemistry, 2001; 58:763–767.

11. Valencia E, Feria M, Diaz JG, Gonzalez A, Bermejo J. Antinociceptive, antiinflammatory, and antipyretic effects of lippidin, a bicyclic sesquiterpene. Planta Med, 1994; 60: 395–399.

12. Singh MM, Agnihotri A, Grag SN, Agarwal SK, Gupta DN, Kashri G, et al. Antifertility and hormonal properties of certain carotene sesquiterpenes of *Ferula jaeschkeana*. Planta Med**,** 1988; 54(6): 492–494.

13. Parkash AO, Pathak S, Mathur R. Postcoital contraceptive action in rats of hexane extract of the aerial parts of *Ferula jaeschkeana*. J Ethnopharmacol. 1991; 34: 221-234.

14. Al-Khalil S, Aqel M, Afifi F, Al-Eisawai D. Effects of an aqueous extract of *Ferula ovina* on rabbit and guinea pig smooth muscle. J Ethnopharmacol. 1990; 30: 35–42.

15. Iranshahy M, Iranshahi M. Traditional uses, phytochemistry and pharmacology of asafoetida (*Ferula assa-foetida* oleo-gum-resin) A review. J Ethnopharmacol. 2011; 134(1): 1-10.

16. Latifi E, Mohammadpour AA, Hafshejani BF, Nourani H. *Ferula assa-foetida* oleo gum resin ethanolic extract alleviated the pancreatic changes and antioxidant status in streptozotocin-induced diabetic rats: A biochemical, histopathological, and ultrastructural study. J Food Biochem. 2022; 46:e 14191. doi.org/10.1111/jfbc.14191

17. Zahra G, Ramin R, Mohammad RA, Mohammad HB. Anti-inflammatory, anti-oxidant, and immunomodulatory activities of the genus *Ferula* and their constituents: a review. Iran J Basic Med Sci. 2021; 24:1613-1623.

18. Arjmand Z, Hamburger M, Dastan D. Isolation and purification of terpenoid compounds

from *Ferula haussknechtii* and evaluation of their antibacterial effects. Nat Prod Res. 2022; / doi.org/10.1080/14786419.2022.2103558

19. Li Q, Li J-J, Bao X-H, Zhang S-Y, Luo Q, Li K-M, et al. Unusual sesquilignans with anti-inflammatory activities from the resin of *Ferula sinkiangensis*. Bioorg Chem. 2022; 127: 105986.

20. Kazemi F, Mohebbati R, Niazmand S, Shafei MN. Antihypertensive effects of standardized Asafoetida: Effect on hypertension ınduced by angiotensin II. Adv Biomed Res. 2020; doi: 10.4103/abr.abr_106_20.

21. Iranshahi M, Rezaee R, Najafi MN, Haghbin A, Jamal Kasaian J. Cytotoxic activity of the genus *Ferula* (Apiaceae) and its bioactive constituents. Avicenna J Phytomed. 2018; 8 (4): 296-312.

22. Doğan HH, Duman R. The anti HRSV activity of *Ferula halophila* Peşmen aqueous and methanol extract by MTT assay. Trak Univ J Nat Sci. 2021; 22(1): 43-48.

23. Zengin G, Uysal A, Diuzheva A, Gunes E, Jekő J, Cziáky Z, et al. Characterization of phytochemical components of *Ferula halophila* extracts using HPLC-MS/MS and their pharmacological potentials: A multi-functional insight. J Pharm Biomed Anal. 2018; 160: 374-382.

24. Kahraman Ç, Baysal İ, Çankaya İ, Göğer F, Kırımer N, Akdemir ZŞ. Acetylcholinesterase inhibitory activities and LC-MS analysis of the antioxidant *Ferula caspica* M. Bieb. and *F. halophila* Peşmen extracts. J Res Pharm. 2019; 23(3): 543-551.

25. Sonigra P, Meena M. Metabolic profile, bioactivities, and variations in the chemical constituents of essential oils of the *Ferula* genus (Apiaceae). Front Pharmacol. 2021; 11:608649. doi: 10.3389/fphar.2020.608649

26. CLSI (NCCLS) M7-A7, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standard, Seventh Edition, 2006.

27. CLSI (NCCLS) M27-A2 Reference Method for broth dilution antifungal susceptibility testing of yeasts; Approved Standard-Second Edition; Vol.22, No. 15, 2002.

28. Chaudhary A, Sood S, Das P, Kaur P, Mahajan I, Gulati A, Singh B. Synthesis of novel antimicrobial aryl himachalene derivatives from naturally occurring himachalenes. EXCLI journal. 2014; 13, 1216.

29. Utegenova GA, Pallister KB, Kushnarenko SV, Özek G, Özek T, Abidkulova KT, et al. Chemical composition and antibacterial activity of essential oils from *Ferula* L. species against methicillin-resistant *Staphylococcus aureus*. 2018; Molecules. 23(7), 1679.

30. Sahpaz S, Neut C, Abedini A, Miski M. Antimicrobial activity of the sesquiterpene esters of *Ferula halophila* Peşmen. Planta Med. 2013; 79(10): PN19.

31. Baldemir A, Coşkun M, Yıldız S. Antimicrobial activity of *Ferula halophila* Peşmen. Fabad J Pharm Sci. 2006; 2(31): 57-61.

Acta Pharm. Sci. Vol 61:(1), 2023
DOI: 10.23893/1307-2080.APS6107

The optimal method to measure polyamines in serum by using HPLC fluorescence detector

Orhan ÇAKAN[1], Neziha HACIHASANOĞLU ÇAKMAK[2], Feyza BAYRAMOĞLU[3], Türkan YİĞİTBAŞI[3*]

[1] Institute for Pharmacology and Toxicology, Giessen University, Germany

[2] Medical Laboratory Techniques, Vocational School of Health Services, Istanbul Medipol University, İstanbul, Türkiye

[3] Department of Basic Medical Sciences, Department of Medical Biochemistry, Faculty of Medicine, Istanbul Medipol University, İstanbul, Türkiye

ABSTRACT

Measurement of polyamines (putrescine, spermidine and spermine) is important in the monitoring of many metabolic events, as well as in cancer prognosis. However, the lack of method standardization and validation for the measurement of polyamines is a major obstacle. In this study, sample preparation, gradient program, chromatography column, sample concentration studies were attempted and an analysis method was developed for the detection of polyamines in serum by using HPLC fluorescence detector in order to reduce the difficulties experienced with the existing methodologies. This method was validated analytically in accordance with EMA and FDA's guidelines. In this study, Waters Nova-Pak C18 A chromatography column with 3.9 mm, 150 mm and 4 μm specifications was used for the first time. Acetonitrile was used instead of methanol. Thanks to the newly developed gradient, peak purity and sufficient peak separation were ensured and accurate, sensitive, reliable and reproducible method was developed and validated.

Keywords: Polyamines, HPLC, derivatization, O-Phthalaldehyde, N-Acetyl-L cysteine

INTRODUCTION

Polyamines are involved in functions related to cell growth and differentiation, such as DNA synthesis and stability, regulation of transcription, ion channel reg-

*Corresponding author: E-mail: tyigitbasi@medipol.edu.tr
ORCIDs:
Orhan Çakan: 0000-0001-8201-3478
Neziha Hacıhasanoğlu Çakmak: 0000-0002-6183-5015
Feyza Bayramoğlu: 0000-0002-0757-9743
TürkanYiğitbaşı: 0000-0002-0675-1839
(Received 02 Aug 2022, Accepted 30 Oct 2022)

ulation and protein phosphorylation. Their role in cancer metabolism and gut maturation, as well as their antioxidant properties and aging are being investigated. Natural polyamines putrescine (Put), spermidine (Spd), and spermine (Spm) are found in all living cells. The biosynthesis of polyamines in mammalian cells begins with putrescine synthesized from L-ornithine. Spermidine is synthesized from putrescine and spermine, from spermidine[1]. Polyamines (putrescin, spermidine, and spermine) are simple aliphatic primary amines that are fully protonated under physiological conditions, essential components of eukaryotic and prokaryotic cells. They are involved in performing many cell functions including cell growth and differentiation and receptor function. They also affect DNA replication, gene expression, protein synthesis, stabilization of lipids, brain development, nerve growth and regeneration [2]. Excessive production or excessive intake of polyamines is toxic to cells and facilitates cell death by oxidative mechanism [3]. These compounds were also suggested as possible tumor markers [4-7].

Because of its recognized importance in clinical diagnosis, it has become a necessity to produce inexpensive, easy, and reproducible methods of polyamine measurement. Various methods for the analysis of polyamines were studied for many years. Thin Layer Chromatography (TLC), enzymatic assay method, benzoyl chloride and dansyl chloride derivatization methods of polyamines using HPLC UV detector were used for the detection of amines [8-14].

The losses experienced in the sample preparation stage in derivatization methods and some limitations in applications cause serious difficulties in terms of obtaining reproducibility and accurate results [15].

LC-MS systems are systems which offer the most selective, sensitive and reproducible results compared to UV and Fluorescent systems. With the development of MS systems in parallel with technological developments, studies were carried out with HPLC/Quadrupole-Time of Flight (Q-TOF)-MS systems. Liu et al. proposed a method to detect the amounts of polyamines in human plasma through benzoyl chloride derivatization process using the HPLC/Q-TOF-MS system [16].

EMA and FDA are currently publishing guidelines for the validation of biological fluids, and these guidelines are used for analytical method validation of biological fluids[17]. Method validity of biological fluids contain the parameters of accuracy, precision, linearity, selectivity, lower limit of quantification (LLOQ), recovery, intra-day and inter-day repeatability and inter-day repeatability [18, 19].

In this study, we aimed to develop a method for the measurement of polyamine in serum, analytically validated with EMA and FDA guidelines by using HPLC fluorescence detector.

METHODOLOGY

Demographics of the Patient and Control Groups

In this study, healthy individuals who came to Medipol University Mega Hospital Laboratory for health checks were informed regarding the study and their blood was taken after receiving their written consent. 40 healthy control serums were obtained from selected individuals according to the exclusion criteria.

Exclusion criteria were determined as being younger than 18 years of age, older than 40 years of age, smoking and use of alcohol, having kidney function disorders, hypertension, heart disease, osteoarthrosis, diabetes, obesity, cancer, polycystic ovarian disease, inflammatory and infectious diseases.

Collection and Storage of Blood Samples

After 12 hours of fasting,10 ml of venous blood was taken from 40 healthy control groups, which was then incubated for 30 minutes at room temperature in vacuum gel tubes and left to coagulate. After coagulation, the tubes were incubated at 1200 g for 15 minutes at room temperature, following which they were centrifuged, and serum separated. For other tests that were not studied on the same day, the sera were transferred to Eppendorf tubes and stored at -80˚ C until the day of study.

Parameters and Methods Examined in Blood Samples

Polyamines levels (putrescine, spermidine and spermine) measured in the REMER laboratory using the HPLC method. Initially, a serum pool was created from the 40 serum samples collected. Selectivity, calibration/standard curve, accuracy, precision, intra-day and inter-day repeatability, recovery, lower limit of test (LLOQ) and stability parameters were performed as specified in the EMA and FDA validation guidelines[18, 19].

Devices and Equipment Used

Chemicals

The following chemicals were used as Merck; Sodium Acetate Trihydrate, Hydrochloric Acid Fuming 37%, Acetonitrile, Potassium Carbonate and Sodium Tetraborate Decahydrate. The following chemicals were used as Sigma Aldrich; Methanol, Tetrahydrofuran, Perchloric Acid, Benzoic Acid, Potassium Tetraborate Tetrahydrate, O-Phthalaldehyde, N-acetyl-L-cysteine, Brij-35 Solution (Brij-L23), Putrescine-2HCI, Spermidine-3HCl and Spermine-4HCI.

Instruments, System Setup and Chromatographic Requirements

The HPLC system was a Waters Alliance 2695 HPLC system equipped with an analytical column (Waters Nova-Pak C18 3.9mm*150mm*4µm). A Waters 2475 fluorescence detector was used to detect the fluorescence each polyamine derivative, with excitation λ set at 340 nm and emission λ at 450 nm. The HPLC conditions for the of separation were as follows; mobile phase A was (0.1 M sodium acetate; pH 7.2): added 27.3 g sodium acetate (trihydrate) and 96 µl of 6 N HCl to 1.6 l LC grade water and on this solution add 180 ml methanol and 10 ml tetrahydrofuran. The final volume was made up of 2 l with HPLC grade water. The solution was mixed thoroughly. Mobile phase B was LC grade acetonitrile. The HPLC was run at a gradient program. The flow rate was 1.0 mL/ min, with the following gradient: 95%-A, 5%-B to 73%-A, 27%-B in 20 min; 73%-A, 37%-B to 30%-A, 70%-B in 1 min; 30%-A, 70%-B from 21 to 25 min; returning to 95%-A, 5%-B from 25 to 26 min and the column was conditioned between 26 and 30 minutes. The total running time for each sample (including column regeneration on the automated system) was 30 minutes. Column temperature was maintained at 40 °C and autosampler temperature was maintained at 4°C. The in-line pre-column derivatization of polyamines was accomplished by setting the "auto addition" option in the sample set method of the software Waters Empower 3. A volume of 10 µl derivatization + 10 µl blank, standards solutions, sample solutions was injected. After mixing in the derivatization loop, an injection (a total volume of 20 µl) was automatically performed with no delay. Separated polyamines were monitored and peak integration and calculations of concentrations against the standard curve were performed using the Waters Empower 3 software, recording and analyzing the HPLC chromatography data.

Preparation of Standard and Sample Solutions

Preparation of 100 ng/ml Polyamine Standard Solution Mixture: Standard amounts of 18.3 mg putrescine-2HCI, 17.5 mg spermidine-3HCl and 17.2 mg spermine-4HCl were precisely weighed and filled into a 10.0 ml plastic balloon flask. Then 5 ml of deionized water was added, and the mixture was was vortexed thoroughly for 2 minutes. Then the mixture was kept in a cold ultrasonic bath for 1 minute, following which the solution was completed to its volume using deionized water. Then we waited for the mixture temperature to reach ambient laboratory conditions. 10.0 µl of this solution was taken into a 1.5 µl eppendorf tube and 990 µl of deionized water was added to it and mixed in vortex for 30 seconds. 10.0 µl of this solution was transferred to a 1.5 ml Eppendorf tube. 940 µl of deionized water and 50 µl of 1.2% (w/v) benzoic acid solution were added and mixed in vortex for 30 seconds. The solution was stored at 0-4 ˚C until used [16, 17].

Sample Solution Preparation

The serum sample pool stored at -80 ˚C were dissolved in laboratory conditions and 100 µl of each solution was taken into a 1.5 ml eppendorf tube. 250 µl of deionized water was added and mixed with vortex for 10 seconds. 100 µl of cold 1.5 M $HCIO_4$ was added to this solution and mixed in vortex for 30 seconds. A cold 50 µl of 2 M K_2CO_3 was added and mixed in vortex for 30 seconds. Tube cap was opened, and gas was released for 20 seconds. Then the solution was centrifuged for 3 minutes at 14000 g and 4 ˚C. 200 µl of the supernatant liquid was transferred to a 1.5 ml Eppendorf tube. 750 µl of deionized water and 50 µl of 1.2% (w/v) benzoic acid solution were added and mixed in vortex for 30 seconds. This solution was transferred to the HPLC vial and then injected [16].

Preparation of Polyamine Spiked Sample Solutions

Preparation of Stock Standard Solution: The solution was prepared as in 100 ng/ml Polyamine standard solution mixture. 25.0 µl of this solution was taken into a 1.5 µl eppendorf tube and 975 µl of deionized water was added to it and mixed in vortex for 30 seconds.

Spike Sample Solution Preparation: The sera of 6 healthy individuals stored at 80 ˚C were dissolved in laboratory conditions and 100 µl of each was taken into a 1.5 ml eppendorf tube. 240 µl of deionized water was added and mixed in vortex for 10 seconds. On top of this solution, 10.0 µl of the stock standard solution and 100 µl of cold 1.5 M $HCIO_4$ were added and mixed in vortex for 30 seconds. Cold 50 µl of 2 M K_2CO_3 was added and mixed in vortex for 30 seconds. Tube caps were opened to enable gas escape. Then the solution was centrifuged for 3 minutes at 14000 g and 4 ˚C. 200 µl of the supernatant liquid was transferred to a 1.5 ml Eppendorf tube. 750 µl of deionized water and 50 µl of 1.2% (w/v) benzoic acid solution were added and mixed in vortex for 30 seconds [16, 17].

Validity Tests of the HPLC Method

The validity test of the HPLC method to be used in the determination of polyamine in serum samples in this study was performed based on the parameters of selectivity, calibration/standard curve, accuracy, precision, intra-day repeatability, inter-day reproducibility, recovery, LLOQ and stability specified in EMA and FDA validation guidelines [18,19].

Selectivity

In the HPLC method, to show that the chemicals in the mobile phase, serum matrix, other impurities and standard peaks do not interfere these solutions were injected separately into the HPLC device calibrated according to polyam-

ine standards. Chromatograms of peak areas versus retention times of the solutions were obtained. With the help of PDA detector, the spectra of the injections were taken between 190 nm and 800 nm. Peak purity was determined from the obtained spectra.

Calibration/Standard Curve (Linearity Range) Study

The linearity of the method was determined through repeated analyzes of 8 standards at each concentration, including the LLOQ, in the concentration range of 0.1-200.0 ng/ml. While selecting the working range in both studies, concentration ranges where acceptable accuracy, precision and linearity were obtained were preferred. For the calibration standards to be used during the study, the concentrations are given in Table 1; 8 standards, including putrescine, spermidine, and LLOQ of spermine at 10.0 ng/ml, 50.0 ng/ml, 80.0 ng/ml, 100.0 ng/ml, 150.0 ng/ml, 200.0 ng/ml and 250.0 ng/ml solution was prepared. All solutions were labeled and stored at -20 ˚C in the dark. Solutions were diluted by dissolving in laboratory conditions before each study. In the linearity study, 3 injections for each were studied and the average was calculated, and the linearity graph was drawn. Calibration curves were derived by plotting the peak area ratios obtained against the concentration of the solution in the concentration ranges specified in the study (n=3). Correct equations of the standard curve and correlation coefficients were obtained by regression analysis of the calibration curves.

Table 1: Concentrations of calibration standards

Solution Level	Concentration of Putrescine (ng/ml)	Concentration of Spermidine (ng/ml)	Concentration of Spermine (ng/ml)
LLOQ	0.2	0.5	1.0
10.0%	10.0	10.0	10.0
50.0%	50.0	50.0	50.0
80.0%	80.0	80.0	80.0
100.0%	100.0	100.0	100.0
150.0%	150.0	150.0	150.0
200.0%	200.0	200.0	200.0
250.0%	250.0	250.0	250.0

Accuracy/Precision Study

Quality control solutions were prepared at three different concentrations (50.0, 100.0 and 150.0 ng/ml) corresponding to the concentration range determined in the linearity study. These solutions were analyzed 6 times in 1 day under the same laboratory conditions using the same method for intraday reproducibility analyses, and 6 times in 3 consecutive days, using the same method for intraday reproducibility analyses, under the same laboratory conditions. Accuracy and precision values were calculated from the obtained results. The mean, standard deviation (SD), relative standard deviation (%RSS) and relative errors (%RE) of the analysis results were determined. Accuracy was given with % relative error (%RE) and precision with % relative standard deviation (%RSS).

Recovery Study

Recovery experiments were performed using standard addition method, comparing analytical responses at three different concentrations (low, medium, high) with non-extracted standards (representing 100% recovery). For recovery, 3 parallel samples of each concentration were prepared at three different concentrations and each sample was analyzed 3 times. Theoretical concentrations (Calculated Concentration) were calculated using the concentration curve equation related to the areas obtained from the injection of the recovery samples, versus the standard areas obtained from the linearity range chromatogram. Theoretical concentrations (added concentration) of the standards added on the samples by the standard addition method were calculated.

Calculation was made using the formula % Recovery = (Calculated Concentration/Added Concentration) x100. Recovery solutions were prepared as serum polyamine concentrations of 50, 100 and 150 ng/ml.

Quantity Determination Lower Limit Study (LLOQ)

As a rule of thumb, the signal-to-noise (S/N) ratio was used in chromatographic measurements. The analyte response on the LLOQ was determined to be at least 5 times the response compared to the blanked response. The analyte peak (response) has been shown to be identifiable, discrete, and reproducible with an accuracy of 20% and an accuracy of 80-120%.

LLOQ values for putrescine, spermidine and spermine were obtained from linearity study. The following conditions were fulfilled in the development of the linearity curve.

- 20% deviation of LLOQ from nominal concentration

- 15% deviation of standards other than LLOQ from nominal concentration

At least four of the six nonzero standards met the above criteria, including the LLOQ and the calibration standard at the highest concentration.

Stability

The stability study was performed on serum samples spiked with three different levels of polyamine. The following methods were used for stability determination.

Short Term Temperature Stability

Three aliquots of each analyte at low, medium, and high concentrations were dissolved at room temperature. They were stored under refrigerator (+4 ˚C) storage conditions and analyzed at 0, 2, 4, 8, 12 and 24 hours.

RESULTS and DISCUSSION

EMA and FDA are currently publishing guidelines for the validation of biological fluids, and these guidelines are used for analytical method validation of biological fluids[17]. In the analytical method validation of the method developed considering the parameters specified in these guidelines, selectivity, linearity, accuracy, precision, recovery, intra-day and inter-day reproducibility, LLOQ and solution stability studies were performed. HPLC methods are widely used due to their high sensitivity and repeatability and ease of automation. Our goal is to separate and quantify polyamines using reverse phase chromatography [18]. HPLC technique is the development of a simple, fast, precise, accurate and precise method. The purpose of all of these studies is to develop and validate a method which is usable in clinical practices as a cheap, fast, reproducible and fast. For this purpose, pre-column in-line derivatization method was preferred by using fluorescent detector and o-phthalaldehyde-N-acetyl-L-cysteine reagents in HPLC system.

HPLC Method Validation Tests

Selectivity Study

No other peaks were observed at the retention time of the polyamine peaks and the peak purity was determined accordingly. No interference was found in the retention times of the polyamine peaks in the chromatograms taken under the chromatographic conditions determined as a result of method development studies.

In the selectivity study, the retention times obtained as a result of the injection of the standard solution with a 30-minute gradient program were approximately 19.2 minutes for putrescine and 18.0 minutes for spermidine and 17.1

min for spermine. The purity angle and purity threshold values obtained from the Empower 3 program were 0.570 and 1.103 for putrescine, 0.361 and 0.406 for spermidine, and 0.361 and 0.406 for spermine. In the method developed according to these data, the peak purity is appropriate, and the peaks were obtained pure. As a result of the 30-minute gradient program they applied in the method, Dai et al. found the retention times to be approximately 12.2 minutes for putrescine, 11.1 for spermidine, and 14.1 for spermine [20].

Calibration/Standard Curve (Linearity Range) Study

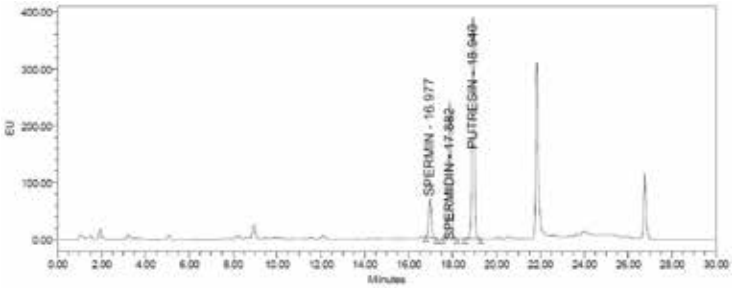
Chromatograms were taken for each of 8 standards, including LLOQ, at concentrations of 0.2-250 ng/ml for putrescine, 0.5-250 ng/ml for spermidine and 1.0-250 ng/ml for spermine, and peak areas were determined. Obtained by HPLC method; Putrescine, spermidine and spermine solution linearity results and statistical analysis values of calibration curve are given in Table 2.

Table 2. Linearity study results of Putrescine, Spermidine and Spermine

Solution Levels	Putrescine Concentration (ng/ml)	Spermidine Concentration (ng/ml)	Spermine Concentration (ng/ml)
LLOQ	0.2	0.5	1,0
10.0%	9,9	10,2	10,0
50.0%	49,6	51,1	50.0
80.0%	79,4	81,7	80.0
100.0%	99,2	102,2	100.0
150.0%	148,8	153,3	150,0
200.0%	198,4	204,4	200.1
250.0%	248.0	255,4	250.1
Linearity Range (ng/ml):	0.2-250.0	0.5 - 250.0	1.0 - 250.0
Slope:	211992.7343	136673.7451	44695.4958
Intercept:	- 163096.5089	- 619833.0382	- 389224.3886
Correlation Coefficient (r^2):	0.9996	0.9981	0.9937

Calibration curves were created by plotting the peak areas obtained against the solution concentrations of putrescine, spermidine and spermine. The correlation coefficient (r^2) found by the least squares method should be ≥0.9950. The r^2 value for putrescine, spermidine, and spermine were 0.9996, 0.9981, 0.9937,

respectively. According to these results, the method; linear for putrescine, spermidine, and spermine. A respective example linearity chromatogram is given below (Figure 1).



	Peak Name	RT	Area	Height
1	SPERMIN	16.977	5902242	680318
2	SPERMIDIN	17.882	20079893	2406922
3	PUTRESIN	18.940	31440893	3912837

Figure 1. Chromatogram of 150% linearity standard

Linearity limits including LLOQ for putrescine, spermidine, and spermine were 0.1-250 ng/ml, 0.5-250 ng/ml, 1-250 ng/ml, respectively. Correlation values were found as 0.9998, 0.9990 and 0.9968, respectively. Liu et al. found linearity limits for each polyamine as 0.4-200.0 ng/ml and correlation values as 0.9941, 0.9939 and 0.9937, respectively [16]. The values found as a result of our study meet the criteria specified in the FDA and EMA validation guidelines [18,19]. LLOQ values for putrescine, spermidine, and spermine were found as 0.2 ng/ml, 0.5 ng/ml and 1.0 ng/ml respectively. Liu et al. found the lower limit of quantitation values in the range of 0.02-0.1ng/ml [16].

Accuracy/Precision Study

Intraday accuracy values for putrescine, spermidine and spermine were 6.3-13.6%, 3.4-8.6%, 1.5-6.0%, respectively. Liu et al. found the intraday accuracy values to be 7.8-9.3%, 5.0-14.0%, 3.9-7.9%, respectively [16]. Intraday precision values for putrescine, spermidine, and spermine were 0.9-1.7%, 0.2-0.3%, and 0.1-0.7%, respectively.

Inter-day accuracy values for putrescine, spermidine, and spermine, were found to be 5.7-12.4%, 6.1-9.1%, 4.5-12.6% respectively. Liu et al. found the intraday accuracy values to be 4.3-9.8%, 7.3-11.1%, and 2.9-3.5%, respectively [16]. Inter-day precision values for putrescine, spermidine and spermine were 6.2-9.7%, 1.6-6.1%, 5.4-13.8%, respectively.

According to FDA criteria, intraday and interday accuracy and precision values should be below 15%[18]. In this study, all values were found below 15% and all criteria were met. Results obtained by HPLC method are given in Table 3 for intra-day and inter-day accuracy and precision values.

Table 3. Intra-day and Inter-day accuracy and precision values

		Intra-day accuracy and precision values			Inter-day accuracy and precision values		
Active Ingredient	**Added Conc. (ng/ml)**	**Calculated Conc. ±SD (ng/ml)**	**RE % (Accuracy)**	**%RSS (Precision)**	**Calculated Conc. ±SD (ng/ml)**	**RE % (Accuracy)**	**% RSS (Precision)**
Putrescine	50	42.6 ±0.4	13.6	0.9	52.7 ±5.1	12.4	9.7
	100	86.1 ±1.5	12.8	1.7	96.0 ±5.9	5.7	6.2
	150	138.7 ±1.3	6.3	0.9	144.6 ±12,9	6.8	8.9
Spermidine	50	49.4 ±0.2	3.4	0.3	49.8 ±3.0	6.1	6.1
	100	93.4 ±0.3	8.6	0.3	87.2 ±1.4	9.1	1.6
	150	147.5 ±0.5	3.8	0.3	128.4 ±3.4	8.5	2.6
Spermine	50	49.9 ±0.06	1.5	0.1	52.6 ±4.5	8.0	8.6
	100	95.3 ±0.7	6.0	0.7	92.1 ±4.8	4.5	13.8
	150	148.6 ±0.3	2.1	0.2	124.3 ±6.7	12.6	5.4

Conc.: Concentration, SD: Standard Deviation, %RSS: Percent Relative Standard Deviation, %RE: Percent Relative Error

Recovery Study

Recovery values were 89.55% for putrescine, 89.62% for spermidine and 88.36% for spermine. Liu et al. found the recovery values to be 80.6% for putrescine, 79.5% for spermidine and 84.0% for spermine [16]. Although 100% recovery of the analyte is required according to FDA guidelines, the degree of recovery of an analyte and internal standard must be consistent, precise, and reproducible [18]. In the study, a high recovery of nearly 100% was achieved and consistent, precise and reproducible results were obtained. These results show that the recovery degree of the method is appropriate. Recovery study results are given in Table 4 for putrescine, Table 5 for spermidine, and Table 6 for spermine.

Table 4. Putrescine recovery study results

Concentration Level	Added Concentration (ng/ml)	Calculated Concentration (ng/ml)	Recovery %
50%	49.33	47.00	95.28
	49.33	47.44	96.17
	49.33	43.45	88.08
100 %	98.67	87.63	88.82
	98.67	82.82	83.93
	98.67	85.86	87.02
150%	148.00	133.24	90.03
	148.00	128.82	87.04
	148.00	132.64	89.62
Average % Recovery Value (ng/ml):		89.55	
Recovery 95% Confidence Interval Limits (ng/ml):		86.53-92.57	
Standard Deviation (ng/ml):		3.93	
Relative Standard Deviation (%):		4.39	

Table 5. Spermidine recovery study results

Concentration Level	Added Concentration (ng/ml)	Calculated Concentration (ng/ml)	Recovery %
50%	51.09	52.31	102.39
	51.09	52.22	102.21
	51.09	48.90	95.71
100 %	102.18	88.13	86.25
	102.18	83.82	82.03
	102.18	86.81	84.96
150%	153.27	130.83	85.36
	153.27	126.93	82.82
	153.27	129.98	84.81
Average % Recovery Value (ng/ml):		89.62	
Recovery 95% Confidence Interval Limits (ng/ml):		83.32-95.92	
Standard Deviation (ng/ml):		8.19	
Relative Standard Deviation (%):		9.14	

Table 6. Spermine recovery study results

Concentration Level	Added Concentration (ng/ml)	Calculated Concentration (ng/ml)	Recovery %
50%	51.46	53.65	104.26
	51.46	49.62	96.43
	51.46	46.71	90.77
100 %	102.92	84.82	82.42
	102.92	81.06	78.76
	102.92	84.79	82.38
150%	154.38	129.61	83.95
	154.38	134.40	87.06
	154.38	137.73	89.22
Average % Recovery Value (ng/ml):		88.36	
Recovery 95% Confidence Interval Limits (ng/ml):		82.23-94.49	
Standard Deviation (ng/ml):		7.98	
Relative Standard Deviation (%):		9.03	

Lower Limit of Quantification (LLOQ) Study

The LLOQ values of the method were determined as 0.2 ng/ml for putrescine, 0.5 ng/ml for spermidine and 1.0 ng/ml for spermine.

Stability Study

For short-term temperature stability study, serum samples with three different levels of polyamine spiked were prepared as described in section stability. They were stored at 4˚C and analyzed at 0, 2, 4, 8, 12 and 24 hours. Areas were determined for each level. The % changes with baseline areas were determined for each study. At the end of the 24th hour, the % change was found to be 9.1% for putrescine, 9.9% for spermidine and 9.8% for spermine. According to these results, solutions of putrescine, spermidine and spermine are stable for 24 hours at 4 ˚C.

As a result, a sensitive, reproducible and reliable method was developed and validated for routine analysis of serum samples in clinical laboratories. This work can be extended to make it applicable to all biological fluids and can be applied for routine analysis.

ETHICAL STATEMENT

Our study was approved by Medipol University local ethics committee (Ethical approval no: 277, date: 28.07.2017)

ACKNOWLEDGMENTS

This article is based on the master's thesis prepared at Istanbul Medipol University, Institute of Health Sciences, Department of Biochemistry.

CONFLICT OF INTEREST STATEMENT

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.

FUNDING SOURCES

There are no any funding sources.

AUTHOR CONTRIBUTIONS

These authors contributed equally.

REFERENCES

1. Williams K. Interactions of polyamines with ion channels. Biochem J.1997; 15(325): 289-97. https:// doi: 10.1042/bj3250289.

2. Alsouz MAK. Separation And Determination Of Benzoylated Polyamines (Spermidine and spermine) Using HPLC Techniqe. Chem. Mater. Res. 2015; 7(1): 33–38 , ISSN 2224- 3224 (Print) ISSN 2225- 0956 (Online)

3. Teti D, Visalli M, McNair H. Analysis of polyamines as markers of (patho)physiological conditions. J Chromatogr B Analyt Technol Biomed Life Sci. 2002; 781(1-2):107-49. https:// doi: 10.1016/s1570-0232(02)00669-4.

4. Ma Y, Liu G, Du M, Stayton I. Recent developments in the determination of urinary cancer biomarkers by capillary electrophoresis. Electrophoresis. 2004; 25(10-11):1473-84.https:// doi:10.1002/elps.200405895.

5. Gerner EW, Meyskens FL. Polyamines and cancer: Old molecules, new understanding. Nat Rev Cancer. 2004; 4(10):781-92. https:// doi: 10.1038/nrc1454.

6. Criss WE. A review of polyamines and cancer. Turkish J. Med. Sci. 2003; 33(4): 195-205.

7. Milovic V. Polyamines in the gut lumen: Bioavailability and biodistribution. Eur J Gastroenterol Hepatol. 2001;13(9):1021-5. https:// doi: 10.1097/00042737-200109000-00004.

8. Peña J, Laespada MEF, Pinto CG, Pavón JLP. Development of a method for the determination of polyamines including N-acetylated forms in human saliva via benzoylation and gas chromatography-mass spectrometry. J Chromatogr A. 2021; 16(1651):462278. https:// doi: 10.1016/j.chroma.2021.462278.

9. Kaneta T. Determination of polyamines by capillary electrophoresis using salicylaldehyde-5-sulfonate as a derivatizing reagent. Methods Mol Biol. 2018; 1694:61-68. doi: 10.1007/978-1-4939-7398-9_5.

10. Vinci G, Maddaloni L, Prencipe SA, Ruggieri R. Natural contaminants in wines: determination of biogenic amines by chromatographic techniques. Int J Environ Res Public Health. 2021; 18(19):10159. doi: 10.3390/ijerph181910159.

11. Seiler N. Identification and quantitation of amines by thin-layer chromatography. J Chromatogr. 1971; 63(1):97-112. doi: 10.1016/s0021-9673(01)85620-x.

12. Isobe K, Yamada H, Soejima Y, Otsuji S. A rapid enzymatic assay for total blood polyamines. Clin Biochem. 1987; 20(3):157-61. doi: 10.1016/s0009-9120(87)80113-3.

13. Sethi R, Chava SR, Bashir S, Castro ME. An Improved High Performance Liquid Chromatographic Method for Identification and Quantization of Polyamines as Benzoylated Derivatives. Am J Anal Chem. 2011; 2: 456-469. https:// doi: 10.4236/ajac.2011.24055.

14. Koski P, Helander IM, Sarvas M, Vaara M. Analysis of polyamines as their dabsyl derivatives by reversed-phase high-performance liquid chromatography. Anal Biochem. 1987; 164(1):261-6. https:// doi: 10.1016/0003-2697(87)90395-2.

15. González O, Blanco ME, Iriarte G, Bartolome L, Maguregui MI, Alonso RM. Bioanalytical chromatographic method validation according to current regulations, with a special focus on the non-well defined parameters limit of quantification, robustness and matrix effect. J Chromatogr A. 2014; 1353:10-27. doi: 10.1016/j.chroma.2014.03.077.

16. Liu R, Bi K, Jia Y, Wang Q, Yin R, Li Q. Determination of polyamines in human plasma by high- performance liquid chromatography coupled with Q-TOF mass spectrometry. J Mass Spectrom. 2012; 47(10):1341-6. doi: 10.1002/jms.3084.

17. Liu R, Li Q, Ma R, Lin X, Xu H, Bi K. Determination of polyamine metabolome in plasma and urine by ultrahigh performance liquid chromatography-tandem mass spectrometry method: Application to identify potential markers for human hepatic cancer. Anal Chim Acta. 2013; 791:36-45. doi: 10.1016/j.aca.2013.06.044.

18. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry, Access.10.07.2021.

19. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf, Access.10.06.2021.

20. Dai Z, Wu Z, Wang J, Wang X, Jia S, Bazer FW, Wu G.Analysis of polyamines in biological samples by HPLC involving pre-column derivatization with o-phthalaldehyde and N-acetyl-L-cysteine. Amino Acids. 2014; 46(6):1557-64. doi: 10.1007/s00726-014-1717-z.