Volume: 5, Issue: 1, June 2022
ISSN: 2619- 9645 (Print)
e-ISSN: 2667- 5722 (On-line, Pdf)

CUPMAP

Current Perspectives on Medicinal and Aromatic Plants
An International Scientific Journal
http://dergipark.gov.tr/cupmap - www.cupmap.org

Current Perspectives on Medicinal and Aromatic Plants

(CUPMAP)

Curr. Pers. MAPs

ISSN: 2619- 9645 (Print) **e-ISSN:** 2667-5722

This journal is international peer-reviewed and published two issues per year.

Corresponding Address
Current Perspectives on Medicinal and Aromatic Plants (CUPMAP),
Nazım Şekeroğlu
Gaziantep University, Faculty of Art and Sciences, 27310, Şehitkamil, Gaziantep - Türkiye
Phone: 0 342-317 19 22
Web: http://www.cupmap.org/
Contact: sekeroglunazim@gmail.com / editor@cupmap.org

Volume 5 **Issue 1** **June, 2022**

Current Perspectives on
Medicinal and Aromatic Plants
Curr. Pers. MAPs

An International Peer Reviewed Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

EXECUTIVE EDITORIAL BOARD

Current Perspectives on Medicinal and Aromatic Plants
Curr. Pers. MAPs

An International Peer Reviewed Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

INTERNATIONAL ADVISORY BOARD

Current Perspectives on Medicinal and Aromatic Plants
Curr. Pers. MAPs

An International Peer Reviewed Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

Current Perspectives on Medicinal and Aromatic Plants
Curr. Pers. MAPs

An International Peer Reviewed Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

JOURNAL INFORMATION

Journal Name	Current Perspectives on Medicinal and Aromatic Plants
Journal Abbreviation	*Curr. Pers. MAPs*
Scope & Subjects	Agriculture, Biology, Molecular Biology & Genetics, Chemistry, Biochemistry, Botany, Ethnobotany, Environmental Science, Forestry, Horticulture, Health Care & Public Health, Nutrition & Food Science, Pharmaceutical Sciences
ISSN	ISSN: 2619-9645 | e-ISSN: 2667-5722
Publisher	Nazım ŞEKEROĞLU
Language	English
Frequency	Biannually (June and December)
Type of Publication	Peer Review Double-Blinded
Publication Fee	Any Submission or Processing Charges
Access type	Open Access Policy
Manuscript Submission System	CUPMAP uses the submission system of TÜBİTAK-ULAKBİM Journal Park Open Journal Systems https://dergipark.org.tr/tr/pub/cupmap
License	Journal is licensed under a Creative Commons Attributions 4.0 International License
Legal Responsibility	Authors are responsible for content of articles that published in Journal.
Indexing and Abstracting	Google Scholar, Index Copernicus International, Worldcat, Elektronische Zeitschriftenbibliothek, Sobiad, Asos Index, Paperity Open Science Aggregated, Crossref, CiteFactor, International Scientific Indexing, Semantic Scholar, Academic Journal Index, Publons
Address	Current Perspectives on Medicinal and Aromatic Plants (CUPMAP), Nazım Şekeroğlu Gaziantep University, Faculty of Art and Sciences, 27310, Şehitkamil, Gaziantep - Türkiye
Web	http://www.cupmap.org/
Contact	Phone: 0 342-317 19 22/2960 E-mail:sekeroglunazim@gmail.com editor@cupmap.org

Current Perspectives on
Medicinal and Aromatic Plants
Curr. Pers. MAPs

An International Peer Reviewed Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

Current Perspectives on Medicinal and Aromatic Plants (CUPMAP)
ISSN: 2619- 9645 (Print) e-ISSN: 2667-5722

Current Perspectives on Medicinal and Aromatic Plants (CUPMAP) is an open access, peer-reviewed and refereed international journal published by MESMAP scientific group. The main objective of the CUPMAP is to provide an intellectual outlook on the scientific researches on Medicinal and Aromatic Plants. CUPMAP have distinguished goals to promote interdisciplinary scientific studies in which results could easily be used in industrial production on MAPs. This international scientific journal publishes research papers related to Medicinal and Aromatic Plants in the fields of science and technology such as Biology, Molecular Biology and Genetics, Chemistry, Agriculture, Biochemistry, Botany, Ethnobotany, Environmental Science, Forestry, Horticulture, Health Care & Public Health, Nutrition and Food Science, Pharmaceutical Sciences, and so on. CUPMAP publishes original research papers, applied studies, and review articles in MAPs science and technology. Special Issues devoted to important topics in the MAPs science and technology could also be published.

CUPMAP Journal publishes **Biannually** (on June and December) in both **print** and **on-line versions**. The publication language of the journal is **English**. Journal of CUPMAP welcomes article submissions and **does not charge any article submission or processing charges**.

Having well known board members distinguished scientists from different disciplines with huge experiences on MAPs all over the world, CUPMAP will be indexed in many databases after first issue. The goal of the journal is to be indexed in Thomson Routers in a short time.

CUPMAP is inviting papers for Volume 5 Issue 2, which is scheduled to be published on December, 2022. Last date of submission: December 16, 2022. However, an early submission will get preference in case of review and publication process. Please submit your manuscripts according to instructions for authors by the Journal online submission system.

Sincerely,
Prof. Dr. Nazım ŞEKEROĞLU
Editor-in-Chief
Current Perspectives on Medicinal and Aromatic Plants (CUPMAP)
Contact: sekeroglunazim@gmail.com / editor@cupmap.org

Current Perspectives on
Medicinal and Aromatic Plants
Curr. Pers. MAPs

An International Peer Reviewed Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

AIM AND SCOPE

Current Perspectives on Medicinal and Aromatic Plants (CUPMAP) is an **open access**, double-blinded **peer-reviewed** and **refereed international** journal published by MESMAP scientific group. The main objective of the CUPMAP is to provide an intellectual outlook on the scientific researches on Medicinal and Aromatic Plants. CUPMAP have distinguished goals to promote interdisciplinary scientific studies in which results could easily be used in industrial production on MAPs. CUPMAP Journal publishes **Biannually** (June and December). The authors should ensure that they have written entirely original works, and if the authors have used the work and/or words of others that this has been appropriately cited or quoted. All submissions are screened by **iThenticate similarity** detection software and our maximum allowed score is **24%** for the document in which the References section truncated.

This international scientific journal publishes high-quality research articles related to Medicinal and Aromatic Plants in the fields of science and technology such as Biology, Molecular Biology and Genetics, Chemistry, Agriculture, Biochemistry, Botany, Ethnobotany, Environmental Science, Forestry, Horticulture, Health Care & Public Health, Nutrition and Food Science, Pharmaceutical Sciences, and so on.

CUPMAP areas of interest include;

- Agricultural Practices of MAPs & NWFPs
- Aromatherapy & Phytotherapy & Phytochemistry
- Biodiversity
- Biology & Biochemistry & Biotechnology
- Botany & Ethnobotany & Ethnopharmacology
- Conservation, Management and Sustainable Uses of MAPs & NWFPs
- Essential Oils & Secondary Plant Metabolites
- Herbal & Traditional Medicines
- Industrial Processing Technologies of MAPs
- Legislations on MAPs & NWFPs
- Literature on MAPS
- Marketing of MAPs and Products
- Molecular Cancer Therapeutics
- Molecular Modeling and Simulations
- Natural Cosmetics
- Non-Governmental & Non-Profit Organizations (NGO & NPO) on MAPs
- Pharmacognosy & Phytopharmacology & Toxicology
- Standardization and Quality of MAP Products
- Traditional & Modern Herbal Products

Current Perspectives on
Medicinal and Aromatic Plants
Curr. Pers. MAPs

An International Peer Reviewed Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

OPEN ACCESS STATEMENT

Current Perspectives on Medicinal and Aromatic Plants (CUPMAP) is a fully open access journal, which means that all articles are **freely available** on the internet without charge to the user or his/her institution.

Except for commercial purposes, users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles, or use them for any other lawful purpose, without asking prior permission from the publisher or the author. This is in accordance with the BOAI definition of open access. All the original articles and review papers published in CUPMAP journal are **free to access** immediately from the date of publication. All authors publishing with the CUPMAP accept these as the terms of publication. Copyright of the layout and design of 'Current Perspectives on Medicinal and Aromatic Plants' articles remains with the journal and cannot be used in other publications.

COPYRIGHT POLICY

Authors who publish with "Current Perspectives on Medicinal and Aromatic Plants (CUPMAP)" agree to the following terms:

Authors transfer the copyright to Current Perspectives on Medicinal and Aromatic Plants (CUPMAP). Authors, however, upon the publication of their work, can republish it in another journal or book provided that they obtain the permission of the Journal. Moreover, they are to include the note indicating that their work has first been published in CUPMAP and the link to their work in the journal.

Authors, upon the publication of their work, can publish it in online environments and/or social networks (personal webpages, course content websites, Facebook, etc.). However, they also provide the link to the work as published in the journal.

Current Perspectives on
Medicinal and Aromatic Plants
Curr. Pers. MAPs

An International Peer Reviewed Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

PUBLICATION CHARGES

No submission, processing, page, colored figures or publication charges are received from authors or elsewhere.

All the original articles and review papers published in CUPMAP journal are **free to access** immediately as early online and on the day of publication on the journal's website. **There is no article processing charges or submission fees for any submitted or accepted articles.**

PEER REVIEW PROCESS

Peer Reviewing Instructions for the "Current Perspectives on Medicinal and Aromatic Plants (CUPMAP)" Journal

Introductions

The primary aims of peer review are to decide whether or not an article should be published (based on quality and relevance to the journal), and to improve the article before publication. All submissions first go through an internal peer review process: an assigned editor makes an initial decision to accept or to reject the manuscript (e.g., topic is outside the scope of the Journal, important flaws in scientific validity, etc.). If the editor believes the article may be of interest, it is sent out for external peer review. The reviewers are selected by area of expertise (reviewers who grant high quality reviews within the requested time are preferred). The editorial board is frequently consulted. Once reviews are obtained, the editor makes a judgment considering the critiques and recommendations from reviewers, and other factors such as relevance to the Journal's aims and usefulness to clinicians or researchers.

Peer Reviewer Selection

Reviewers are selected according to their background and experience in some aspect of the subject. The most desirable reviewers identify the strengths and weaknesses of the submitted paper, and analyze it from different viewpoints. The peer reviewers are asked to read and analyze the assigned manuscript and provide a written opinion of its quality, novelty, relevance and suitability for publication in the "Current Perspectives on Medicinal and Aromatic Plants (CUPMAP)" Journal. Peer reviewers also make suggestions to assist the authors in improving the article. Reviewers must not only analyze and comment on the paper, but also provide opinions about general concerns such as clarity and quality of the writing, validity of scientific approach, and whether the article provides new information.

Current Perspectives on
Medicinal and Aromatic Plants
Curr. Pers. MAPs

An International Peer Reviewed Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

Ethical Guidelines for Journal Peer Reviewers

When a selected individual accepts a peer reviewing assignment, the reviewer implicitly agrees to the ethical standards that are commonly accepted in biomedical publishing. Ethical guidelines for reviewers, authors, and editors are reported by the International Committee of Medical Journal Editors in the 'Uniform Requirements for Manuscripts Submitted to Biomedical Journals' available from: www.icmje.org

Reviewers for the "Current Perspectives on Medicinal and Aromatic Plants (CUPMAP)" Journal must agree to:

Produce as careful and objective a review as possible Respect the editor's deadline. Consider with an open mind innovations or approaches different from those of one's own.

Provide a balanced critique targeted not only to identify the strengths and weaknesses of the paper, but also to provide useful feedback to the authors to improve their manuscript, without being overly critical of minor points.

Avoid scientific misconduct such as the misappropriation of intellectual property.

Each manuscript should be treated as an extremely confidential document.

The privacy of the authors' ideas must always be guaranteed.

Direct comments about ethical concerns confidentially to the editors.

Contacting an author with questions about the manuscript is not allowed.

All critiques, including the latter, must be reported in the written critique.

Declare any conflict of interest (real or perceived) identified to the editor before the end of review. Not every potential conflict necessitates a rejection.

Reviewers are encouraged to discuss potential conflicts with the editors if they believe they can provide a fair review.

Reject an assignment if the following conflicts are present: Financial interests (e.g. paid consultancies, stock holdings), significant professional or personal relationships or rivalries, antipathy toward study question/approach, political or special interest affiliations (e.g. religious or deep convictions that conflict with the manuscript topic).

Current Perspectives on
Medicinal and Aromatic Plants
Curr. Pers. MAPs

An International Peer Reviewed Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

Reviewer Guidelines

Potential reviewers are contacted by e-mail, which contains the manuscript title, abstract, and assignment deadline. The selected reviewer accepts or declines the assignment within 7 days. Failure to reply within the prescribed time will be treated as an implicit rejection. It is acceptable to propose an extended deadline when the given deadline (usually 4 weeks from the task acceptance date) cannot be met. The selected reviewers usually have extensive experience as faculty members, researchers, and published authors. Sometimes reviewers from other specific areas are selected. This selection is always well thought-out, and we encourage such potential reviewers to consider the assignment if they can make a contribution to some aspect of the work. The following points must be provided by the reviewers in the written response:

General Overview

Organized Critique

Assessment of Strengths and Weaknesses: the following should be evaluated: Literature review is up-to-date; Methods align with study purpose or research questions; Methods described in sufficient and appropriate detail; Research design or study approach is adequate; Approach to data analysis is appropriate; Thoughtful consideration given to the study limitations; Manuscript provides new information that is likely to be of interest to our readers.

Possible Improvements

Commonly Overlooked Areas: Reviewers should carefully note: title, abstract, tables and figures, references.

Editor's Final Decision

After the peer review process has ended and an adequate number of reviews has been received, the assigned editor makes the final decision about the manuscript (accept, invite a revision, or reject) based on a consideration of all the reviewer comments, general critique, and other external factors (e.g. the article is consistent with the Journal purpose, similar articles recently published, number of accepted articles awaiting publication, potential impact of the article, etc.). Editors may consult with each other when making the decision. A decision summarizing the opinions of editors and reviewers will be sent to the corresponding author.

Current Perspectives on
Medicinal and Aromatic Plants
Curr. Pers. MAPs

An International Peer Reviewed Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

ETHIC RULES AND PLAGIARISM

Publishers Ethic Rules

"Current Perspectives on Medicinal and Aromatic Plants (CUPMAP)" is an international journal, which publishes at the highest scientific level on original research articles dealing with Medicinal and Aromatic Plants in the fields of science and technology such as Biology, Molecular Biology and Genetics, Chemistry, Agriculture, Biochemistry, Botany, Ethnobotany, Environmental Science, Forestry, Horticulture, Health Care & Public Health, Nutrition and Food Science, Pharmaceutical Sciences, and so on. Originality, high scientific quality, and citation potential are the most important criteria for a manuscript to be accepted for publication. Manuscripts submitted for evaluation should not have been previously presented or already published in an electronic or printed medium. The journal should be informed of manuscripts that have been submitted to another journal for evaluation and rejected for publication. The submission of previous reviewer reports will expedite the evaluation process. Manuscripts that have been presented in a meeting should be submitted with detailed information on the organization, including the name, date, and location of the organization. All authors submitting their works to "Current Perspectives on Medicinal and Aromatic Plants (CUPMAP)" for publication as original articles attest that the submitted works represent their authors' contributions and have not been copied or plagiarized in whole or in part from other works. It is necessary to agree upon standards of expected ethical behavior for all parties involved in the act of publishing: the author, the journal editor, the peer reviewer and the publisher. "Current Perspectives on Medicinal and Aromatic Plants (CUPMAP)" ethic statements are based on COPE's Best Practice Guidelines for Journal Editors.

Ethic Committee Report

The studies have to be prepared according to scientific rules and ethics. The results of any research (especially biology, veterinary, aquaculture and zootechnics) to be published as a manuscript need to have the report of a committee of ethic and that report's copy should be attached with the manuscript itself. Inclusion of the approval letter from the relevant Ethics Committee or Institution's Review Board regarding the research protocol and the rights of the subjects. The author or all the authors whose studies are to be published in the journal will own all kind of liabilities in terms of their manuscripts.

Publication Decisions

The editor is responsible for deciding which of the articles submitted to the "Current Perspectives on Medicinal and Aromatic Plants (CUPMAP)" should be published. The editor may be guided by the policies of the CUPMAP's editorial board and constrained by

such legal requirements as shall then be in force regarding libel, copyright infringement and plagiarism. The editor may confer with other editors or reviewers in making this decision.

Fair Play

An editor at any time evaluate manuscripts for their intellectual content without regard to race, gender, sexual orientation, religious belief, ethnic origin, citizenship, or political philosophy of the authors.

Confidentiality

The editor and any editorial staff must not disclose any information about a submitted manuscript to anyone other than the corresponding author, reviewers, potential reviewers, other editorial advisers, and the publisher, as appropriate.

Disclosure and Conflicts of Interest

Unpublished materials disclosed in a submitted manuscript must not be used in an editor's own research without the express written consent of the author.

Duties of Reviewers

Contribution to Editorial Decisions

Peer review assists the editor in making editorial decisions and through the editorial communications with the author may also assist the author in improving the paper.

Promptness

Any selected referee who feels unqualified to review the research reported in a manuscript or knows that its prompt review will be impossible should notify the editor and excuse himself from the review process.

Confidentiality

Any manuscripts received for review must be treated as confidential documents. They must not be shown to or discussed with others except as authorized by the editor.

Standards of Objectivity

Reviews should be conducted objectively. Personal criticism of the author is inappropriate. Referees should express their views clearly with supporting arguments.

Acknowledgement of Sources

Reviewers should identify relevant published work that has not been cited by the authors. Any statement that an observation, derivation, or argument had been previously reported should be accompanied by the relevant citation. A reviewer should also call to the editor's attention any substantial similarity or overlap between the manuscript under consideration and any other published paper of which they have personal knowledge.

Disclosure and Conflict of Interest

Privileged information or ideas obtained through peer review must be kept confidential and not used for personal advantage. Reviewers should not consider manuscripts in which they have conflicts of interest resulting from competitive, collaborative, or other relationships or connections with any of the authors, companies, or institutions connected to the papers.

Duties of Authors

Reporting Standards

Authors of reports of original research should present an accurate account of the work performed as well as an objective discussion of its significance. Underlying data should be represented accurately in the paper. A paper should contain sufficient detail and references to permit others to replicate the work. Fraudulent or knowingly inaccurate statements constitute unethical behavior and are unacceptable.

Data Access and Retention

Authors are asked to provide the raw data in connection with a paper for editorial review, and should be prepared to provide public access to such data (consistent with the ALPSP-STM Statement on Data and Databases), if practicable, and should in any event be prepared to retain such data for a reasonable time after publication.

Originality and Plagiarism

The authors should ensure that they have written entirely original works, and if the authors have used the work and/or words of others that this has been appropriately cited or quoted. All submissions are screened by a similarity detection software. We are using iThenticate plagiarism solution and our maximum allowed score, for the document in which the References sections truncated, is 24%. Higher scores are not allowed and the

author will be given the plagiarism report and the manuscript will be returned to the authors.

Multiple, Redundant or Concurrent Publication

An author should not in general publish manuscripts describing essentially the same research in more than one journal or primary publication. Submitting the same manuscript to more than one journal concurrently constitutes unethical publishing behavior and is unacceptable.

Acknowledgement of Sources

Proper acknowledgment of the work of others must always be given. Authors should cite publications that have been influential in determining the nature of the reported work.

Authorship of the Paper

Authorship should be limited to those who have made a significant contribution to the conception, design, execution, or interpretation of the reported study. All those who have made significant contributions should be listed as co-authors. Where there are others who have participated in certain substantive aspects of the research project, they should be acknowledged or listed as contributors. The corresponding author should ensure that all appropriate co-authors and no inappropriate co-authors are included on the paper, and that all co-authors have seen and approved the final version of the paper and have agreed to its submission for publication.

Hazards and Human or Animal Subjects

If the work involves chemicals, procedures or equipment that have any unusual hazards inherent in their use, the author must clearly identify these in the manuscript.

Disclosure and Conflicts of Interest

All authors should disclose in their manuscript any financial or other substantive conflict of interest that might be construed to influence the results or interpretation of their manuscript. All sources of financial support for the project should be disclosed.

Fundamental Errors in Published Works

When an author discovers a significant error or inaccuracy in his/her own published work, it is the author's obligation to promptly notify the journal editor or publisher and cooperate with the editor to retract or correct the paper.

Current Perspectives on
Medicinal and Aromatic Plants
Curr. Pers. MAPs

An International Peer Reviewed Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

CUPMAP INSTRUCTIONS FOR THE AUTHORS

- Please read all of the following manuscript preparation instructions carefully.
- Manuscript should be written in **English.**
- Maximum length for articles is **15 pages**. Articles over 15 pages in length can only be considered on an exceptional basis.
- Manuscripts can only be submitted through the CUPMAP **online** manuscript submission and evaluation system https://dergipark.org.tr/tr/pub/cupmap
- The journal management requires that all authors register to **ORCID** at http://orcid.org/register and get a free ORCID ID.
- All submissions are screened by **iThenticate** similarity detection software and our maximum allowed score is **24%** for the document in which the References section truncated.

Manuscript Types

Original Articles: This is the most important type of article since it provides new information based on original research. The manuscript should include an abstract with the following subheadings: "Introduction", "Materials and Methods", "Results and Discussion", and "Conclusion".

Short Communications: Short communication is for a concise to present scientific reports related to scope of the journal. Short communication is not intended to publish preliminary results, but if these results are of exceptional interest and are particularly topical and relevant will be considered for publication. It should include an abstract with the following subheadings: "Introduction", "Materials and Methods", "Results and Discussion", and "Conclusion".

Review Articles: Reviews prepared by authors who have extensive knowledge on a particular field and whose scientific background has been translated into a high volume of publications with a high citation potential are welcomed.

Current Perspectives on
Medicinal and Aromatic Plants
Curr. Pers. MAPs

An International Peer Reviewed Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

CUPMAP STRUCTURE OF THE MANUSCRIPT

Font
Word document, Cambria, 12 point, single line space. Page margins are 2.5 for all sides.

Length
Maximum length for articles is 15 pages. Articles over 15 pages in length can only be considered on an exceptional basis.

Title
A concise title of the paper, avoid Abbreviations and formulae where possible.

- Use bold 14-point Cambria font. Use title uppercase, and make title in centered.
- Name(s) and SURNAME(s) of author(s) [Use centered, bold 12-point Cambria font, Use uppercase for surnames.]
- Affiliation(s) of author(s) [Use 10-point Cambria font.]
- E-mail address of the corresponding author [Use 10-point Cambria font.]
- ORCID(s) of author(s) [Use 10-point Cambria font.]

Abstract
Each article is to be preceded by a succinct abstract, of up to 250 words, that highlights the objectives, methods, results, and conclusions of the paper. The abstract should state briefly the purpose of the research, the principal results and major conclusions. The abstract body is typed in Cambria, 10 pt.

Key Words
Provide a maximum of 6 (six) key words or phrases in order of importance, separated by commas and typed in Cambria, 10 pt.

Headings
Use bold, uppercase, 12 Cambria font for headings.

Introduction
This should define the problem and, if possible, the frame of existing knowledge. Please ensure that people not working in that particular field will be able to understand the intention. The word length of the introduction should be 150 to 300 words.

Materials and Methods
Materials and methods should be clearly presented to allow the reproduction of the experiments.

Results and Discussion

A combined Results and Discussion section is often appropriate. Results should be clear and concise and give the significance of the results of the work. Data must not be repeated in figures and tables. Implications for further studies or application may be discussed.

Conclusion

A short Conclusions section should be added if results and discussion are combined.

Tables and Figures

- Tables should have a short descriptive title.
- The unit of measurement used in a table should be stated.
- Tables should be numbered consecutively.
- Figures should be prepared in GIF, TIFF, JPEG or PowerPoint.
- Tables and Figures should be appropriately cited in the manuscript.

Acknowledgements

Acknowledgements of financial support, advice or other kind of assistance should be given at the end of the text under the heading "Acknowledgements". The names of funding organizations should be written in full.

Author Contribution

The authors should indicate the contribution of the authors.

Conflict of Interest

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

References

They should be grouped at the end of the paper in surname order of appearance (alphabetically ordered). Abbreviated titles of periodicals are to be used according to APA references, but names of lesser-known journals should be typed in full.

References should be styled and punctuated according to the following examples:

Original Papers
Surname, N.N., and Surname, N.N., Year. The full title of the article. Journal Full Name, volume (issue), first and last page. DOI number
Book or Proceeding
Surname, N.N., and Surname, N.N., Year. Title of the contribution. In: Title of the book or proceeding. Volume (Publisher, City), pp. first and last page
Thesis
Surname, N.N., Year. Title of the thesis, University and Faculty, City. pages.

Current Perspectives on
Medicinal and Aromatic Plants
Curr. Pers. MAPs

An International Peer Reviewed Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

CURRENT PERSPECTIVES ON MEDICINAL AND AROMATIC PLANTS (CUPMAP)

An International Scientific Journal - ISSN: 2619- 9645 (Print)

Volume: 5, Issue: 1, June 2022

Current Perspectives on Medicinal and Aromatic Plants
Curr. Pers. MAPs

An International Peer Reviewed Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

---------- Gaziantep University, Publishing House ----------
June, 2022 - GAZİANTEP

Research article
Curr. Pers. MAPs, (2022); 5(1): 1-11

Current Perspectives on Medicinal and Aromatic Plants

An International Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

Physician's acceptance and knowledge in herbal medicine: A cross-sectional study in Northwest Algeria

Amal HELALI*, Khadidja BENCHACHOU, Mostefa TERBECHE, Sid Ahmed YAGOUB

Department of Pharmacy, Faculty of Medicine, University ABOU BEKR BELKAID, 13000, Tlemcen, Algeria,
*Corresponding author: amal.helali@univ-tlemcen.dz

https://doi.org/10.38093/cupmap.1047618

Received: 26/12/2021
Accepted: 23/02/2022

Abstract

The use of medicinal plants in Algeria is done in an anarchic, uncontrolled, and unregulated way. Serious consequences can affect patients and compromise their good care, especially since this use is often associated with the lack of training and knowledge of a large part of Algerian doctors on the benefits and risks of medicinal plants. This study aims to determine the relationship between the knowledge of Algerian physicians on medicinal plants and their acceptance of herbal medicine practice. A cross-sectional study was conducted in different health institutions in two regions (Tlemcen and Aïn-Témouchent). The main tool that was used to collect the required data was a self-administered questionnaire that was specifically developed and approved by the researchers to meet the study objectives. The data collected was processed by R++. The study revealed that 54.85% of the physicians had below average knowledge about plants, and more than half of them acquired their knowledge through self-training. In addition, the results show that 30% of physicians use herbal medicines, while only 12% prescribe them to their patients. Interestingly, a large majority (81.4%) of the participants expressed a desire to improve their knowledge of herbal medicines and there was a highly significant relationship between physician's knowledge and their acceptance of herbal medicine (Spearman's test: *p*-value=0.00066). Almost all physicians (87.55%) agreed that knowledge of medicinal plants is important to them and should be included in the general medical curriculum.

Keywords: Physicians, Herbal medicine, Knowledge, Acceptance, Algeria.

1. Introduction

Over 80% of the population in developing countries rely on traditional medicine, which includes herbal remedies, to manage their health (Zhang & Who, 2002; Mohammed et al., 2021; Pehlivan et al., 2021). There has been an unprecedented explosion in the popularity of herbs in recent decades, particularly in developed countries (Tindle et al., 2005; Kına et al., 2021). This has led to considerable public health concerns among physicians who are sometimes uncertain about the safety of herbs, especially when used with other allopathic medications (Risberg et al., 1999) (Hyodo et al., 2003). Despite these concerns, the global prevalence of herbal use continues to increase, with patients self-medicating with or without informing their physicians (Zhang & Who, 2002). In this context, physicians's knowledge and acceptance of herbal medicine impact the physician-patient

relationship and the overall quality of health care, particularly about possible adverse effects of herbs and herbal-drug interactions (Sardesai, 2002) (Klepser & Klepser, 1999) (Fugh-Berman, 2000) (Brazier & Levine, 2003) (Izzo & Ernst, 2001). Therefore, the physician needs to have a minimum of data on herbal medicine to better advise and manage the management of his or her patient and avoid potentially serious complications.
The use of medicinal plants is important in Algeria (Izzo & Ernst, 2001) (Gardner et al., 2000). Recent studies conducted throughout the national territory, and particularly in the western region, have shown a relatively high prevalence of the use of medicinal plants for the treatment of hypertension (Hassaïne et al., 2019), the therapeutic management of thyroid disorders (Taïbi et al., 2021) and the management of diabetes mellitus (Hamza et al., 2019). This high prevalence of use necessitates the implementation of the most appropriate intervention strategies to facilitate improved health care delivery, including physician knowledge of herbal medicines.

Herbal medicine is not well known to Algerian doctors, and no notion of medicinal plants is part of the medical curriculum, unlike in other countries such as Germany, China, or many Arab countries such as Bahrain, Saudi Arabia, and Jordan, where courses on alternative and complementary medicine have been integrated (Silverstein & Spiegel, 2001). Thus, no previous study has focused on the knowledge and perception of Algerian physicians of herbal medicine.
This study was undertaken mainly to assess the level of acceptance and knowledge of herbal medicine by physicians in Tlemcen and Aïn Témouchent hospitals.

2. Material and Methods

2.1. Type, place and period of the study

Descriptive cross-sectional observational study which was conducted mainly at the University Hospital Center Dr. Tidjani Damerdji - Tlemcen, the Hospital Establishment (EH) Dr. Benzerdjeb - Aïn Témouchent and in a few local public health establishments (EPSP) and a few private medical practices in the two areas. The study was spread over 5 months, from February 2021 to June 2021.

2.2. Study population

For the constitution of the sample of our study, the only inclusion criterion was to be a medical practitioner i.e. holder of a degree “Doctor of Medicine” and practicing as a generalist, resident or specialist either in a public hospital structure or in a private practice.

2.3. Data collection and study process

The collection of data from doctors was done using a questionnaire presented in the APPENDIX which was inspired from several similar surveys (Risberg et al., 1999) (Clement et al., 2005) (Hilal & Hilal, 2017) and subsequently validated by a group comprising three teacher-researchers from the Faculty of Medicine -Tlemcen. The questionnaire contained twenty-nine questions covering three major themes:

The physician's profile: Nine questions concerning the physician's gender, age, field of work, qualification, hospital structure for physicians working in the public domain, department, region, work environment, and number of years of experience.

Physician's acceptance of herbal medicine: this part comprised four questions scored on a scale of 0 to 3 that assessed physician’s use of herbal medicine or herbal products in terms of personal use, prescription or recommendation. The item descriptors were never, rarely, often, and always.

Physician's knowledge of herbal medicine: Sixteen questions:

- Two questioning the physician on the level of his knowledge in herbal medicine and how this knowledge was acquired;

- Three YES/NO questions exploring the physician's knowledge of some medicinal plants, some uses of medicinal plants, some adverse effects associated with medicinal plants or possible interactions between medicinal plants and conventional drugs;

- Eight five-choice, single-answer quiz questions assessing the physician's knowledge of herbal medicine. The choice of these questions and of the 31 plants mentioned in the statements or among the proposals was made based on a literature review of ethnobotanical surveys carried out in Algeria, and particularly in the western region, concerning the plants used in traditional medicine and their toxicity. These eight questions were also scored: the value 1 corresponds to a correct answer, while the value 0 corresponds to either a false answer or a blank answer.

- Finally, three questions were asked about the physician's opinion regarding the lack of training in herbal medicine and whether this constitutes a barrier to its use by physicians, whether he/she would like to improve his/her knowledge in this field, and whether he/she would like to see courses and/or lectures on herbal medicine and its risks integrated into the general medicine training curriculum.

Physicians were asked to respond on the spot if they had time and they were encouraged to respond without a literature search to ensure the accuracy of the results.

2.4. Statistical analysis of data:

The analysis was performed in two steps:
- In the first step, the data were entered using LibreOffice Calc (Free Alternative to Microsoft Excel) and exported in CSV format;
- In a second step, the data were imported, visualized, analyzed and interpreted using R++ (statistical tool based on the programming language for statistics R).

Results were expressed as percentages for categorical variables and as mean ± standard deviation for quantitative variables.
Bivariate analysis was also performed using R++.
The tests used were the following nonparametric statistical tests: Fisher exact, Kruskal-Wallis, Wilcoxon, Spearman and the choice of test depended on the type of variables involved in the bivariate analysis.
The significance levels chosen were 1% and 5%:,
- p-value < 0.01 is very highly significant
- 0.01 < p-value < 0.05 is highly significant
- p-value > 0.05 is not significant.

2.5. Ethical aspect

The questionnaire was filled out anonymously, without access to the answers and without infringing on the health services intended for the patients. All the physicians were informed of the study's nature and interest.

3. Results and Discussion

240 physicians from both areas agreed to take part in the study and to answer our questionnaire (Table 1).

Table 1. Physician's demographic background.

Characteristic	%
Age 39 ± 10.5 years	
Gender	
Men	41.67%
Women	58.33%
Geographic region	
Urban	95%
Rural	5%
Type of institution	
Public	69.17%
Private	30.83%
Qualification	
Generalist	35.15%
Resident	19.66%
Specialist	45.19%
Experience	
Less than 5 years	32%
Between 5 and 10 years	19%
Over 10 years	49%

3.1. Physician's acceptance of herbal medicine

The mean acceptance score was equal to 3.11 ± 1.86 (Maximum score = 12). Half of the physicians did not exceed a score equal to 3 (median) and 75% had a score equal to or less than 4 points, whereas the best score obtained by the respondents was equal to 10 and 13 physicians had a score equal to 0 (Figure 1).

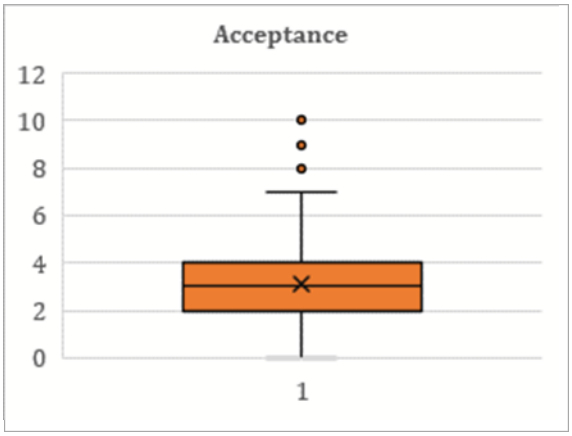


Figure 1: Distribution of physicians by total acceptance score.

Socio-demographic factors did not influence the acceptance scores (Table 2). Approximately 15% of physicians reported almost never having used herbal medicine for personal use. 73.53% never asked their patients about herbal use and 87.45% almost never recommended a plant or herbal product, let alone an herbalist. This result diverges from the study of Clement et al. (2005) in Trinidad and Tobago where most physicians (55.7%) reported having asked their patients about their use of herbal medicines as part of the history taking and 27.1% of the respondents had already recommended the use of herbal medicines to their patients mainly for the management of diseases such as gastric ulcers, prostate enlargement and hepatitis, which supported their acceptance of this modality. And, on the contrary, agrees with that of Hilal et al. (2017) study where the use of herbs by Bahraini physicians was found to be limited. Specifically, approximately 18.8% of the taking part physicians never used herbs, while most of the participants rated their herbal use as rare (Hilal & Hilal, 2017) (Clement et al., 2005). Furthermore, the results presented suggest that physicians tend to use herbal medicines rather than prescribe them to their patients.

However, approximately 13% of our respondents had ever advised their patients about herbal medicines, which is in full agreement with the results of the previously cited study (Clement et al.) and those of another Norwegian study, where only 12% of the physicians supported the idea that herbs could improve symptoms or speed up healing (Clement et al., 2005) (Risberg & Kolstad, 2003). According to the same study, the main reason for rejecting the use of herbs was the lack of scientific information from clinical trials to support the safety and efficacy of herbs in health care management. Physicians even indicated that they did not believe that herbs were safe or beneficial and that their efficacy is not scientifically proven and may give false hope to patients (Clement et al., 2005). To a lesser extent,

another reason for rejecting herbs was the lack of transmission of this knowledge during their medical training. Indeed, physicians rarely question their patients about herbal medicine. This may be because asking a patient about his or her consumption of plants is not necessarily a habit. The problem is not that the physician is uninterested in the subject, nor that he or she deliberately does not want to talk about it, but because it is not his or her habit. Over the years, each doctor has created a standard questioning scheme to be systematic in questioning his patients. This line of questioning was often taught during medical school and became instinctive, and the question "Do you use plants or plant-based products?" was not necessarily the question that doctors learned to ask. However, given the reality that, with or without the help of the attending physician, patients are turning to plants, it would be interesting to be able to accompany them in the best possible way, to be alerted in the event of a change in the state of health or the biological balance of a patient taking plants, more particularly elderly patients, multi-pathological patients, patients with multiple medications, pregnant women and children.

3.2. Physician's knowledge of medicinal plants

64.13% of the physicians surveyed reported having good or moderately good knowledge of herbal medicine and that this knowledge was acquired, generally, through self-study or with experience (55.35% and 40.93% respectively) versus only 3.72% via qualifying training. This diverges and agrees with both the study by Hilal et al. (2017) where half of the participants, with basic knowledge of medicinal plants, indicated that experience was the main source of this knowledge, followed by academic studies (28.1%) and other resources such as the Internet (21.9%) (Hilal & Hilal, 2017). Percentages of 27.5% and 22.92% of the physicians were able to answer correctly to both 2 questions regarding medicinal plants and their traditional uses. Only 17.08% could answer the question on interactions and a higher proportion could answer the questions on toxicity and adverse effects. This result coincides slightly with that found in the study by Clement et al. (2005) in Trinidad and Tobago where 50% of respondents were able to identify at least two Caribbean medicinal plants and their traditional uses, most respondents were unable to identify at least one contraindication and only 15.1% were able to correctly identify a known interaction between a plant and a drug (Clement et al., 2005).

Overall, physicians who reported having a better knowledge of medicinal plants, their traditional uses, drug-plant interactions and adverse or toxic effects had better answers to the different questions of the quiz. This finding is in complete contrast to the results of Jeffrey R et al. (2004) and Clement et al. (2005) where only 12.4% of the 54.7% of physicians who reported knowledge of the Caribbean Pharmacopoeia and medicinal plants were able to identify at least one (Clement et al., 2005) (Suchard et al., 2004). On a scale of 8, the average knowledge score (obtained by answering the quiz questions) was equal to or less than 2.50 ± 1.93 (Maximum score = 8). Half of the physicians did not exceed a score equal to 2 (median) and 75% had a score equal to 4. The highest score obtained by the respondents was equal to 7 and 53 physicians did not answer any question correctly (Figure 2).

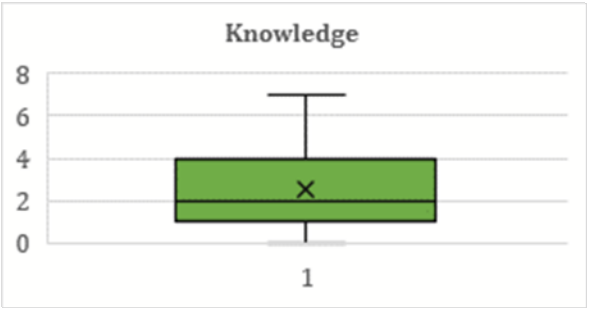


Figure 2: Distribution of physicians by total knowledge score.

Jeffrey R et al. (2004) found similar results in their study in Orange and Los Angeles counties in California with a mean score of 4.63 (on a scale of 16) significantly different from that obtained with a random sample. The same result was also observed in another study conducted in Trinidad and Tobago by Clement et al. (2005), which used open-ended questions as an approach to assess physician's knowledge, where the average score did not exceed 15% (Clement et al., 2005) (Suchard et al., 2004).

There was no significant relationship between demographic factors and knowledge (Table 2). This result is consistent with those of Clement et al. (2005) regarding gender, hospital site and specialty, and with Jeffrey R et al. (2004) regarding experience. But diverges from Hilal et al. (2017) and Clement et al. (2005) regarding years of experience. Indeed, in both studies, a trend toward a moderate increase in knowledge with years of medical experience was observed, but it was not statistically significant (Hilal & Hilal, 2017) (Clement et al., 2005) (Suchard et al., 2004).

Table 2. Association between demographic factors and knowledge and acceptance of herbal medicine.

Profile	Number (%)	Knowledge mean score (Maximum = 8)	*p*-value knowledge	Acceptance mean score (Maximum = 12)	*p*-value Acceptance
Doctors combined	240 (100)	2.50 ± 1.93		3.11 ± 1.86	
Women	140 (58.33)	2.52 ± 1.83	0.792601	3.28 ± 1.87	0.104202
Men	100 (41.67)	2.48 ± 2.08		2.88 ± 1.84	
Age	222 (92.5)		0.560416		0.258613
Public	166 (69.17)	2.51 ± 1.91	0.974757	3.11 ± 1.75	0.600454
Private	74 (30.83)	2.50 ± 1.99		3.10 ± 2.12	
Generalist	84 (35.15)	2.61 ± 1.98		3.33 ± 2.17	
Specialist	108 (45.19)	2.57 ± 2.02	0.547628	3.04 ± 1.65	0.578465
Resident	47 (19.67)	2.21 ± 1.60		2.83 ± 1.69	
Urban	228 (95.80)	2.49 ± 1.94	0.775219	3.09 ± 1.84	0.46805
Rural	10 (4.20)	2.60 ± 1.84		3.80 ± 2.53	
Less than 5 years	77 (32.35)	2.49 ± 1.92		3.03 ± 1.57	
Between 5 and 10	46 (19.33)	2.59 ± 1.80	0.892474	3.13 ± 1.76	0.977285
Over 10 years	115 (48.32)	2.50 ± 2.01		3.18 ± 2.09	

3.3. Knowledge and acceptance of herbal medicine: what relationship?

The correlation between knowledge and acceptance was significant. The more physicians had knowledge of herbal medicine, the more they accepted its practice (p-value = 0.00066 highly significant with Spearman's test) (Figure 3).

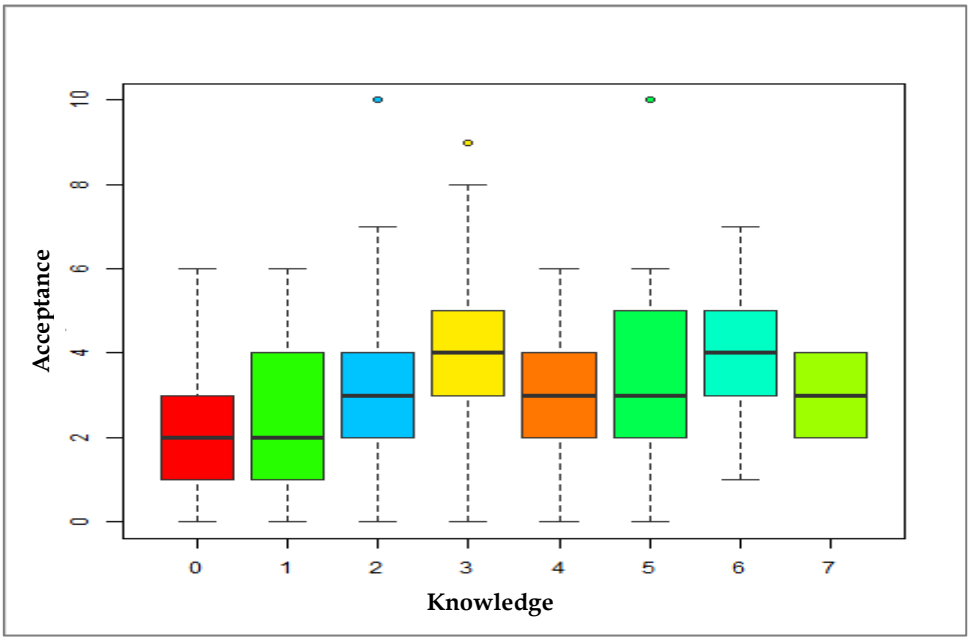


Figure 3: Distribution of physicians according to their knowledge and acceptance scores.

This result is very different compared to the study of Clement et al. (2005) where a marked disparity was shown between acceptance and knowledge of herbs by physicians who had relatively high levels of acceptance with poor knowledge. It is totally in agreement with the results of Hilal et al. (2017) where participants believe that having the knowledge is the main factor that would encourage physicians to use plants (Hilal & Hilal, 2017) (Clement et al., 2005).

3.4. Doctors and plants: a training to develop in Algeria

A large proportion of the respondents to our questionnaire agreed that the lack of training in herbal medicine is a barrier to its use by physicians. Similarly, the majority (95.95%) expressed their desire to improve their knowledge of herbal medicine and were in favor of integrating some courses/lectures on medicinal plants and their risks into the general medicine training curriculum (Table 3).

Our results are consistent with those reported by other studies such as Awodele et al. (2012) for resident physicians in Nigeria, Ghia and Jha (2012) for health professionals in India, Clement et al. (2005) for physicians in Trinidad and Tobago and Hilal et al. (2017) for physicians in Bahrain where most of the physicians surveyed (81.3% and 91.7% respectively for the latter 2 studies) felt that continuing education in herbal medicine is important in facilitating greater physician-patient interaction in this growing area of health care management (Hilal & Hilal, 2017) (Clement et al., 2005) (Afolabi et al., 2012) (Amin & Fattouh, 2017).

Table 3. Distribution of doctors according to their responses on questions 27,28 and 29.

Question	%
Question 27: In your opinion, the lack of training in herbal medicine is a hindrance to its use by Algerian doctors?	
Strongly disagree	3.98
Somewhat disagree	3.10
Neither agree nor disagree	11.50
Somewhat agree	31.42
Strongly agree	50.00
Question 28: Do you want to improve your knowledge of herbal medicine?	
Yes	95.95
No	4.05
Question 29: Do you think that adding some courses/lectures about herbal medicine and its risks in the medical curriculum is necessary to improve this knowledge?	
Strongly disagree	2.15
Somewhat disagree	3.43
Neither agree nor disagree	6.87
Somewhat agree	31.76
Strongly agree	55.79

From the above, it seems clear that a lack of knowledge of herbal medicine may make the physician more reluctant to accept its use. Indeed, while pharmacists receive basic training in herbal medicine during their studies, this is not the case for medical students. Therefore, offering herbal medicine and pharmacognosy education during medical school could interest physicians. Studies have shown that educational interventions on herbal medicine, taught as structured programs through different media, significantly improve physician's knowledge, confidence and interactions with patients. The goal in this case is not to achieve comprehensive training in herbal medicine or to know how to recommend herbs or prescribe herbal products, but to be aware of the major risks associated with herbs and herbal products and therefore be able to consider possible self-medication and assess the risk of drug-herb interactions. Continuing education programs are also recommended so that practicing physicians enhance their knowledge in this rapidly expanding field, which is an important public health issue. In the meantime, physicians should be equipped with books of reputable herbs in their regions, consult trusted journals and electronic websites to further their knowledge of herbs and answer questions that arise during clinical practice.

4. Conclusion

As far as we know, this study is the first one carried out in Algeria on physicians' knowledge of medicinal plants and their acceptance of the practice of herbal medicine. Our results showed that the physicians interviewed had basic knowledge of herbal medicine, knowledge that was not influenced by socio-demographic factors, and that there was a significant relationship between this knowledge and the physician's acceptance of herbal medicine. Almost physicians showed a desire to improve their knowledge of herbal medicine and supported the idea of integrating courses/lectures on this discipline into the curriculum of graduate medical studies. This is not surprising, as they fully agreed that the lack of information about herbal medicine is a barrier to its use in medicine. This creates an interesting scenario where the lack of knowledge, and

the desire to improve, provides an ideal opportunity to facilitate the introduction of educational programs and policies that would increase the knowledge base of health care professionals. Well-informed physicians would be more confident in their interactions with patients, which would improve the quality of health care delivery, as more meaningful communication about important issues such as adverse effects and herbal-drug interactions would be facilitated. The trend toward increased use of herbs is expected to continue for the foreseeable future, and the improved knowledge of physicians will benefit patients who will appreciate being able to discuss their health needs in a judgment-free environment.

Acknowledgements

This work was supported by the Departement of pharmacy, Faculty of Medecine, ABOU BEKR BELKAID University, Tlemcen, Algeria. The authors of this manuscript would like to acknowledge their thanks and appreciation to all the physicians who agreed to willingly take part in this study and to the lecturers in the department of Pharmacy more especially Pr Mustapha Kamel DALI-YAHIA, Pr Mohammed Adil SELKA, Dr Sihem BABA AHMED and Dr Souâd GUENDOUZ for the ethical approval of the research proposal.

Author Contribution

Concept and design: A.H., K.B. Data Collection and processing: M.T., S.A.Y. Data analysis and interpretation: A.H., K.B., M.T. Literature Search: A.H., K.B. Writing: A.H.

Conflicts of Interest

The authors declared no conflict of interest.

References

1. Afolabi, S. O., Akindele, A. J., Awodele, O., Anunobi, C. C., & Adeyemi, O. O. (2012). A 90 day chronic toxicity study of Nigerian herbal preparation DAS-77 in rats. BMC Complementary and Alternative Medicine, 12(1), 79. https://doi.org/10.1186/1472-6882-12-79
2. Brazier, N. C., & Levine, M. A. H. (2003). Drug-herb interaction among commonly used conventional medicines: A compendium for health care professionals. American Journal of Therapeutics, 10(3), 163–169. https://doi.org/10.1097/00045391-200305000-00003
3. Fugh-Berman, A. (2000). Herb-drug interactions. Lancet (London, England), 355(9198), 134–138. https://doi.org/10.1016/S0140-6736(99)06457-0
4. Gardner, J. M., Grant, D., Hutchinson, S., & Wilks, R. (2000). The use of herbal teas and remedies in Jamaica. The West Indian Medical Journal, 49(4), 331–335.
5. Hamza, N., Berke, B., Umar, A., Cheze, C., Gin, H., & Moore, N. (2019). A review of Algerian medicinal plants used in the treatment of diabetes. Journal of Ethnopharmacology, 238, 111841. https://doi.org/10.1016/j.jep.2019.111841
6. Hassaïne S, Saïdi A, Belhadj OA (2019) Ethnobotanical study of medicinal plants used in the treatment of high blood pressure in the region of Tlemcen (Northwestern Algeria). J Pharm Pharmacogn Res 7(1): 1–11.
7. Hilal, M., & Hilal, S. (2017). Knowledge, attitude, and utilization of herbal medicines by physicians in the Kingdom of Bahrain: A cross-sectional study. Journal of the Association of Arab Universities for Basic and Applied Sciences, 24, 325–333. https://doi.org/10.1016/j.jaubas.2016.11.001
8. Hyodo, I., Eguchi, K., Nishina, T., Endo, H., Tanimizu, M., Mikami, I., Takashima, S., & Imanishi, J. (2003). Perceptions and attitudes of clinical oncologists on complementary and alternative medicine: A nationwide survey in Japan. Cancer, 97(11), 2861–2868. https://doi.org/10.1002/cncr.11402
9. Izzo, A. A., & Ernst, E. (2001). Interactions between herbal medicines and prescribed drugs: A systematic review. Drugs, 61(15), 2163–2175. https://doi.org/10.2165/00003495-200161150-00002
10. Kına, E., Uysal, İ., Mohammed, F. S., Doğan, M., & Sevindik, M. (2021). In-vitro antioxidant and oxidant properties of Centaurea rigida. Turkish Journal of Agriculture-Food Science and Technology, 9(10), 1905-1907.
11. Klepser, T. B., & Klepser, M. E. (1999). Unsafe and potentially safe herbal therapies. American Journal of Health-System Pharmacy: AJHP: Official Journal of the American Society of Health-System Pharmacists, 56(2), 125–138; quiz 139–141. https://doi.org/10.1093/ajhp/56.2.125
12. Mohammed, F. S., Kına, E., Sevindik, M., Doğan, M., & Pehlivan, M. (2021). Antioxidant and antimicrobial activities of ethanol extract of Helianthemum salicifolium (Cistaceae). Indian Journal of Natural Products and Resources (IJNPR)[Formerly Natural Product Radiance (NPR)], 12(3), 459-462.
13. Pehlivan, M., Mohammed, F. S., Şabik, A. E., Kına, E., Dogan, M., Yumrutaş, Ö., & Sevindik, M. (2021).

Some Biological activities of ethanol extract of Marrubium globosum. Turkish Journal of Agriculture-Food Science and Technology, 9(6), 1129-1132.
14. Risberg, T., & Kolstad, A. (2003). [Alternative medicine—Attitudes and use among physicians, nurses and administrative staff employed in hospitals in northern Norway]. Tidsskrift for Den Norske Laegeforening: Tidsskrift for Praktisk Medicin, Ny Raekke, 123(5), 604–606.
15. Risberg, T., Kolstad, A., Johansen, A., & Vingerhagen, K. (1999). Opinions on and use of alternative medicine among physicians, nurses and clerks in northern Norway. In Vivo (Athens, Greece), 13(6), 493–498.
16. Sardesai, V. M. (2002). Herbal medicines: Poisons or potions? The Journal of Laboratory and Clinical Medicine, 139(6), 343–348. https://doi.org/10.1067/mlc.2002.124582
17. Silverstein, D. D., & Spiegel, A. D. (2001). Are Physicians Aware of the Risks of Alternative Medicine? Journal of Community Health, 26(3), 159–174. https://doi.org/10.1023/A:1010303528081
18. Suchard, J. R., Suchard, M. A., & Steinfeldt, J. L. (2004). Physician knowledge of herbal toxicities and adverse herb-drug interactions. European Journal of Emergency Medicine: Official Journal of the European Society for Emergency Medicine, 11(4), 193–197. https://doi.org/10.1097/01.mej.0000134721.72356.f7
19. Taïbi, K., Ait Abderrahim, L., Helal, F., & Hadji, K. (2021). Ethnopharmacological study of herbal remedies used for the management of thyroid disorders in Algeria. Saudi Pharmaceutical Journal: SPJ: The Official Publication of the Saudi Pharmaceutical Society, 29(1), 43–52. https://doi.org/10.1016/j.jsps.2020.12.004
20. Tindle, H. A., Davis, R. B., Phillips, R. S., & Eisenberg, D. M. (2005). Trends in use of complementary and alternative medicine by US adults: 1997-2002. Alternative Therapies in Health and Medicine, 11(1), 42–49.
21. Zhang, X., & Who, W. H. O.-. (2002). Traditional medicine strategy 2002□2005. http://whqlibdoc.who.int/hq/2002/WHO_EDM_TRM_2002.1.pdf

APPENDIX . Questionnaire.

Acceptance and knowledge of Algerian doctors in phytotherapy

Profile:

1- **Gender:** Woman ☐ -Man ☐
2- **Age:** .....
3- **Domain:** -Public ☐ -Private ☐
4- **Structure**:
-EHU ☐ -CHU☐ -ESH☐ -EPH☐ -EPSP ☐
5- **Qualification:** -Generalist -Specialist -Resident
6- **Service:** .....
7- **Wilaya:** - Tlemcen ☐ - Aïn-Témouchent ☐
8- **Working environment**: -Urban ☐ -Rural ☐
9- **Experience:** -Less than 5 years ☐ -Between 5 and 10 years ☐ -Over 10 years ☐

Acceptance of herbal medicine:

10- **Do you use herbal medicine on your own?**
-Never ☐ -Rarely ☐ -Often ☐ -Always ☐
11- **During a consultation, do you ask your patients if they use medicinal plants before prescribing a treatment?**
-Never ☐ -Rarely ☐ -Often ☐ -Always ☐
12- **Have you ever recommended or prescribed a herbal medicine or product to your patients?**
-Never ☐ -Rarely ☐ -Often ☐ -Always ☐
13- **Have you ever recommended to your patients to go to an herbalist?**
-Never ☐ -Rarely ☐ -Often ☐ -Always ☐

Knowledge in herbal medicine:

14- **How would you describe your knowledge of herbal medicine?**
-Not at all good ☐ -Moderately good ☐ -Fairly good ☐ -Excellent ☐
15- **How did you acquire this knowledge?**
Self-training ☐ - Qualifying training ☐ -Experience ☐
16- **Do you know some medicinal plants and their uses?** -Yes ☐ -No ☐
17- **Are you aware of any interactions between certain medicinal plants and conventional drugs?** -Yes ☐ -No ☐
18- **Are you aware of any undesirable and/or toxic effects related to the use of certain plants?** -Yes ☐ -No ☐
19- **Milk thistle is a plant known for its activity:**
-Hypoglycemic ☐ -Antiviral ☐
-Hepatoprotective ☐
-Antimicrobial ☐ -Antispasmodic ☐
20- **One of the following plants decreases the absorption of certain substances: iron, zinc, calcium, magnesium, vitamin B12, drugs... which one?**
-Black cumin ☐ -White horehound ☐
-Basil ☐ -Flaxseeds ☐ -Ricin ☐
21- **One of the following plants is used to treat diabetes and is known to be toxic:**
-Thyme ☐ -Coloquint ☐ -Poulliot mint ☐
-Verbena ☐ -Spearmint ☐
22- **The root of a common plant in the Mediterranean region is the cause of serious life-threatening poisoning:**
-Ginger ☐ -Poppy Mint ☐ -Glue Thistle ☐
-Verbena ☐ -Spearmint ☐
23- **One of these plants stimulates the release of LH, which one?**
-Atlas cypress ☐ -Sage ☐
-Celery ☐ -Fenugreek ☐ -Verbena☐
24- **Orally, one of these plants is contraindicated in people with bile duct obstruction:**
-Turmeric ☐ -Caraway ☐ -Laurel ☐
-Thyme ☐ -Licorice ☐
25- **A plant with expectorant and antispasmodic action can induce high blood pressure if consumed for a long time and in high doses:**
-Sesame ☐ -Carob ☐ -Wormwood ☐
-Cinnamon ☐ -Licorice ☐
26- **Prolonged use of any of the following plants may cause hypokalemia and disruption of heart function:**
-Celery ☐ -Senna ☐ -Arbutus ☐
-Flaxseed ☐ -Cinnamon ☐
27- **In your opinion, the lack of training on herbal medicine is a hindrance to its use by Algerian doctors?**
-Strongly disagree ☐ -Somewhat disagree ☐
-Neither agree nor disagree ☐
-Somewhat agree ☐ -Strongly agree ☐
28- **Do you want to improve your knowledge of herbal medicine?** -Yes ☐ -No ☐
29- **Do you think that adding some courses/lectures about herbal medicine and its risks in the medical curriculum is necessary to improve this knowledge?**
-Strongly disagree ☐ -Somewhat disagree ☐
-Neither agree nor disagree ☐ -Somewhat agree ☐ -Strongly agree ☐

Research article
Curr. Pers. MAPs, (2022); 5(1): 12-18

Current Perspectives on Medicinal and Aromatic Plants

An International Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

In vitro Shoot Regeneration of *Lysimachia nummularia* L. in Solid and Liquid Culture Medium

Muhammet DOĞAN*

Department of Nutrition and Dietetics, Faculty of Health Sciences,
Karamanoglu Mehmetbey University, 70200, Karaman, Turkey

*Corresponding author : mtdogan1@gmail.com

https://doi.org/10.38093/cupmap.1057290

Received: 13/01/2022
Accepted: 19/02/2022

Abstract

Lysimachia nummularia L. is a perennial medicinal and aromatic plant. In this study, the effects of solid and liquid culture media, and plant growth regulators on micropropagation of *L. nummularia* were investigated. Nodes were used as an explant. Different combinations of thidiazuron (TDZ: 0.05-0.80 mg L^{-1}) and indole-3-butyric acid (IBA: 0.10 mg L^{-1}) were used as growth regulators in Murashige and Skoog (MS) basal medium. In solid and liquid media experiments, the highest number of shoots was obtained from MS nutrient media supplemented with 0.40 mg L^{-1} TDZ + 0.10 mg L^{-1} IBA. The shoot length was higher in both liquid and solid culture medium supplemented with 0.05 mg L^{-1} TDZ + 0.10 mg L^{-1} IBA. The best results for number of shoots and regeneration ratio were determined in the solid nutrient media according to results . On the other hand, the best results for shoot length were found in the liquid culture media. Increasing doses of TDZ adversely affected shoot length in both culture media. The regenerated plants were successfully acclimatized to aquatic conditions. The results of this study may help the large-scale propagation of *L. nummularia* by tissue culture.

Key Words: *In vitro* propagation, MS medium, Node Explant, Tissue culture

1. Introduction

Medicinal plants have a great value in the treatment of diseases, nutrition and cosmetics industry since ancient times (Solomou et al., 2016). They are used commercially in many industrial sectors today. In addition, studies on medicinal and aromatic plants are carried out in different disciplines such as production of medicinal plants and the extraction of herbal products from them (Kala, 2015).

Lysimachia nummularia L. is a perennial plant belonging to the *Primulaceae*. This herb is known as Creeping Jenny or Moneywort. It is a plant native to Eurasia. It

can live in aquatic or humid environmental conditions (Kodela and Jobson, 2016). *L. nummularia* has been preferred since ancient times for the cure of many disease symptoms. It has also been reported to have antimicrobial properties (Podolak et al., 2013).

Plant tissue culture applications allow the production of many economically valuable plants, especially medicinal and aromatic plants. Recently, it was reported that many medicinal plants such as *Broussonetia papyrifera* L'Hér. Ex Vent (Lin et al., 2021), *Eupatorium glandulosum* L. (Nithya and Kamalam, 2021) and *Mondia whitei* (Hook. F.) Skeels (Patricia et al., 2021) produced by tissue culture.

There are a few studies on the production of *L. nummularia* by tissue culture. (Dogan, 2018; Dogan, 2019a). It is important to ensure optimization in the production of plants via tissue culture methods. Therefore, in this study, the effects of liquid and solid nutrient media and plant growth regulators on the *in vitro* propogation of *L. nummularia* were investigated.

2. Material and Methods

L. nummularia was purchased from aquarium store. Sterilization of the plants was achieved using bleach (5.7% active chlorine-NaOCI-ACE) for 10 min. After rinsing 3 times for 5 min, the node explants were placed to MS (Murashige and Skoog 1962) medium without plant growth regulators.

4.4 g L^{-1} MS basal salts (Duchefa), 30 g L^{-1} sucrose (Duchefa) and 6.5 g L^{-1} agar (Duchefa) were added to the solid MS medium. 4.4 30 g L^{-1} MS basal salts (Duchefa) and 30 g L^{-1} sucrose (Duchefa) were added to the liquid MS medium (no agar). Thidiazuron (TDZ:0.05-0.80 mg L^{-1}) and indole-3-butyric acid (IBA:0.10 mg L^{-1}) were added in both solid and liquid nutrient media at different combinations as growth regulators (Table 1). Propagation studies were completed at the end of eight weeks.

The pH of the culture media was adjusted to 5.7±0.1 via 1N NaOH and 1N HCl and then sterilized in an autoclave (120 °C for 20 min). The sterile nutrient media were poured into sterile plastic petri dishes. The node explants were placed in the petri dishes. These culture dishes were kept under white light emitting diodes (1500 lux) in a photoperiod of 16 hours light and 8 hours dark. The room temperature was set to 24°C.

The rooting experiments were not performed because dense roots were formed in the *in vitro* propagation experiments. For this reason, the stage of acclimatization of the regenerated plants to external conditions was started.

The MS nutrient medium on the regenerated plants was carefully removed. The plants were then placed to an aquatic environment (aquarium conditions were set at 24°C temperature and 16 hours of light).

Experiments were carried out in petri dishes with 6 replications. Data were reviewed with SPSS 21 for the Windows and Duncan was used for Post Hoc tests.

Table 1. The growth regulators used for *in vitro* shoot regeneration

TDZ (mg L^{-1})	IBA (mg L^{-1})
0.05	0.10
0.10	0.10
0.20	0.10
0.40	0.10
0.80	0.10

3. Results and Discussion

3.1. Solid media experiments

The node explants were cultured in solid medium supplemented with TDZ (0.05-0.80 mg L^{-1}) + IBA (0.10 mg L^{-1}). After two weeks from beginning of the culture callus formations and three weeks later shoot formations were observed in the MS media. After eight weeks, multiple shoots were obtained (Figure 1a and b) from cultures.

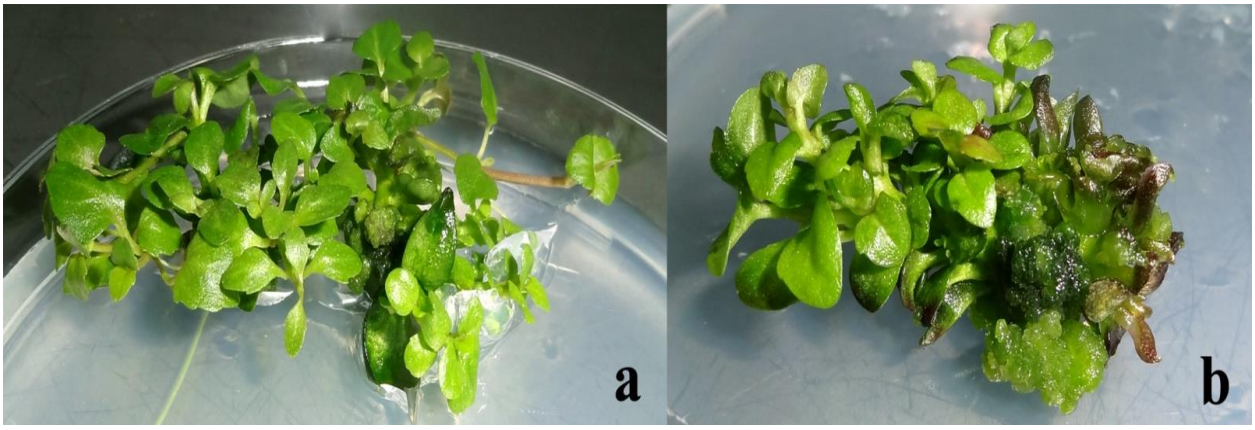


Figure 1. Shoot formations in the solid MS medium supplemented with IBA + TDZ. **(a)** Multiple shoot formations in the solid medium supplemented with 0.20 mg L^{-1} TDZ + 0.10 mg L^{-1} IBA **(b)** 0.40 mg L^{-1} TDZ + 0.10 mg L^{-1} IBA

Table 2. The effect of liquid medium supplemented with IBA + TDZ on *in vitro* shoot formation.

Growth regulators (mg L^{-1})		Regeneracy (%)	Average shoot numbers	Shoot lengths (cm)
TDZ	**IBA**			
0.05	**0.10**	100^{a}	5.39^{d}	1.28^{a}
0.10	**0.10**	77.78^{a}	9.94^{bc}	1.24^{ab}
0.20	**0.10**	72.22^{a}	12.30^{ab}	1.03^{ab}
0.40	**0.10**	94.44^{a}	15.16^{a}	0.95^{ab}
0.80	**0.10**	94.44^{a}	8.38^{cd}	0.81^{b}

Values shown with different letters in the same column are important at the $p<0.01$ level.

As seen on Table 2, shoot regeneration rate was recorded between 72.22 and 100%. 100% regeneration was achieved in the MS medium supplemented with 0.05 mg L^{-1} TDZ.

The number of shoots in MS medium supplemented with TDZ and IBA were recorded as statistically significant at the $p<0.01$ level. The best result in terms of shoot number (15.16 shoots/explant) was achieved in MS medium including 0.40 mg L^{-1} TDZ + 0.10 mg L^{-1} IBA, while the least poor result (5.39 shoots/explant) was obtained in MS medium with 0.05 mg L^{-1} TDZ + 0.10 mg L^{-1} IBA. The least count of shoots was determined at the lowest rates of TDZ (0.05 mg L^{-1}).

Shoot lengths were generally short in MS media including TDZ and IBA. The longest shoot (1.28 cm) was obtained in MS medium with 0.05 mg L^{-1} TDZ + 0.10 mg L^{-1} IBA, while the shortest shoot (0.81 cm) was reached on the MS medium with 0.80 mg L^{-1} TDZ + 0.10 mg L^{-1} IBA. Increasing TDZ dose negatively affected shoot lengths.

3.2. Liquid media experiments

The node explants were placed in liquid nutrient medium including 0.05-80 mg L^{-1} TDZ + 0.10 mg L^{-1} IBA. Multiple shoot formations were observed in the second week (Figure 2 a, b and c). The experiment was terminated in the eighth week of culture. Data were analyzed statistically (Table 3).

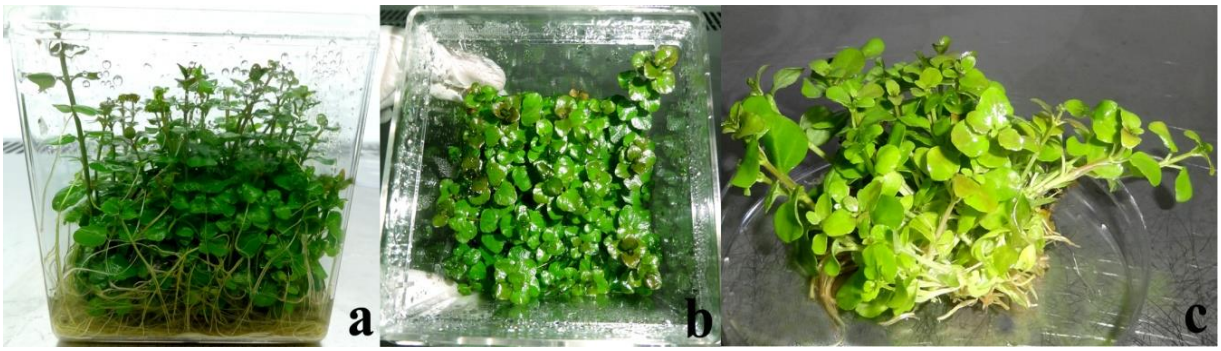


Figure 2. Shoot formations in the in liquid MS medium with IBA + TDZ. After eighth week of culture **(a**, **b**, **c)** Multiple shoot formation in MS medium including 0.40 mg L^{-1} TDZ + 0.10 mg L^{-1} IBA

Table 3. The effect of liquid medium with IBA + TDZ on *in vitro* shoot formations.

Growth regulators (mg L^{-1})		Regeneracy (%)	Average shoot counts	Shoot lengths (cm)
TDZ	**IBA**			
0.05	**0.10**	66.66^{a}	3.88^{c}	3.53^{a}
0.10	**0.10**	66.66^{a}	7.16^{bc}	3.42^{a}
0.20	**0.10**	88.89^{a}	10.05^{ab}	2.25^{ab}
0.40	**0.10**	83.33^{a}	12.33^{a}	1.94^{b}
0.80	**0.10**	72.22^{a}	5.84^{c}	1.79^{b}

Values shown with different letters in the same column are important at the $p<0.01$ level.

Regeneration values in liquid culture medium ranged from 66,66 to 88,89% (Table 3). 88.89% regeneration was reached in MS medium including 0.20 mg L^{-1} TDZ + 0.10 mg L^{-1} IBA. The least regeneration was reached in nutrient medium including 0.05 and 0.10 mg L^{-1} TDZ + 0.10 mg L^{-1} IBA.

The count of shoots per explant in liquid MS nutrient media was recorded between 3.88-12.33 and was recorded to be statistically important ($p<0.01$). The most count of shoots (12.33) was achieved in MS medium including 0.40 mg L^{-1} TDZ + 0.10 mg L^{-1} IBA. Whereas, the lowest shoot count (3.88) was obtained in MS medium including 0.05 mg L^{-1} TDZ + 0.10 mg L^{-1} IBA.

Shoot longs were between 1.79 and 3.53 cm. While the longest shoot (3.53 cm) was obtained in MS medium with 0.05 mg L^{-1} TDZ + 0.10 mg L^{-1} IBA, the shortest shoot (1.79 cm) was recorded in MS medium with 0.80 mg L^{-1} TDZ + 0.10 mg L^{-1} IBA.

Since root formations were obtained in liquid and solid propagation media, *in vitro* rooting study was not carried out. The regenerated plants were transferred to aquariums. In the fourth week, the plantlets were acclimatized to *ex vitro* conditions**.**

4. Discussion

Propagation of plants by tissue culture provides many advantages. For this reason, the transition from traditional production to biotechnological production has accelerated. In this study, *in vitro* production of *L. nummularia* in solid and liquid MS media was investigated.

Preparation of the nutrient medium as liquid or solid/semi-solid is a parameter that affects the regeneration value of explants (Moniruzzaman et al., 2021; Ab Aziz et al., 2021). Similarly, solid/semi-solid and liquid nutrient media trials have been carried out for *Urginea altissima* (Lf) Baker (Baskaran et al., 2018), *Dendrocalamus strictus* (Khare et al., 2021), *Ficus carica* L. (Moniruzzaman et al., 2021), *Begonia pavonina* (Ab Aziz et al., 2021) and *Ananas comosus* (L) Merr (Hamad et al., 2021) before.

In the current study, trials were carried out with different combinations of TDZ + IBA. TDZ is widely used in tissue culture studies. In recent years, tissue culture studies including TDZ have been carried out in some species such as *Ceratophyllum demersum* L. (Emsen and Dogan, 2018), *Pterocarpus marsupium* Roxb. (Tippani and Thammidala, 2021) and *Plumbago europaea* L. (Beigmohamadi et al., 2021).

When compared in terms of shoot regeneration values, higher regeneration values were obtained in solid nutrient medium. These results showed that solid media increased the regeneration abilities of explants compared to liquid media. When the average shoot numbers were compared, the best results were reached in nutrient medium including 0.40 mg L^{-1} TDZ + 0.10 mg L^{-1} IBA in both nutrient media. The most shoot counts in solid and liquid nutrient media were determined as 15.16 and 12.33 shoots/explant, respectively. These results revealed that solid nutrient medium is much more efficient than liquid nutrient medium.

Similarly, Gupta et al. (2020) transferred the nodal explants of *Tylophora indica* (Burm. f) Merrill to liquid and solid MS nutrient medium including different doses of BAP for *in vitro* propagation. They found the best results in terms of shoot number in solid media (13.00 ± 0.25). On the other hand, Yimam (2018) conducted a study for *in vitro* shoot regeneration of *Plectranthus edulis* in liquid and solid nutrient media. More shoots were obtained in the liquid environment than in the solid medium. The greatest number of shoots in liquid and solid nutrient media was recorded as 6.07 and 5.85, respectively. Shekhawat et al. (2015) investigated the *in vitro* micropropagation of *Morinda citrifolia* L. in liquid and semisolid (with agar) MS environment including different doses of BAP, Kinetin and IAA. The most count of shoots was obtained in 2.0 mg L^{-1} BAP + 1.0 mg L^{-1} Kinetin in liquid and semi-solid environments, respectively, as 11.4 ± 0.47 and 10.6 ± 0.17. The number of regenerated shoots of *Ajuga multiflora* Bunge was found to be higher in liquid medium compared to solid medium (Sivanesan et al., 2016).

When the shoot lengths were compared, it was seen that the liquid medium had higher length values than the solid medium. The maximum length was determined as 3.53 cm in liquid nutrient medium and 1.28 cm in solid nutrient medium. Shoots almost 3 times longer were obtained in liquid nutrient medium. This may be due to the fact that the liquid medium is more favourable for the elongation of the plant. Since *L. nummularia* is an aquatic plant, it may have given longer shoots in liquid

medium. Similarly, Rezali et al. (2017) conducted experiments at different MS levels (1/2, 1/4 and Full) and in liquid and solid media for *in vitro* multiproduction of *Typhonium flagelliforme*. The longest shoots at all MS levels were obtained in liquid nutrient medium. On the other hand, Yimam (2018) obtained longer shoots in solid medium than in liquid medium. Shekhawat et al. (2015) compared the lengths of regenerated shoots of *M. citrifolia* in semi-solid and liquid media and determined the longest shoot in solid MS medium.

Considering the effect of TDZ on shoot lengths, shoot lengths decreased with increasing TDZ concentration in both culture media. Similarly, the negative effects of TDZ on shoot length values have been reported in *Rauvolfia tetraphylla* (L.) (Hussain et al., 2018), *C. demersum* (Dogan, 2019b).

4. Conclusion

In this study, *in vitro* shoot regeneration of *L. nummularia* was investigated in solid and liquid nutrient media. Multiple shoots were obtained successfully in both culture media. The best results in terms of shoot number and regeneration values were determined in solid MS medium. The best results for shoot lengths were determined in liquid MS medium. In addition, the negative effects of high values of TDZ on shoot length were also recorded. These results may help the efficient production of *L. nummularia*, a medicinal and aromatic plant, by tissue culture. In addition, this study may contribute to the production of secondary compounds by the multiple productions of *L. nummularia*.

Acknowledgements

This work was supported by the Scientific and Technological Research Council of Turkey (TUBITAK) under Grant (Project no: 2130190).

Author Contribution

Muhammet DOĞAN designed the experiments and wrote the paper.

Conflicts of Interest

No potential conflict of interest was reported by the author.

References

1. Ab Aziz, R., Kandasamy, K. I., Qamaruz Zaman, F., Namasivayam, P., (2021). *In vitro* shoot proliferation of *Begonia pavonina*: a comparison of semisolid, liquid, and temporary immersion medium system. Journal of Academia, 9, 39-48.
2. Baskaran, P., Kumari, A., Van Staden, J., (2018). *In vitro* propagation via organogenesis and synthetic seeds of *Urginea altissima* (Lf) Baker: a threatened medicinal plant. 3 Biotech, 8(1), 1-8.
3. Beigmohamadi, M., Movafeghi, A., Jafari, S., Sharafi, A., (2021). Efficient *in vitro* organogenesis, micropropagation, and plumbagin production in *Plumbago europaea* L. *In Vitro* Cellular Developmental Biology-Plant, 57(5), 820-830.
4. Dogan, M., (2018). *In vitro* micropropagation from nodal explants of the medicinal plant *Lysimachia nummularia* L. Journal Of Agriculture and Nature, 21(6), 875-881.
5. Dogan, M., (2019a). Farklı ışık yayan diyotlar (LED) altında tıbbi sucul bitki *Lysimachia nummularia* L.'nın boğum eksplantlarından çoklu sürgün rejenerasyonu. Eurasian Journal of Biological and Chemical Sciences, 2(1), 11-16.
6. Dogan, M., (2019b). Multiple shoot regeneration via indirect organogenesis from shoot tip and nodal meristem explants of *Ceratophyllum demersum* L. Journal of Animal and Plant Sciences, 29(2), 568-577.
7. Emsen, B., Dogan, M., (2018). Evaluation of antioxidant activity of in vitro propagated medicinal *Ceratophyllum demersum* L. extracts. Acta Scientiarum Polonorum-Hortorum Cultus, 17(1), 23-33.
8. Gupta, V., Guleri, R., Gupta, M., Kaur, N., Kaur, K., Kumar, P., ... Pati, P.K., (2020). Anti-neuroinflammatory potential of *Tylophora indica* (Burm. f) Merrill and development of an efficient *in vitro* propagation system for its clinical use. Plos ONE, 15(3), e0230142.
9. Hamad, A. M., (2021). Effect of pH, Saccharose Concentrations and Medium States on *in vitro* Root-ing of Pineapple (*Ananas comosus* (L) Merr) cv Queen. Al-Mukhtar Journal of Sciences, 36(2), 135-147.
10. Hussain, S. A., Ahmad, N., Anis, M., (2018). Synergetic effect of TDZ and BA on minimizing the post-exposure effects on axillary shoot proliferation and assessment of genetic fidelity in

Rauvolfia tetraphylla (L.). Rendiconti Lincei. Scienze Fisiche e Naturali, 29(1), 109-115.
11. Kala, C. P., (2015). Medicinal and aromatic plants: Boon for enterprise development. Journal of Applied Research on Medicinal and Aromatic Plants, 2(4), 134-139.
12. Khare, S. R., Kharate, P. S., kumar Sahu, R., Jha, Z., (2021). The rapid *in-vitro* micropropagation of Bamboo (*Dendrocalamus strictus*) and its genetic fidelity testing using ISSR markers. Environment Conservation Journal, 22(3), 69-77.
13. Kodela, P., Jobson, R. W., (2016). *Lysimachia nummularia* (Primulaceae) Naturalised in New South Wales, Australia. Telopea, 19, 153-157.
14. Lin, J., Zou, J., Zhang, B., Que, Q., Zhang, J., Chen, X., Zhou, W., (2021). An efficient in vitro propagation protocol for direct organogenesis from root explants of a multi-purpose plant, *Broussonetia papyrifera* (L.) L'Hér. ex Vent. Industrial Crops and Products, 170, 113686.
15. Moniruzzaman, M., Yaakob, Z., Anuar, N., (2021). Factors affecting in vitro regeneration of Ficus carica L. and genetic fidelity studies using molecular marker. Journal of Plant Biochemistry and Biotechnology, 30(2), 304-316.
16. Murashige, T., Skoog, F., (1962). A revised medium for rapid growth bioassay with tobacco tissue culture. Physiologia Plantarum, 15, 473–497.
17. Nithya, V., Kamalam, M., (2021). Standardization of a protocol for micropropagation of *Eupatorium glandulosum* L. an important medicinal plant. Plant Cell, Tissue and Organ Culture (PCTOC), 146, 339–344.
18. Patricia, D., Stephen, B., John, A., (2021). Shoot organogenesis from leaf discs of the African ginger (*Mondia whitei* (Hook. f.) Skeels), an endangered medicinal plant. In Vitro Cellular Developmental Biology-Plant, 57(3), 493-498.
19. Podolak, I., Koczurkiewicz, P., Michalik, M., Galanty, A., Zajdel, P., Janeczko, Z., (2013). A new cytotoxic triterpene saponin from *Lysimachia nummularia* L. Carbohydrate research, 375, 16-20.
20. Rezali, N. I., Sidik, N. J., Saleh, A., Osman, N. I., Adam, N. A. M., (2017). The effects of different strength of MS media in solid and liquid media on *in vitro* growth of Typhonium flagelliforme. Asian Pacific Journal of Tropical Biomedicine, 7(2), 151-156.
21. Shekhawat, M. S., Kannan, N., Manokari, M., Ravindran, C. P., (2015). Enhanced micropropagation protocol of *Morinda citrifolia* L. through nodal explants. Journal of Applied Research on Medicinal and Aromatic Plants, 2(4), 174-181.
22. Sivanesan, I., Saini, R. K., Noorzai, R., Zamany, A. J., Kim, D. H., (2016). In vitro propagation, carotenoid, fatty acid and tocopherol content of *Ajuga multiflora* Bunge. 3 Biotech, 6(1), 91.
23. Solomou, A. D., Martinos, K., Skoufogianni, E., Danalatos, N. G., (2016). Medicinal and aromatic plants diversity in Greece and their future prospects: A review. Agricultural Science, 4(1), 9-21.
24. Tippani, R., Thammidala, C., (2021). TDZ induced plant regeneration from immature cotyledons of *Pterocarpus marsupium* Roxb. and validation of genetic homogeneity using ISSR markers. Vegetos, 34(1), 144-152.
25. Yimam, T., (2018). Effect of solid and liquid media on *in vitro* propagation of *Plectranthus edulis* (Vatke) (Agnew). Journal of Biology, Agriculture and Healthcare, 8(3), 23-28.

Research article
Curr. Pers. MAPs, (2022); 5(1): 19-35

Current Perspectives on Medicinal and Aromatic Plants

An International Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

Qualitative and Quantitative Phytoconstituent Determination, DPPH Free Radical Lowering Effect and *in vitro* Hypoglycemic Activity Study by Alpha-amylase Enzyme Assay along with Membrane Diffusion Technique

Rishiram BARAL[1,2*], Laxman SUBEDI[1,3], Monica GURUNG[1]
Sabita OJHA[1], Basanta SHRESTHA[1], Nirmala JAMMARKATTEL[1]

[1] School of Health and Allied Sciences, Faculty of Health Sciences, Pokhara University, Pokhara-30, Dhungepatan, Kaski, Nepal
[2] Research Institute of Pharmaceutical Sciences, Department of Pharmacy, Kyungpook National University, Daegu, South Korea
[3]College of Pharmacy and Natural Medicine, Mokpo National University, Mokpo, South Korea

*Corresponding author: rishirambaral1996@knu.ac.kr; nirmalajk@hotmail.com

https://doi.org/10.38093/cupmap.1111518
Received: 30/04/2022
Accepted: 31/05/2022

Abstract

Diabetes mellitus, a physiological disorder is characterized by low secretion of insulin due to the attack in insulin producing beta cell (Type I) or the body cell become insulin resistance (Type II). This study was designed to evaluate the DPPH lowering effect, in-vitro alpha amylase and glucose diffusion inhibition of the selected medicinal plants. Five different plant sample Amomum subulatum, Choerospondias axillaris, Musa sp, Myrica esculenta and Nephrolepis cordifolia were taken for the study. From the result it was revealed that the methanol extracts of Myrica esculenta stem bark and small branches showed potent DPPH free radical scavenging activity with the IC50 value of 4.23 µg/ml and 3.14 µg/ml respectively which is almost comparable to standard Ascorbic acid taken. Meanwhile, alpha-amylase inhibitory study showed that Myrica esculenta stem bark showed potent subsidiary effect on methanol extracts with IC50 value of 0.96 mg/ml which is comparable to standard volgibose taken. Lastly, membrane diffusion study with glucose and plant sample showed that Amomum subulatum seed and Choerospondias axillaris fruit have potent glucose diffusion inhibition with highest GDRI %. From the result, it could be correlated that the free radical scavenging activity and glucose lowering effect of these plant extracts is due to the presence of phytoconstituents like phenolics, flavonoids, alkaloids, terpenoids, glycosides saponins etc. as well as several other uncompetitive modes of inhibition.

Keywords: Diabetes mellitus, free radical scavenging, alpha amylase, glucose diffusion, phytochemicals

1. Introduction

Diabetes mellitus is a group of physiological disorder which is characterized by low secretion of insulin due to irreversible damage of insulin producing β-cell of pancreas due to of autoimmune disorder (Type I). Likewise, target cell of insulin become insulin resistance (Type II) or excessive glucagon secretion which results in elevation of blood glucose level (Blair, 2016). This increment in the level of blood glucose leads to several other life threatening complications such as damage to small and large blood vessels, cardiovascular disease (CVD), neuropathy, retinopathy and nephropathy (Zarkogianni et al., 2015). According to IDF diabetes atlas, the global prevalence of diabetes in adult is 8.8% and it is predicted to rise up to 10.4% until 2040 with around 16.2% live birth associate with hyperglycemia during pregnancy (Whiting et al., 2011). Similarly, vascular complications during diabetes are particularly caused by oxidative stress. Oxidative stress is particularly elevated in diabetic patient due to inability of endogenous antioxidant system such as catalase, superoxide dismutase and glutathione peroxidase enzyme to neutralize the increased production of Reactive Oxygen Species (ROS). Therefore, targeting ROS induced oxidative stress is also an effective strategy for the treatment of Diabetes Mellitus (Asmat et al., 2016).

Among, various enzymes present in human body as a part of digestive system, pancreatic alpha amylase is one of the calcium metalloenzymes which cannot function in the absence of calcium (Agarwal et al., 2016). Alpha-amylase catalyzes hydrolysis of the α-1, 4 glycosidic linkages in starch, amylopectin, amylose, glycogen and numerous maltodextrins in polysaccharides (Nair et al., 2013). Hydrolysis of such complex polysaccharides leads to the formation of smaller monosaccharides which are absorbable by different modes of passive and active transport through the intestinal barrier and reach the blood circulation (Ernest et al., 2012). This leads towards the rapid increment in the level of blood glucose causing Post Prandial Hyperglycemia (PPH) which is the indicator of Type II diabetes mellitus. Therefore, for controlling the Type II diabetes mellitus two major enzyme alpha-amylase and alpha glucosidase are the effective targets to inhibit their catalytic activity in the breakdown of complex polysaccharides to smaller monosaccharides (Dastjerdi et al., 2015).

Meanwhile, the diffusion of glucose across the intestinal membrane to the blood circulation can be inhibited with the use of complex polysaccharides and various dietary fibers. With the inhibition of such glucose diffusion from the intestinal lumen to the blood leads to the decrement of postprandial blood glucose level (Shadhan et al., 2017).

Plants have always been an exemplary sources of drugs and many of the currently available drugs have been derived directly or indirectly from them (Arumugam et al., 2013). Meanwhile, for the reduction of blood glucose level various medical plants are being used either as a dietary adjuvant or in the form of crude extract. Some plant secondary metabolites like alkaloids, carbohydrates, glycosides, flavonoids, steroids, terpenoids, peptides, phenolics have shown blood glucose lowering effect revealing their anti-diabetic potential. The antidiabetic effect shown by herbal medicine is either due to the insulin like activity or affecting insulin secreting beta cell or by modifying glucose utilization (Gulati et al., 2012a).

Despite widespread use of herbal medicine in Nepal for treatment of various bodily ailments, scientific study to prove the safety, efficacy, quality control and other aspects are limited (Kunwar et al., 2010). In this study five different medicinal plants which are being used as herbal remedies by different indigenous group of Nepal for treatment of diabetes complication were

evaluated. The plants are Greater Cardamom or Large Cardamom (*Amomum subulatum* Roxb.), Lapsi (*Choerospondias axillaris* Roxb.), Banana (*Musa* sp.), Box berry or Kafal (*Myrica esculenta* Buch. - Ham.ex D Don), Pani Amala (*Nephrolepis cordifolia* (L). C. Presl).

Greater cardamom or large cardamom (*Amomum subulatum* Roxb.) member of Gingeberaceae family is a well-known flavoring spice, used to treat various ailments in different medicinal system all over (Verma et al., 2012). *Amomum subulatum* is used traditionally for analgesic properties, antimalarial properties, antimicrobial properties, antioxidants, anti-diabetic and anthelmintic activities (Verma et al., 2012). Lapsi (*Choerospondias axillaris* Roxb.) belonging to family Anacardiaceae is a popular fruit tree of Nepal and many other Asian countries. In a study by Puja et al. 2018, it was reported that *Choerospondias axillaris* fruit juice is being used traditionally for the glucose lowering effect by various indigenous group of Nepal (Shrestha et al., 2018).

Musa sp. (Musaceae) called Banana in English, are one of the interesting tropical plants which have been consumed since centuries by human and animals as a nutritious food. In a study on *Musa sapientum*, mainly used in Indian folk medicine for the treatment of diabetes mellitus, oral administration of chloroform extract of the banana flowers in alloxan induced diabetic rats for 30 days resulted in a significant reduction in blood glucose, glycosylated hemoglobin and an increased in total hemoglobin (Orhan, 2001a). *Myrica esculenta* (Myricaceae) commonly known as box berry or Kafal is an important Asian medicinal plant. It is found in foothill track of eastern Himalayas Meghalayas, Nepal, China, and Pakistan. Stem bark of *M. esculenta* is reported for various pharmacological activities like radical scavenging, antioxidant, anti-diabetic, anxiolytic, anti-bacterial, anti-helminthic, allergic, anti-inflammatory, anti-microbial, mass cell stabilizing and anti-asthamatic (Srivastava et al., 2016).

Nephrolepis cordifolia is a member belonging to the Nephrolepidaceae family. It grows on hillside, riverside etc. It is adaptable and abundant in resources. It is traditional folk medicine. Modern medical experiment shows that the water extracts from the tuber of *N. cordifolia* possess significant blood glucose lowering activity. It opens up broad prospects for this kind of medicine for having anti-diabetic activity (Chai et al., 2015). These plants were collected from the Kaski district of Nepal. This study is focused for qualitative and quantitative determination of phytoconstituents, DPPH radical scavenging effect, digestive enzyme inhibitory study along with membrane permeability study of glucose in presence of plant extracts.

2. Materials and Methods

Chemicals and Reagents: Positive control inhibitor Volgibose IP was provided from Asian Pharmaceuticals Pvt. Ltd, Bhairahawa, Nepal as bounteous gift. Purified anhydrous Dextrose, Potassium sodium tartrate tetra hydrate, Di-sodium hydrogen phosphate, Benzene, Copper sulphate pentahydrate, Sodium anhydrous, 1-napthol were bought from Merck specialties, India. Central drug house, India provided 3,5-Dinitrosalicyclic acid while 1,1 Diphenyl-2 picryl hydrazyl radical (DPPH) was procured from Tokyo Chemical Industry, Japan. Dimethyl sulfoxide (DMSO), Benedict's reagent, Hydrochloric acid, Mercuric chloride, Sodium hydroxide pellets and starch were purchased from Thermo Fischer Scientific, India. Himedia Laboratories, India delivered the dialysis membrane and L-ascorbic acid on placement of order. All chemicals and reagents used were of analytical reagent grade.

Collection and identification of plant samples: Medicinal plants listed on Table 1

were gathered from numerous locations of western Nepal. Botanist of National Herbarium and Plant Laboratories, Godawari, Kathmandu, Nepal helped in the plant identification after observing the prepared herbaria. Crude drug museum of School of Health and Allied Sciences, Pokhara University in housed the voucher specimen of all collected medicinal plants. Plants samples were cleaned, chopped into small pieces, and left in shed for drying. Moisture was removed from the samples by placing the samples in hot air oven at 40˚C. Weight variation test determined the complete moisture removal and fine powdering was done with the help of grinder.

Table 1. List of plants

Scientific Name	Family	Local Name	Parts Used	Crude Drug Voucher No.	Sample No.
Amomum subulatum Roxb.	Zingiberaceae	Alaichi	Seed	PUCD-2019-12	S1
			Outer cover	PUCD-2019-13	S2
Choerospondias axillaries (Roxb.) B.L. Burrt	Anacardiaceae	Lapsi	Fruits	PUCD-2019-14	S3
Musa sp.	Musaceae	Kera	Leave	PUCD-2019-15	S4
			Leaves vein	PUCD-2019-15	S5
Myrica esculenta Buch. - Ham.ex D Don	Myricaceae	Kafal	Stem bark	PUCD-2019-16	S6
			Small branches	PUCD-2019-17	S7
Nephrolepis cordifolia (L). C. Presl	Nephrolepidaceae	Pani amala	Fruits	PUCD-2019-18	S8
			Leaves	PUCD-2019-19	S9

Sample Extraction: Single maceration for 24 hours was done for the extraction of crude drugs. The sample was macerated with hexane and methanol extracts separately and ratio of sample and solvents is 1:5 (w/v). Subsequently, filtration of the extract was followed by the Soxhlet extraction to obtain concentrated extracts after removing the residual solvent.

Qualitative Phytochemical Screening: Phytochemical screening was done as described by Anthony et al. 2013 to detect presence/ absence of secondary metabolite such as Alkaloid, Carbohydrate, Glycoside, Saponins, Phenolics, Flavonoids, Tannins, Terpenoids in the plants extract (Adegor et al., 2013).

Quantitative Phytochemical screening

Total phenolic content (TPC): TPC was determined by the Folin-Ciocalteu method as reported earlier by Juan Carlos et al. 2013, (Abreu-Villela et al.). The experiment employed the mixing of 5 ml of distilled water in test tubes containing 1 ml of sample along with 1 ml of Folin reagent. The tubes were incubated for 5 minutes and 1 ml of 10% 10% Na_2Co_3 was dropped. This was further followed by the incubation for another 1 hour in the dark at room temperature. Absorbance was measured at wavelength 725 nm. Experiments were performed in triplicate (n=3) and mean along with the standard deviation values were considered during the calculation. µg of Gallic acid equivalent per mg (GAE/mg) of extract expressed the TPC.

Total flavonoid content (TFC): Aluminum chloride method was employed to determine TFC as given by Mooza et al. 2014. 0.2 ml of 5% sodium nitrite solution was mixed with 4 ml of distilled water

which was subsequently added to the test tube containing 1 ml of plant extract. The tubes were incubated in dark for 5 minutes. Then, 0.2 ml of 10% of aluminum chloride and 2 ml of 1 M sodium hydroxide was added. Followingly, the absorbance was measured with UV-Vis spectrophotometer at λ = 510 nm. Experiments were performed in triplicate (n=3) and mean along with the standard deviation values were considered during the calculation. µg of quercetin equivalent per mg (QE/mg) of the plant extract expressed the TFC.

Antioxidant Activity Study

DPPH Free Radical Scavenging Activity: The method described Marinova et al. 2011 was taken as reference protocol to study the DPPH free radical scavenging activity (Marinova et al., 2011). To describe it in brief, 2ml plant sample of different concentration ranging from 1 µg/ml to 100 µg/ml was mixed with 2 ml of DPPH solution of concentration 60 µM. Reaction was completed after the incubation of the mixture for 30 minutes in dark. At the end, the absorbance obtained at 517 nm was taken to calculate the relative free radical scavenging effect at each sample concentration. Finally, following formula converted the relative scavenging effect into the %DPPH Scavenging activity:

$$\%\ DPPH\ Scavenging\ activity = \frac{Abs_{\ control} - Abs_{sample}}{Abs_{\ control}} \times 100\%$$

Where,

$Abs_{control}$ = Absorbance of control

Abs_{sample} = Absorbance of sample

Alpha- Amylase Inhibition Assay: The protocol defiend by Nyambe-Silavwe et al. 2015 was taken as reference for Alpha-inhibition assay by starch iodine method (Nyambe-Silavwe et al., 2015). Briefly describing, plant samples of concentration ranging from 0.25 mg/ml upto 2 mg/ml was mixed with alpha amylase solution of concentration 0.025 mg/ml. With the 10 minute incubation at 37°C, 1 % starch solution was added and the sample was incubated again for 1 hour in dark. Addition of 1% iodine solution was followed with the dilution of test tube with 5 ml of distilled water. Absorbance at 565 nm was converted into the relative alpha amylase enzyme inhibition effect of the plant extract. Volgibose IP was taken as reference standard. Test tubes containing buffer instead of enzyme was used as a background signal. Test tubes containing buffer instead of starch but having enzyme were taken as tracer control. Following formula converted the absorbance into % inhibition of enzyme activity.

$$Inhibition\ of\ enzyme\ activity(\%) = \frac{(A - C)}{(B - C)} * 100\%$$

Where,

A= Absorbance of sample

B= Absorbance of blank (no alpha amylase)

C= Absorbance of control (no starch)

Glucose Diffusion Inhibition Assay

Measurement of Glucose Diffusion Inhibition Activity: Few modifications on the protocol of Archit et al. 2013 was done to determine the glucose diffusion inhibitory activity of the plant samples (Archit et al., 2013). Dialysis membrane (12000 MW cutoff) of 5 cm was taken for the study purpose after the removal of the sulphate content of the membrane by boiling in water for 25-30 minutes. The membrane was then loaded with dextrose solution 5 % containing 0.15 M NaCl and different concentration of plant extracts. Both ends of the membrane were tied by the nylon thread. RO water replaces the plant extracts in negative control. 250 ml conical flasks containing 40 ml of 0.15 M NaCl, and 10 ml of RO water were prepared for each sample inside the membrane. NaCl aided in the equalization of the internal and external strength. Water bath shaker was

maintained at temperature of 37°C and 100 rpm and prepared conical flasks with samples were placed over it. Sample was withdrawn from each conical flask on regular time interval and glucose concentration was determined on every half an hour time interval. The process was repeated thrice to determine the reproducibility of the experiment.

3,5 Dinitro salicylic Acid (DNSA) Method to determine the reducing sugar from sample withdrawn 500 µl of samples were mixed with 1 ml of DNSA reagent and 2 ml of deionized water. To initiate the reaction between the glucose present in sample and DNSA, the tubes were placed on 95 °C for 5 minutes. Color change from yellow to reddish is the indicator of the reaction process. This was followed by cooling the sample thoroughly with the addition of RO water of 3.5 ml to each tube again. Absorbance measured at 540 nm gave the glucose content in the solution after subtracting the background signal. The natural glucose present on the plant sample might cause the experimental outcome to be errorful. Therefore, plant sample and DNSA was mixed outside the membrane and the resulted absorbance was converted to determine the glucose content on the plant samples. The glucose concentration determined was converted into Glucose Diffusion Retardation Index by using following formula:

$$GDRI\% = 100 - \frac{glucose\ content\ with\ addition\ of\ sample}{glucose\ content\ of\ control} * 100\%$$

Standard calibration curve was plotted with different concentration of dextrose in the range from 0.1% to 10%. This curve was taken as reference standard to determine the concentration of glucose as dextrose equivalent from the obtained graph.

3. Results

Qualitative Phytochemical Screening: Secondary metabolite (phytoconstituents) present in Hexane and methanol plant samples were determined by qualitative method and the result obtained are listed in **Table 2** and **Table 3.** From, the result it is seen that phenol, saponin and flavonoids are the secondary metabolites present in Hexane extract. The result shows that **S8** and **S9** possesses most of the phytoconstituents and leave extract of **S9** contains alkaloid in it. The result shows that Methanol extracts of plant samples contain almost all the phytoconstituents. Mayer and Wagner test revealed that all plant samples except **S4** and **S5** have alkaloid in them. Similarly, most of the methanolic plant extracts possesses glycoside, saponin, phenol, flavonoid, tannin and terpenoid. *Myrica esculenta* (bark and small branches), *Amomum. subulatum* and *Musa sp.* have almost all tested phytoconstituents.

Table 2. Phytochemical Analysis of Hexane Extracts of Plants Samples

Phytochemical Constituents	Specific Tests	S1	S2`	S3	S4	S5	S6	S7	S8	S9
Alkaloid	Mayer	-	-	-	-	-	-	-	-	+
	Wagner	+	+	-	-	-	-	-	+	+
Carbohydrate	Molish	-	-	-	+	-	-	-	+	-
	Benedict	-	-	-	-	-	-	-	-	-
Glycoside	Modified Borntrager	-	-	-	-	-	-	-	-	-
Saponin	Foam	+	+	+	+	+	+	+	+	+
Phenol	Ferric Chloride	+	+	-	+	+	+	-	+	-
Flavonoid	Alkaline Reagent	-	+	+	+	-	+	+	+	-
Tannin	Gelatin	-	-	-	-	-	-	-	-	-
Terpenoid	Salkowaski	+	+	+	-	+	-	-	-	-

Table 3. Phytochemical Analysis of Methanol Extracts of Plants Samples

Phytochemical Constituents	Specific Tests	S1	S2	S3	S4	S5	S6	S7	S8	S9
Alkaloid	Mayer	+	+	+	+	-	+	+	+	+
	Wagner	+	+	+	+	-	+	+	+	+
Carbohydrate	Molish	+	-	-	+	+	+	+	-	-
	Benedict	-	-	-	-	-	-	-	+	+
Glycoside	Modified Borntrager	+	+	-	+	+	+	+	+	-
Saponin	Foam	+	+	+	+	+	+	+	+	+
Phenol	Ferric Chloride	-	-	+	+	+	+	+	+	-
Flavonoid	Alkaline Reagent	+	+	-	+	+	+	+	-	-
Tannin	Gelatin	+	+	+	+	+	+	+	+	+
Terpenoid	Salkowaski	+	+	+	+	+	+	+	+	+

Quantitative phytochemical screening

Total Phenolic content (TPC)

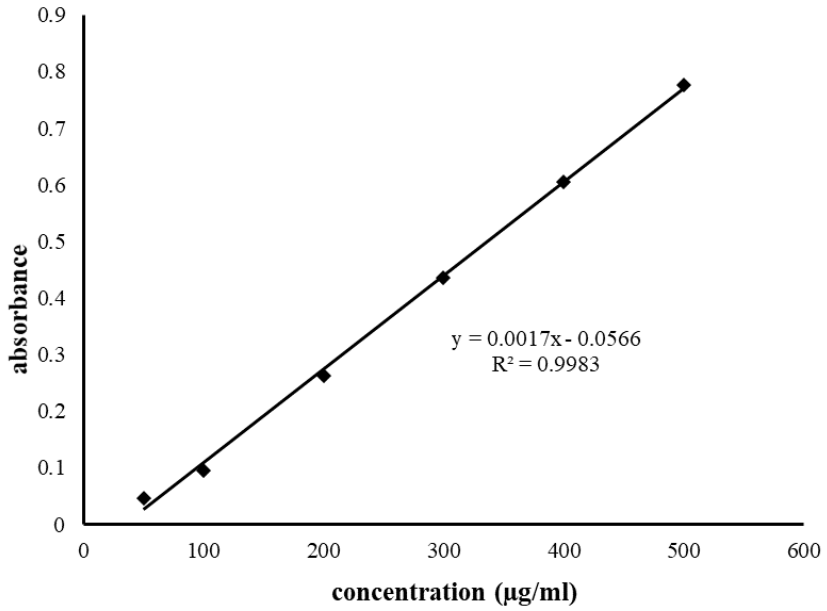


Figure 1. Calibration curve of gallic acid for total phenolic content.

Table 4. Total phenolic expressed as µg GAE/mg extracts

Sample	Total phenolic content (µg GAE/ mg ± SD) of plant extracts	
	Hexane	Methanol
S1	23.02±0.12	56.31±1.89
S2	12.56±0.46	101.25±2.41
S3	27.19±0.78	160.32±1.63
S4	63.41±0.29	168.58±1.57
S5	45.21±1.29	178.42±1.48
S6	72.09±0.95	180.21±0.79
S7	18.73±1.59	154.21±0.83
S8	24.10±0.72	174.61±0.79
S9	14.21±0.87	89.21±2.01

Data are expressed as mean ± standard deviation (n=3).

Results of TPC contents are expressed as µg gallic acid equivalent per mg of extract. Among the selected plant samples the methanol extracts of **S6** showed the highest TPC of 180.42±1.48 µg GAE/mg of extracts which is followed by the 178.58±1.57 µg GAE/mg of extracts of **S5**. The calibration curve of different concentration of gallic acid is shown in **Figure 1** and the phenolic content of each plant samples is given in **Table 4**.

Total flavonoid content: Results of TFC are expressed as µg quercetin equivalent per mg of extract. Among the selected plant sample the sample **S6** showed the maximum content of total flavonoid given as 301.51±1.89 µg QE/mg of extracts which is followed by 296.42±2.58 µg QE/mg of extracts in **S5**. The calibration curve of different concentration of gallic acid is shown in **Figure 2** and the flavonoid content of each plant samples is given in **Table 5**.

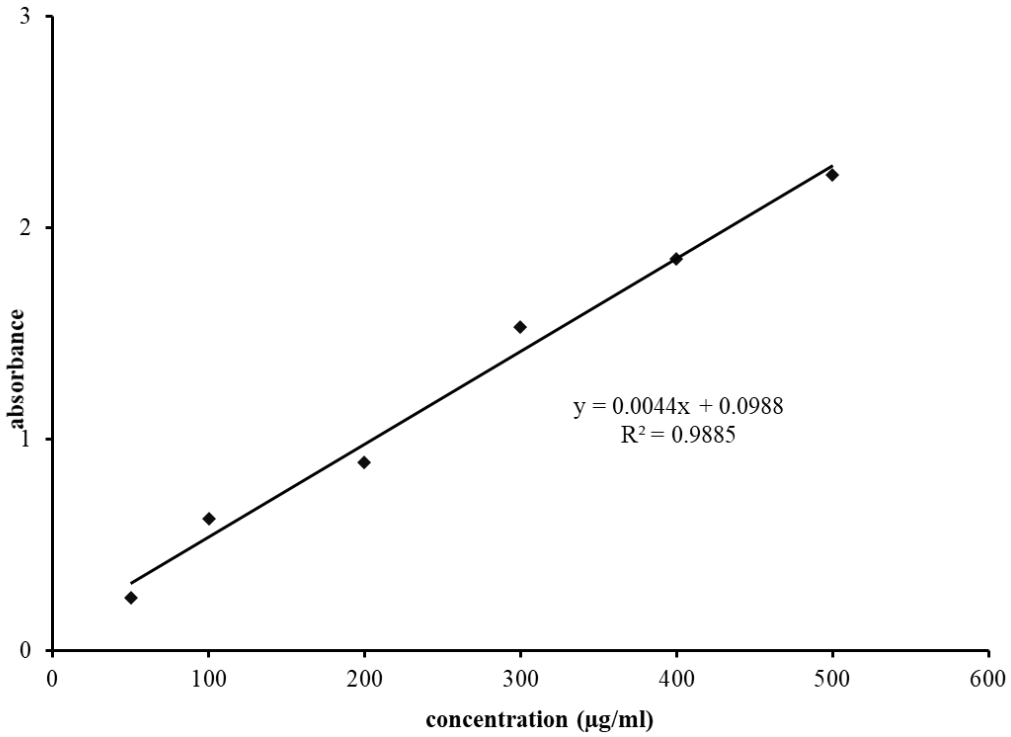


Figure 2. Calibration curve of quercetin for total flavonoid content.

Table 5. Total flavonoid content expressed as µg QE/mg extracts

Sample	Total flavonoid content (µg QE/ mg ± SD) of plant extracts	
	Hexane	Methanol
S1	30.21±1.47	264.53±1.28
S2	42.19±7.28	278.21±4.51
S3	51.28±1.49	156.31±2.81
S4	64.78±1.75	289.51±1.89
S5	54.72±0.47	296.42±2.58
S6	81.24±5.26	301.72±1.01
S7	78.21±2.15	294.58±2.51
S8	57.96±1.24	142.21±1.21
S9	37.41±0.78	124.63±1.15

Data are expressed as mean ± standard deviation (n=3).

DPPH Free Radical Scavenging Activity: DPPH free radical scavenging method evaluated the electron contributing ability or the free hydrogen atom. The scavenging effects of plant extracts at different concentration are shown in **Table 6** and **Table 7**. Hexane extracts showed very poor DPPH free radical scavenging activity. **S3** fruits showed maximum inhibition of DPPH free radical which is 62.23±2.03 at 100µg/ml with IC_{50}value of 80.06 µg/ml.

Table 6. DPPH Free Radical Scavenging Activity and IC_{50} of Hexane Extracts of Selected Plants

Plants	Concentration			IC_{50}
	1 µg/ml	10 µg/ml	100 µg/ml	
S1	1.25±0.76	4.31±0.5	12.76±0.25	>100
S2	2.69±1.27	5.03±0.5	9.89±1.27	>100
S3	3.23±2.03	7.01±1.27	62.23±2.03	80.06
S4	0.36±0.5	2.87±2.54	9.53±3.3	>100
S5	4.49±0.76	9.17±1.78	12.05±0.25	>100
S6	4.86±0.25	10.25±2.79	51.61±0.76	96.48
S7	1.07±0.5	2.33±1.78	12.41±1	>100
S8	1.8±0.5	3.78±1.78	2.48±2.7	>100
S9	0.71±0.5	7.73±1.27	9.1±5.95	>100
Ascorbic acid	23.96±4.26	96.34±0.22	97.62±0.22	>4.23

Data are expressed as mean ± standard deviation (n=3).

Table 7. DPPH Free Radical Scavenging Activity and IC_{50} of Methanol Extracts of Selected Plants

Plants	Concentration			IC_{50}
	1 µg/ml	10 µg/ml	100 µg/ml	
S1	20.48±4.71	26.34±0.89	40.15±5.61	>100
S2	16.98±2.02	19.52±0.67	39.04±2.69	>100
S3	21.11±2.46	40.95±1.34	89.52±0.44	26.77
S4	26.34±3.59	43.33±1.12	84.92±2.91	24.43
S5	16.5±0.44	18.41±1.34	25.87±1.57	>100
S6	24.92±1.12	94.76±1.57	96.03±0.22	4.23
S7	36.35±5.19	93.8±0.22	94.44±0.22	3.13
S8	26.98±2.69	34.29±0.44	95.07±0.22	33.26
S9	18.09±0	26.98±2.69	73.68±4.04	54.36
Ascorbic acid	23.96±4.26	96.34±0.22	97.62±0.22	4.23

Data are expressed as mean ± standard deviation(n=3)

The result revealed that most of the methanolic plant extracts are potent DPPH free radical scavenger. Methanol extract of S6 and S7 showed IC50 value of 4.23 µg/ml and 3.14 µg/ml respectively which is comparable to ascorbic acid standard. S8, S3 and S4 also showed potent inhibitory activity with IC_{50} value of 33.26 µg/ml, 26.76 µg/ml, 24.43 µg/ml, respectively.

In vitro Alpha-amylase Inhibition Activity: *α*- amylase inhibition assay was performed using spectrophotometric assay at 540nm. The inhibitory effects of hexane and methanol extracts at different concentration and solvents are shown in **Table 8** and **Table 9**. Hexane extracts showed minimum alpha-amylase inhibition at the concentration up to 2mg/ml**.**

Table 8. Alpha-amylase Inhibitory Activity and IC_{50} Value of Hexane Extracts of Selected Plant Samples

Plants	Concentration				IC_{50}
	0.25 mg/ml	0.5 mg/ml	1 mg/ml	2 mg/ml	
S1	16.85±0.23	20.83±0.92	35.28±1.96	61.61±3.17	1.55
S2	2.71±1.11	8.52±1.19	12.9±0.83	21.7±1.26	>2
S3	18.92±0.36	28.55±1.46	42.13±1.19	57.07±1.26	1.52
S4	14.97±0.77	26.32±1.14	35.68±2.22	55.79±2.11	1.71
S5	4.03±0.59	12.11±1.03	22.26±0.65	28.63±0.73	>2
S6	15.77±1.09	21.62±1.68	37.19±0.88	57.46±1.09	1.68
S7	5.97±2.12	8.72±0.97	12.31±0.83	34.33±2.05	>2
S8	2.27±1.26	7.37±1.82	9.96±0.81	19.39±0.92	>2
S9	12.86±0.92	19.43±0.65	33.930.47	51.41±2.86	1.91
Volgibose	48.71±1.08	69.16±4.14	82.2±4.15	94.31±2.45	0.26

Data are expressed as mean ± standard deviation(n=3)

Table 9. Alpha-amylase Inhibitory Activity and IC_{50} Value of Methanol Extracts of Selected Plant Samples

Plants	Concentration				IC_{50}
	0.25 mg/ml	**0.5 mg/ml**	**1 mg/ml**	**2 mg/ml**	
S1	11.95±0.9	35.05±0.73	45.28±0.66	66.03±0.84	1.22
S2	3.5±0.54	12.18±1.09	18.08±0.01	22.93±1.31	>2
S3	11.38±0.9	28.04±0.73	36.16±0.54	60.09±1.46	1.57
S4	14.69±1.31	15.53±0.66	34.01±0.3	50.77±1.13	1.95
S5	0.43±1.01	7.08±0.53	8.44±0.6	25.24±0.95	>2
S6	15.69±0.93	32.41±0.26	51.17±1.06	55.43±0.97	0.96
S7	2.11±0.61	9.04±0.65	10.51±0.63	22.98±0.65	>2
S8	1.39±0.36	5.49±0.43	7.13±0.48	20.35±0.48	>2
S9	14.29±0.6	15.49±1.19	31.1±0.84	50.37±1.04	1.98
Volgibose	48.71±1.08	69.16±4.14	82.2±4.15	94.31±2.45	0.26

Data are expressed as mean ± standard deviation(n=3)

Among all the hexane extracts only **S3** showed considerable inhibition of alpha amylase with IC50 value of 1.52 mg/ml. Methanol extract of **S6** showed potent alpha-amylase inhibition with IC_{50} value of 0.96 mg/ml which is comparable to volgibose. *Nephrolepis cordifolia* leaves and *Musa* sp. fruit even shows potent alpha-amylase inhibition with IC50 below 2 mg/ml.

Glucose Diffusion Inhibition Study

Studies have revealed that, mainly glucose diffusion inhibition could be achieved due to the presence of fibrous compound in the samples. Hexane is a non-polar solvent in which resinous or fibrous compounds are extracted in least quantity. Therefore, glucose diffusion inhibition study was performed only with methanol extracts where fibrous and resinous compounds are expected to get extracted in substantial amount. **Fig 3** represented the standard calibration curve. Based on this curve as referencing sample, the amount of glucose lied exterior of the dialysis membrane was calculated.

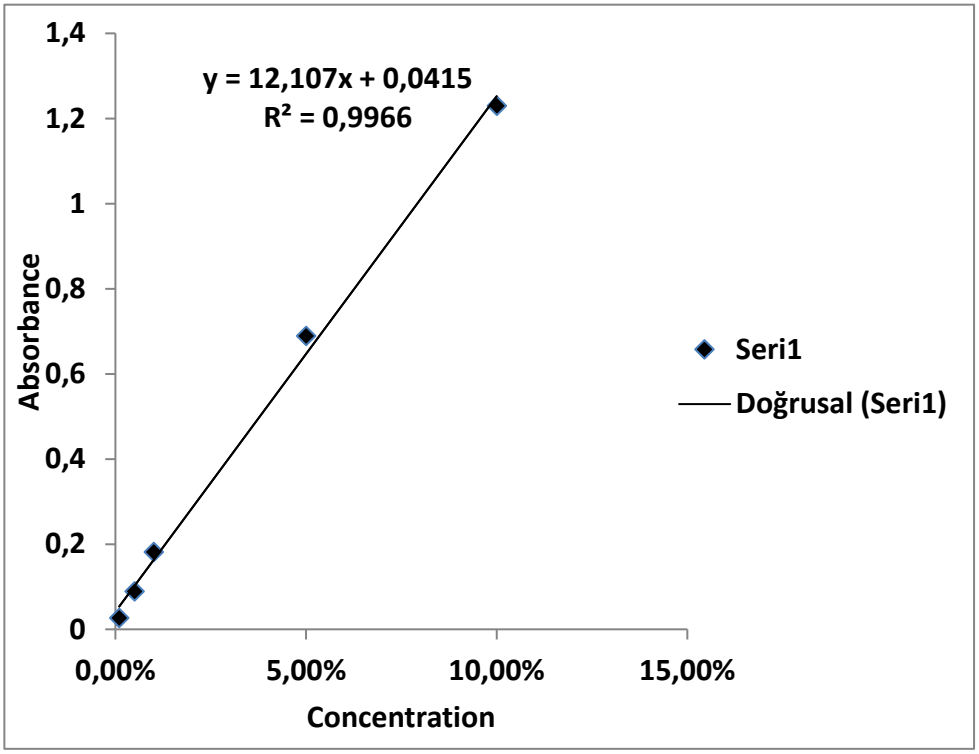


Figure 3. Calibration curve of dextrose exterior to the dialysis membrane

From the result, 20 mg/ml methanol extract of **S2** showed potent GDRI% from 120-180 minutes, with maximum GDRI% of 22.5 at 150 minutes. Followingly, the GDRI index of **S5** is 20% within first 90 minute. Meanwhile, **S7** also revealed potent GDRI% at 180 minutes.

When the concentration of sample was increased by two times with methanol extract, the GDRI% of **S1** was found maximum i.e., 49% as shown in **Fig 5**. This proved that **S1** was found highly potent at 40 mg/ml. **S3** was proved potent in all time intervals with 31% GDRI in 150-minute time.

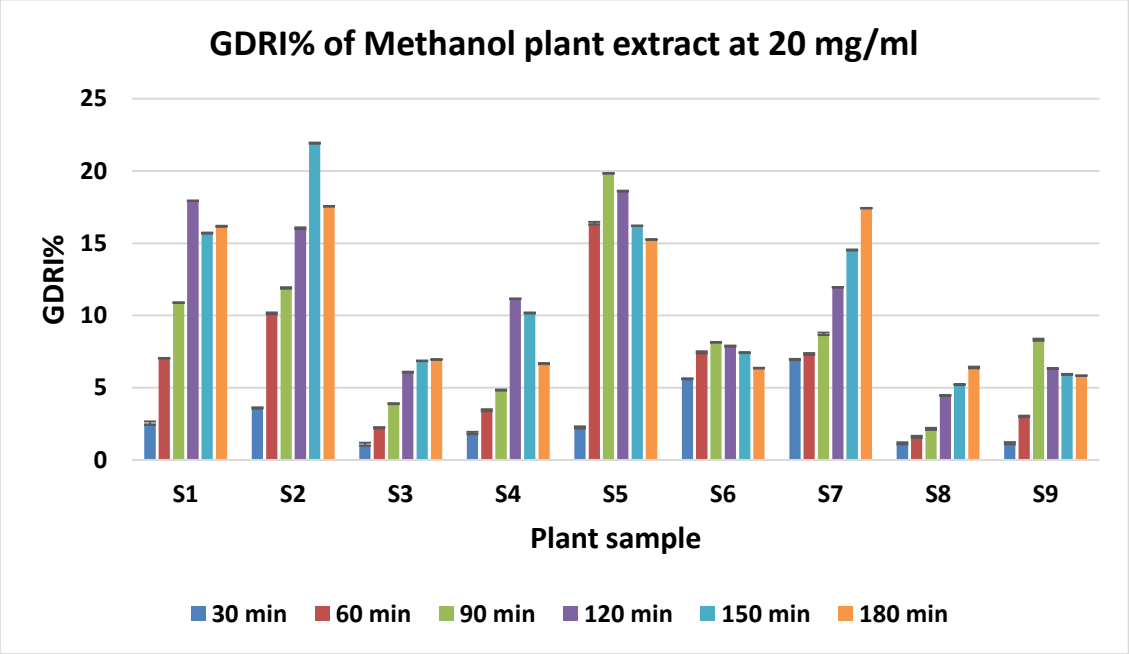


Figure 4. GDRI% of Methanol Extracts at 20 mg/ml

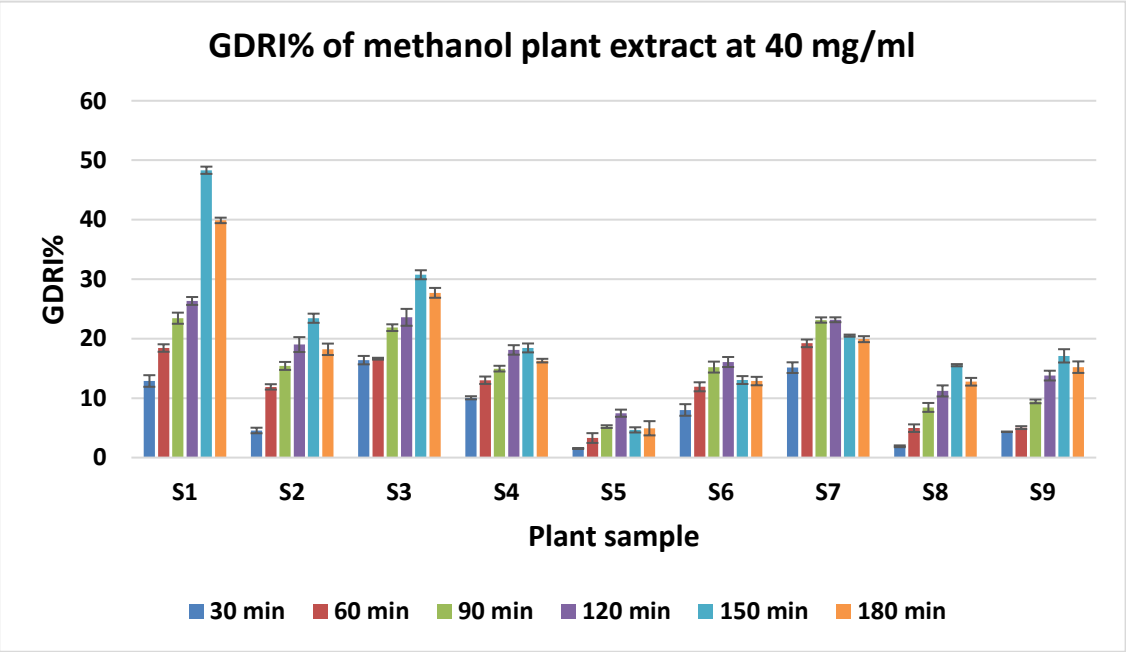


Figure 5. GDRI% of Methanol Extracts at 40 mg/ml

4. Discussion

Based on the local and ethnomedicinal utilization as hypoglycemic agents, different parts of five different medicinal plants were selected. From the phytochemical result obtained, it was determined that hexane extracts have very low phytoconstituents or secondary metabolites present on it. But in contrast, methanol extracts are found to be rich in phytoconstituents like glycosides, saponins, flavonoids, tannins and terpenoids which can be further correlated

with the positive in-vitro alpha amylase and glucose diffusion inhibition activity.

Phenol and polyphenolic compounds are proved to be potent free radical scavenger acknowledged to protect cell against oxidative stress (Rasouli et al., 2017). In a study by Daniil et al. 2014, it was shown that phenolic phyto substances were proved as the perspective natural compounds which exhibited antidiabetic activity as well (Olennikov et al., 2014). Common phenolic compounds present in plants which shows pharmacological activity are gallic acid, caffeic acid, ferulic acid, protocatechuic acid, coumaric acid, L-DOPA, ellagic acid, resveratrol, quercetin, rosmarinic acid etc. (Lin et al., 2016). According to Sandeep et al., 2011, *Myrica esculenta* revealed anti-oxidant properties due to the phenolic compound present on it. More specifically, gallic acid which is efficiently absorbed in the body contributed more to scavenge the free radicals present in them. Oxidative injuries in epithelial layer of various smooth muscles could be overcome by the phenolic constituents like chlorogenic acid, catechin and P-coumaric acid(Rawat et al., 2011).

On the other hand, the DPPH free radical scavenging effect of the *Choerospondias axillaries* fruit is correlated with the high presence of Vitamin C on plant extracts (Labh et al., 2015). In accordance with previous study, it has been reported that *Musa sp.* can protect itself from oxidative stress producing large amount of antioxidants thus is considered as good source of natural antioxidant due to the high presence of poly phenolic compounds (Orhan, 2001b). Not only phenolic compounds but also the presence of alkaloids, flavonoids, tannins, terpenoids, saponin and glycoside are also linked towards the scavenging properties of free radical. The results from our study revealed that the methanolic extracts of S6 and S7 showed potent DPPH free radical scavenging effect with the IC50 of 4.23 µg/ml and 3.13 µg/ml respectively. The result could be correlated with the presence of maximum amount of TPC and TFC in those plant extracts.

Meanwhile, natural products from plant sources are also gaining interest nowadays for inhibition of digestive enzymes such as alpha-amylase and alpha-glucosidase to control the blood glucose level. This is mainly due to the minimal side effect and therapies based on natural compounds compared to other hypoglycemic agent currently available (Gulati et al., 2012a). In a study by Nguyen et al. 2018, it was seen that isolated flavonoids showed the most active alpha amylase and alpha glucosidase inhibitory activity with IC50 value of 112.8 ± 15.1 and 785.9 ± 12.7 µg/mL respectively. This results showed the correlation between flavonoids and alpha-amylase inhibitory activity (Thao et al., 2018). Glucose lowering ability of epigallocatecin gallate, epicatechin and flavonoids are highly pronounced. Study from Uddin et al., 2014, revealed that alpha-amylase inhibitory activity of medicinal plants is more credited for the role of terpenoids, steroids and saponins to inhibit the respective enzyme. The study even reported that the hydrolysis is the major step for the breakdown of carbohydrate into glucose which is catalyzed by both alpha-amylase and alpha glucosidase enzyme. This step could be prevented even with the natural polyphenols(Uddin et al., 2014). In our experiment, S6 showed potent alpha-amylase inhibition on methanol extracts with IC_{50} value of 0.96 mg/ml which can be correlated with the presence of natural flavonoids, saponins, polyphenols and terpenoids. The quantitative phytochemical data revealed that the TPC in S6 is 180.21±0.79 µg GAE/mg of extracts and TFC in S6 is 301.72±1.01 µg QE/mg of extracts. This high amount of phenolic and flavonoid content could be correlated with the potent alpha-amylase inhibitory activity of the S6.

Likewise, in a study by Roberta et al 2011, spices and herbs commonly used in Italian cuisine were studied thoroughly for their combine anti-oxidant and alpha amylase

inhibitory activity and results revealed that these herbs and spices can improve the condition of non-insulin-dependent diabetes patients for their anti-oxidant activity (Cazzola et al., 2011). Phenolic acids such as hydroxycinnamic acid and gallic acid are subsequently found in plants which have both glucose lowering as well as free radical scavenging properties (Gulati et al., 2012b). Reactive oxygen species (ROS) formed with the oxidation of glucose and denaturation of glycated proteins consecutively results on oxidative stress. This is also a major cause for diabetic complication in human. So, in our study as well we could predict that the alpha amylase inhibitory activity of S6, S3, S9 and S4 is due to the potent free radical scavenging effect. Aside from the competitive binding study of plant extract with alpha amylase enzyme, uncompetitive mode of inhibition was also reported. The study suggested that binding strength of the enzyme for the substrate given by Km and rate of reaction given by Vm could also be decreased by the plant extracts (Picot et al., 2014b). Therefore, the alpha amylase inhibitory activity of our methanolic plant extracts can be correlated with uncompetitive mode of inhibition as well.

Complex polysaccharides such as oats, gum, psyllium husk and guar are revealed for their unique ability to lower the blood glucose level. Diffusion of glucose from the biological membrane in-vivo is predicted due to the physical boundary created by insoluble fiber particles. The fibrous network is supposed to entrap glucose moieties with in the networking residue preventing the upsurge of postprandial glucose level. Downsurge in glucose uptake in blood after crossing the biological semi-permeable membrane as well as the prolongation of gastric emptying time are the major outcome of viscous matrix. The accessibility of glucose molecule to the epithelial layer of intestine got decreased due to viscous gel which minimize the rise in postprandial glucose peaks (Gallagher et al., 2003). GDRI is a useful in-vitro index to predict the effect of fiber to decrease delay in glucose diffusion in the intestinal tract. In our study, we used a normal dialysis membrane to test the glucose diffusion inhibition by the plant sample at two different concentration 20 mg/ml and 40 mg/ml. We prepared glucose in NaCl solution as Glucose need carrier molecule to diffuse across cells. Our result showed that 20 mg/ml methanol extract of S1 showed potent GDRI which can be matched with the presence of insoluble fibers and complex polysaccharides in the plant extracts. S3, S6 and S7 even showed time dependent increment in GDRI% . The resinous network of the insoluble fiber particles is the cause glucose molecule to be entrapped resulting in the downfall of glucose diffusion to exterior of membrane (Bhutkar et al., 2017). Furthermore, several studies even highlighted that decrease in alpha-amylase enzyme activity is also directly correlated to the glucose diffusion retardation through semi permeable membrane. Subsequent decrement in the release of glucose from starch is the final output. On the other hand, the encapsulation of starch and enzyme within the resinous matrix as well as the action of the inhibitors might be the other cause for the inhibition of alpha amylase(Picot et al., 2014a).

5. Conclusion

The result of present study indicates that out of the five plants taken Methanol extract of stem bark and small branches of *Myrica esculenta, Nephrolepis cordifolia* fruit, *Choerospondias axillaris* fruit and *Musa sp.* leaves showed potent DPPH free radical scavenging effect. Similarly, the maximum alpha-amylase inhibition was shown by *Myrica esculenta* stem bark followed by *Choerospondias axillaris* fruits, *Nephrolepis cordifolia* leaves and *Musa sp.* leaves.
Similarly, among the plants taken *Amomum subulatum* and *Choerospondias axillaries* fruit showed maximum glucose diffusion inhibition. Above all, the local and traditional utilization of the selected medicinal plants to lower the blood glucose

level was substantially supported by the outcomes of this study. Conclusively, isolation of the active phytocompounds, investigation on the molecular level to determine the receptor-ligand binding affinity could be further encouraged with the outcome of the study.

Acknowledgement

The platform for the research was provided by School of Health and Allied Sciences, Faculty of Health Science, Pokhara University, Pokhara-30, Nepal. Our immense gratitude towards National Herbarium and plant laboratory, Godawari, Kathmandu Nepal for providing the plant dentification letter with correct verification.

Authors Contribution

Conceptualization, Rishiram BARAL, Nirmala JAMMARKATTEL; methodology, Rishiram BARAL, Laxman SUBEDI, Sabita OJHA; investigation, Rishiram BARAL, Laxman SUBEDI, Sabita OJHA, Monika GURUNG, Basanta SHRESTHA; writing-original draft preparation, Rishiram BARAL, writing-review and editing, Rishiram BARAL, Nirmala JAMMARKATTEL; supervision and project administration, Nirmala JAMMARKATTEL.

Funding

The authors declare no competing financial interest.

Conflict of Interest

The author declares no conflict of interest.

References

1. Abreu-Villela, R. et al., 2018. Early stages of drug crystallization from amorphous solid dispersion via fractal analysis based on chemical imaging. 133, 122-130.
2. Adegor, E. C. & Lawrence, E. O., 2013. Preliminary phytochemical screening, analgesic and anti-inflammatory properties of Celosia isertii. European Journal of Medicinal Plants 369-380. http://www.sciencedomain.org/review-history.php?iid=223&id=13&aid=1296.
3. Agarwal, P. & Gupta, R., 2016. Alpha-amylase inhibition can treat diabetes mellitus. Res. Rev. J. Med. Health Sci, 5(4), 1-8.
4. Archit, R., Gayathri, M. & Punnagai, M., 2013. An in vitro investigation into the mechanism of anti-diabetic activity of selected medicinal plants.International Journal of Drug Development, 9(2), 2.
5. Arumugam, G., Manjula, P. & Paari, N., 2013. A review: Anti diabetic medicinal plants used for diabetes mellitus.Journal of Acute Disease, 2(3), 196-200. https://doi.org/10.1016/S2221-6189(13)60126-2.
6. Asmat, U., Abad, K. & Ismail, K., 2016. Diabetes mellitus and oxidative stress—A concise review.Saudi pharmaceutical journal, 24(5). 547-553. https://doi.org/10.1016/j.jsps.2015.03.013.
7. Bhutkar, M. A., Bhinge, S. D., Randive, D. S. & Wadkar, G. H., 2017. Hypoglycemic effects of Berberis aristata and Tamarindus indica extracts in vitro. Bulletin of Faculty of Pharmacy, Cairo University 55(1). 91-94. https://doi.org/10.1016/j.bfopcu.2016.09.001.
8. Blair, M., 2016. Diabetes Mellitus Review. Urologic nursing 36(1).
9. Cazzola, R., Camerotto, C. & Cestaro, B., 2011. Anti-oxidant, anti-glycant, and inhibitory activity against α-amylase and α-glucosidase of selected spices and culinary herbs.International Journal of Food Sciences and Nutrition 62(2). 175-184. https://doi.org/10.3109/09637486.2010.529068.
10. Chai, T.-T. et al., 2015. Evaluation of glucosidase inhibitory and cytotoxic potential of five selected edible and medicinal ferns.Tropical Journal of Pharmaceutical Research. 14(3). 449-454. http://dx.doi.org/10.4314/tjpr.v14i3.13.
11. Dastjerdi, Z. M., Namjoyan, F. & Azemi, M. E., 2015. Alpha amylase inhibition activity of some plants extract of Teucrium species. European Journal of Biological Sciences. 7(1). 26-31. 10.5829/idosi.ejbs.2015.7.01.91127.
12. Ernest, V., Shiny, P., Mukherjee, A. & Chandrasekaran, N., 2012. Silver nanoparticles: a potential nanocatalyst for the rapid degradation of starch hydrolysis by α-amylase. Carbohydrate Research, 35260-35264.
13. Gallagher, A., Flatt, P., Duffy, G. & Abdel-Wahab, Y., 2003. The effects of traditional antidiabetic plants on in vitro glucose diffusion.Nutrition research, 23(3), 413-424. https://doi.org/10.1016/j.nutres.2020.12.024.
14. Gulati, V., Harding, I. H. & Palombo, E. A., 2012a. Enzyme inhibitory and antioxidant activities of traditional medicinal plants: potential application in the management of hyperglycemia.Bmc complementary and

alternative medicine 12(1), 77. http://www.biomedcentral.com/1472-6882/12/77.
15. Gulati, V., Harding, I. H. & Palombo, E. A., 2012b. Enzyme inhibitory and antioxidant activities of traditional medicinal plants: potential application in the management of hyperglycemia.BMC complementary and alternative medicine, 12(1). 77.
16. Kunwar, R. M., Shrestha, K. P., Bussmann, R. W. J. J. O. E. & Ethnomedicine, 2010. Traditional herbal medicine in Far-west Nepal: a pharmacological appraisal.Journal of Ethnobiology and Ethnomedicine 6(1), 35. http://www.ethnobiomed.com/content/6/1/35
17. Labh, S. N., Shakya, S. R. & Kayasta, B. L., 2015. Extract of Medicinal lapsi Choerospondias axillaris (Roxb.) exhibit antioxidant activities during in vitro studies.ournal of Pharmacognosy and Phytochemistry, 4(3), 194.
18. Lin, D. et al., 2016. An overview of plant phenolic compounds and their importance in human nutrition and management of type 2 diabetes.Molecules, 21(10), 1374.
19. Marinova, G. & Batchvarov, V., 2011. Evaluation of the methods for determination of the free radical scavenging activity by DPPH.Bulgarian Journal of Agricultural Science, 17(1), 11-24.
20. Nair, S. S., Kavrekar, V. & Mishra, A., 2013. In vitro studies on alpha amylase and alpha glucosidase inhibitory activities of selected plant extracts.European Journal of Experimental Biology, 3(1). 128-132.
21. Nyambe-Silavwe, H. et al., 2015. Inhibition of human α-amylase by dietary polyphenols.Journal of Functional Foods, 19, 723-732. http://dx.doi.org/10.1016/j.jff.2015.10.003.
22. Olennikov, D. N. & Kashchenko, N. I., 2014. Componential profile and amylase inhibiting activity of phenolic compounds from Calendula officinalis L. leaves.The Scientific World Journal, https://doi.org/10.1155/2014/654193 .
23. Orhan, I., 2001a. Biological activities of Musa species.Ankara Üniversitesi Eczacılık Fakültesi Dergisi, 30(1). 39-50. 10.1501/ Eczfak.
24. Orhan, I., 2001b. Biological activities of Musa species.Fac. Pharm, 30(1). 39-50. 10.1501/Eczfak_0000000607.
25. Picot, C., Subratty, A. H. & Mahomoodally, M. F., 2014a. Inhibitory potential of five traditionally used native antidiabetic medicinal plants on α-amylase, α-glucosidase, glucose entrapment, and amylolysis kinetics in vitro. Advances in Pharmacological Sciences, https://doi.org/10.1155/2014/739834.
26. Picot, C., Subratty, A. H. & Mahomoodally, M. F. J. a. I. P. S., 2014b. Inhibitory potential of five traditionally used native antidiabetic medicinal plants on α-amylase, α-glucosidase, glucose entrapment, and amylolysis kinetics in vitro.2 https://doi.org/10.1155/2014/739834.
27. Rasouli, H. et al., 2017. Differential α-amylase/α-glucosidase inhibitory activities of plant-derived phenolic compounds: a virtual screening perspective for the treatment of obesity and diabetes.Food Function, 8(5). 1942-1954. 10.1039/c7fo00220c.
28. Rawat, S. et al., 2011. Assessment of antioxidant properties in fruits of Myrica esculenta: A popular wild edible species in Indian Himalayan region.Evidence-Based complementary and alternative medicineS, 2011(10.1093/ecam/neq055.
29. Shadhan, R. M. & Bohari, S. P. M. J. a. P. J. O. T. B., 2017. Effects of Hibiscus sabdariffa Linn. fruit extracts on α-glucosidase enzyme, glucose diffusion and wound healing activities.Asian Pacific Journal of Tropical Biomedicine, 7(5), 466-472. https://doi.org/10.1016/j.apjtb.2017.01.023.
30. Shrestha, P., Jamarkattel-Pandit, N. J. J. O. H. & Sciences, A., 2018. Survey on Medicinal Plants used for Anti-diabetic Activity in Kaski District, Nepal.Journal of Health and Allied Sciences 7(1), 1-7. 10.1055/s-011-50210.
31. Srivastava, B. et al., 2016. Evaluation for substitution of stem bark with small branches of Myrica esculenta for medicinal use–A comparative phytochemical study.Journal of Ayurveda and integrative medicine, 7(4), 218-223. https://doi.org/10.1016/j.jaim.2016.08.004.
32. Thao, N. P. et al., 2018. α-Amylase and α-glucosidase inhibitory activities of chemical constituents from Wedelia chinensis (Osbeck.) Merr. leaves.Journal of analytical methods in chemistry, 2018, https://doi.org/10.1155/2018/2794904.
33. Uddin, N. et al., 2014. In vitro α–amylase inhibitory activity and in vivo hypoglycemic effect of methanol extract of Citrus macroptera Montr. fruit. Asian Pacific journal of tropical biomedicine, 4(6), 473-479. https://doi.org/10.12980/APJTB.4.2014C1173.
34. Verma, S. K., Jain, V. & Singh, D. P., 2012. Effect of greater cardamom (Amomum subulatum Roxb.) on blood lipids, fibrinolysis and total antioxidant status in patients with ischemic heart disease.Asian Pacific Journal of Tropical Disease, 2(S739-S743. https://doi.org/10.1016/S2222-1808(12)60255-2.
35. Whiting, D. R. et al., 2011. IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030.Diabetes research and clinical practice, 94(3), 311-321. http://dx.doi.org /10.1016/j.diabres.2011.10.029.
36. Zarkogianni, K. et al., 2015. A review of emerging technologies for the management of diabetes mellitus.IEEE Transactions on Biomedical Engineering, 62(12), 2735-2749. 10.1109/TBME.2015.2470521.

Research article
Curr. Pers. MAPs, (2022); 5(1): 36-49

Current Perspectives on Medicinal and Aromatic Plants

An International Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

Formulation and Control of a Topical Emulsion, Containing Algerian Vitis vinifera L. Leaves Extract

Mohammed Adil SELKA [1] *, Amel CHENAFA [2], Mohammed Yacine ACHOURI [2], Nazim BELLIFA [2]

[1] Department of Pharmacy, TOXICOMED laboratory, Faculty of Medicine, University of Tlemcen,13000, Algeria.E-mail: ad.selka@gmail.com ORCID ID: 0000-0003-3060-3809
[2] Department of Pharmacy, Faculty of Medicine, University of Sid Bel Abbès, 22000, Algeria, E-mail: amel.ph2007@yahoo.fr, ORCID ID: 0000-0002-9295-0913.
[2] Department of Pharmacy, Faculty of Medicine, University of Sid Bel Abbès, 22000, Algeria, E-mail: yac.achouri@gmail.com, ORCID ID: 0000-0003-3765-8642.
[2] Department of Pharmacy, Faculty of Medicine, University of Sid Bel Abbès, 22000, Algeria, E-mail: belifa.nazim@gmail.com, ORCID ID: 0000-0002-4247-1850.

* Corresponding Author. ad.selka@gmail.com

https://doi.org/10.38093/cupmap.1118538

Received: 20/05/2022
Accepted: 27/06/2022

Abstract

Topical emulsions find their applications in wrinkle reduction (skin aging), acne treatment and, sebum secretions regulation. For various dermatological affections, many topical formulations like sunscreens and anti-aging creams are prepared using plant-based ingredients
The objective of this study was to formulate an emulsion containing *Vitis vinifera L.* leaves extract, and to evaluate its stability and antioxidant activity in vitro.A spectrophotometric assay of the main phenolic groups for 10 *Vitis vinifera L.* leave samples was performed.O/W emulsions were prepared using a nonionic surfactant polysorbate 80.1,1-diphenyl-2-picrylhydrazyl assay was performed to evaluate plant extracts and emulsions antioxidant activity.
The Physical stability of the emulsions stored at 25 ºC and 40 ºC for 60 days was assessed based on various physico-chemical characteristics including color, creaming, liquefaction, centrifugation pH, and electrical conductivity.
The emulsions showed good physical properties and pharmaceutical stability. The polyphenol-rich-plant-derived extract and the emulsion showed good antioxidant activities.
this research allowed the development of an emulsion based on *Vitis vinifera L.* extract, which can be proposed for topical use. However, in vivo studies are recommended to confirm the antioxidant action of this cream.

Key Words: Plant, emulsion, antioxidant, stability, parameters.

1. Introduction

Emulsions are thermodynamically unstable dispersed systems, of two immiscible phases, one of them dispersed in the other as fine droplets by using an interface surfactant (Seiller and Martini 1996). They have potential applications in paint, food, cosmetic and pharmaceutical industries (Al-Achi

2020). They have received particular interest as Drugs Delivery Vehicle because they increase their bioavailability (Abdulbaqi and Rajab 2020; Zhao et al. 2020). Emulsions are the most frequently used galenic form in dermocosmetology, due to their physical, chemical, and biological compatibility with the skin. Their main advantage is that they increase solubility, bioavailability, and skin absorption of active substances (Lu and Gao 2010). Oil-in-water or O/W emulsions are commonly used as bases for water-soluble substances in cosmetology (Lim et al. 2020). A benefit value can be brought to these formulations by including components with a specific cosmetic effect, especially those containing antioxidants as an active ingredient (Noon et al. 2020). Due to their biological and therapeutic properties, plant extracts and their derived products are often included in emulsions and several other pharmaceutical preparations (Huma et al. 2020). In the last decade, there has been a lot of interest in the antioxidant potential of natural polyphenol in health field (Pandey and Rizvi 2009).

Red vine, one of the most widespread fruit crops, is a plant rich in polyphenols (Baydar et al. 2004). Several studies have reported that red vine leaves contain organic acids, phenolic acids, flavonols, tannins, procyanidins, anthocyanins, (Orhan et al. 2009; Selka et al. 2019). Most therapeutic properties of this plant and especially of its leaves are related to phenolic compounds (Xia et al. 2010). The use of plant phenolic extract in new cosmetic product development such as emulsions or creams has already been mentioned (Zillich et al. 2015; Selka et al. 2016). Plant polyphenols can be used as sunscreens, whitening, and anti-aging agents (González et al. 2008).

Emulsion stability is an important factor that determines the safety of its use, this type of formulation is thermodynamically unstable, so it may be subject to deterioration over time because of various physicochemical mechanisms such as creaming, flocculation, coalescence, phase inversion and/or Ostwald ripening (Ushikubo and Cunha 2014, Ralla et al. 2020). This study aimed to develop a *Vitis vinifera L.* leaf extract emulsion based, and to study its physicochemical stability and its in vitro antioxidant property.

2. Material and Methods

2.1. Apparatus: Centrifuge Sigma 4-16K, Germany; Spectrophotometer OPTIZEN 3220, South Korea; Vortex mixer VWR, Germany; Rotary Evaporator Buchi RII, Germany; Homogenizer ultra-turrax T18, Germany; Microscope Leica DM300, Germany; pH meter Inolab WTW level 2, Germany; Conductivity meter Inolab WTW level 1, Germany.

2.2. Plant material: Red vine leaves sampling was carried out in autumn 2020 (September-October), during this period *Vitis vinifera L.* leaves begin to take a reddish color, and their polyphenol content is at its highest level (Katalinic et al. 2013). The collection was carried out in various areas of Algeria; region selection was based on the international organization of oenology and viticulture (OIV) report, classifying the most known regions for viticulture through the northern Algerian area from East to West (OIV 2017)

The region of El-Bayadh does not appear on The OIV report, it was introduced following the recent development that knows the viticulture in this region despite its particular climate. Geographical situations and bioclimatic stages of the different stations are represented in Table 1.

Table 1. Geographical situations and bioclimatic stages of the sampling stations

Region	Sample code	Collecting date	Latitude (North)	Longitude (West)	Altitude (M)	Sector	Bioclimatic stage
Terga – Aïn Temouchent	E3	October 2015	35.41’	-1°17’	28	Oranese interior plain : O2	Semi-arid
Bir El Djir – Oran	E1	October 2015	35.72’	-0°55’	164	western coastline : O1	Sub-humid
Dellys – Boumerdes	E6	October 2015	36.87’	3° 93’	184	central coastline : A1	Sub-humid
Aïn Soltane - Bordj-Bou-Arreridj	E5	October 2015	36.07’	4° 81’	946	Constantine High Plains H2	Semi arid
Stidia – Mostaganem	E4	October 2015	35.83’	0° 006’	26	western coastline : O1	Sub-humid
Dhayet El-Bagra El-Bayadh	E10	October 2015	32.35’	0° 60’	810	Saharian : SS1	Saharian
Si Mahdjoub - Medea	E2	October 2015	36.28’	2° 76'	789	Tellian Atlas : A2	Sub-humid to humid
El Bouni – Annaba	E9	October 2015	36.85’	7° 74’	20	Numidian : K3	Sub-humid to humid
Beni Chougrane – Mascara	E7	October 2015	35.39’	0° 14’	563	Tellian Atlas : O3	Sub-humid to humid
Mitidja - Blida	E8	October 2015	36.49’	2° 84’	199	central coastline : A1	Sub-humid

The collected samples were identified by Dr M.Chelghoum in the botany laboratory of Sidi Bel Abbes pharmacy department in Algeria and confirmed by the Botany Institute of Liege University in Belgium.Harvest materials were shade-dried, at room temperature. The drying time was about 10 days for the different samples, which were afterwards stored in Kraft paper bags.

2.3. Microscopic observation of leaves powder: Grapevine leaves powder was placed separately on slides, each slide was mounted 2-3 drops of chloral hydrate, and each slide was covered with a coverslip, and then examined under a microscope at different magnifications. The found elements were noted and photographed.

2.4. Phenolic compound extraction: 40 g of crushed leaves were placed in a flask

containing 100 ml of methanol-water mixture (80 - 20) v/v and 0.1 ml/ml of 37% hydrochloric acid to avoid polyphenols oxidation. Extraction was performed by reflux at a temperature of 60°C for 30 minutes. The extracts were filtered and centrifuged at 3000 rpm for 20 min at 25°C, the supernatants were concentrated using a rotary evaporator at room temperature, and the crude extract was kept at low temperature in amber glass vials until use (Benmeziane et al. 2014).

2.5. Determination of the total phenolic content: The total polyphenol content of leaves extracts was determined by the Folin-Ciocalteu method.400 microliters of methanolic extract were mixed with 1.6 ml of 7,5% sodium carbonate solution (Na2CO3) and with 2 ml of freshly prepared Folin-Ciocalteu reagent (diluted (1:10). The mixture was vortexed and incubated at room temperature for 90 min, the absorbance was measured at 765 nm. A calibration curve was obtained using gallic acid solution at different concentrations. The result was expressed as mg Gallic acid equivalents/ g dry plant material. All operations were performed in triplicate (Benmeziane et al. 2014).

2.6. Determination of the total flavonoid content: Flavonoid content was determined using the method described by Ying and Wan (2012).1 ml of diluted extract was mixed with 3 ml of distilled water and with 300 µl of 7% sodium nitrite (NaNO2) solution. This was incubated for 5 min. Later, 300 µl of 10% aluminum chloride (AlCl3), and 1 ml of sodium hydroxide (1N) were added to the mixture. After 6 min of incubation, the total mixture was placed in a visible spectrophotometer and the absorbance reading was taken at a wavelength of 510 nm. A calibration curve was obtained using catechin solution at different concentrations. The result was expressed as mg catechin equivalents/ g dry plant material. All operations were performed in triplicate (Ying and Wan 2012).

2.7. Determination of condensed tannins content: Condensed tannins content was estimated using the vanillin HCl method.50 µl of diluted methanolic extract was mixed with 1500 µl of vanillin/methanol solution (4%, w/v) and with 750 µl of 37% hydrochloric acid (HCl). After 20 minutes of incubation at room temperature, the absorbance of the mixture was measured at 500 nm. A calibration curve was obtained using catechin solution at different concentrations. The result was expressed as mg catechin equivalents/ g dry plant material. All operations were performed in triplicate (Julkunen-Tiitto 1985).

2.8. Preparation of emulsions: A galenic formulation, following the work of Rasul and Akhtar (2012) and Khan, Akhtar et al (2013) was carried, based on grapevine leaves extract (Rasul and Akhtar 2012; Khan et al. 2013).The extract used for emulsion development was chosen according to the crude extract amount and its polyphenol content.Two O/W emulsions type (control formulation and formulation containing the extract) were prepared according to the work of Khan, Akhtar et al. (2013), the qualitative and quantitative composition of the emulsions is summarized in Table 2.

The preparation was carried out in four steps:

-Oily phase excipients mixing (liquid paraffin, stearic acid, cetostearyl alcohol, beeswax, sorbitan monooleate) at a temperature of 70°C.

-Aqueous phase excipients mixing and addition of grapevine leaves extract at the temperature of 70°C.

-Addition of the aqueous phase to the oil phase on a drop-to-drop basis under continuous stirring at 1000 rpm for 10 min.

Control emulsion was also prepared by the same method above but without plant extracts (the active ingredient). Homogenization of the emulsion with a homogenizer at 13000 rpm for 2 min (Khan et al. 2013)

Table 2. Composition of emulsions weight by weight (%,w/w)

Ingredients	Role	Control emulsion	Active emulsion
liquid paraffin	Lipophilic phase	27%	27%
Stearic acid	emulsifier	5%	5%
Sorbitan monooleate	Lipophilic surfactant	1.2%	1.2%
Beeswax	thickener	4%	4%
Cetostearyl alcohol	viscosifier	5%	5%
Polysorbate 80	Hydrophilic surfactant	6.8%	6.8%
Plant extract	active ingredient	-	5%
Water	Hydrophilic phase	51%	46%

(Rasul and Akhtar 2012; Khan et al. 2013)

2.9. Characterization of emulsions: Various controls were carried out on the freshly prepared emulsions:

-Macroscopic examination: organoleptic characteristics (appearance, odor, color, and consistency) physical stability (creaming, sedimentation, and phase separation) (Smaoui et al. 2017; Huma et al. 2020)
-Emulsion Type: using the dye method where two dyes were used including methylene blue as a water-soluble dye and Sudan red III as a lipophilic dye.
-Microscopic examination: colored emulsion drop was analyzed using an optical microscope to study the globule size homogeneity and to detect flocculation and coalescence phenomena (Khan et al. 2013)
-Centrifugation stability: centrifugation test was done by adding 2 g of the colored emulsion into centrifugation tubes to be centrifuged at 25°C and speed of 3000 rpm for 30 min.
-pH measurement: emulsions pH was measured using a calibrated pH meter, after dilution (1:10) in neutral distilled water, (Wehrlé 2012; Brossard et al. 2016).
-Conductivity measurement: this test was performed to highlight a potential phase change. The electrical conductivity was measured in µS/cm using a calibrated conductivity meter.
-Macroscopic examination, pH, and conductivity measurements were performed during 60 days on the emulsions kept at 25°C and 40°C. These tests were carried out on D1, D7, D14, D21, D30, D40, and D60 (Khan et al. 2013).

2.10. Mathematical analysis: The percentage changes for the individual values of pH and conductivity, taken every week, were calculated by the following formula;

Percentage Change = [(A - B) / B]*100
Here; A = Individual value of any parameter on D1, D7, D14, D21, D30, D40, D60
B = Zero hour value of that parameter (freshly prepared emulsions)

2.11. Evaluation of the Antioxidant Activity: Extract and emulsions Antioxidant Activity was evaluated by DPPH (2,2-diphenyl-1-picrylhydrazil) standard methods . 50 µl of different concentrations of the extract was added to 1.950 ml of freshly prepared DPPH- methanolic solution (0.025 g/l). At the same time, a negative control was prepared by mixing 50 µl of methanol with 1.95 ml of the methanolic DPPH solution. After 30 min of incubation in the dark at room temperature, the absorbance was measured at 515 nm (OPTIZEN 3220, South Korea). All operations were performed in triplicate.

The radical scavenging activity was

calculated as a percentage of DPPH discoloration using the following equation: DPPH radical scavenging % = [(A0 – A1)/A0] × 100.

Where A0 is the absorbance of the DPPH solution and A1 is the absorbance of the sample. The antiradical activity was expressed as IC50 (mg/mL), the extract dose required to cause a 50% decrease of the absorbance at 515 nm. A lower IC50 value corresponds to a higher antioxidant activity. Emulsion antioxidant activity was determined by the same method. 50 µl of different concentrations of each emulsion (base and with extract) previously diluted (1:100) were mixed with 1.950 ml of freshly prepared DPPH- methanolic solution (0.025 g/l). The negative control was prepared the same way as for the extract (50 µl of methanol with 1.95 ml of the DPPH-methanolic solution) (Sánchez-Moreno 2002; Khan et al. 2013). This operation aimed to define if the emulsion was able to keep the antioxidant activity of the extract compared to control formulation**.**

3. Results and Discussion

3.1. Microscopic observation

Microscopic observation of leaves powder revealed the following characteristic elements (Figure 1): Unicellular long and flexuous trichome (A), Parenchymatous cells containing calcium oxalate raphides (sharp needle shape) (B) fragments of cutinized epidermis with calcium oxalate star-shaped druses (C).

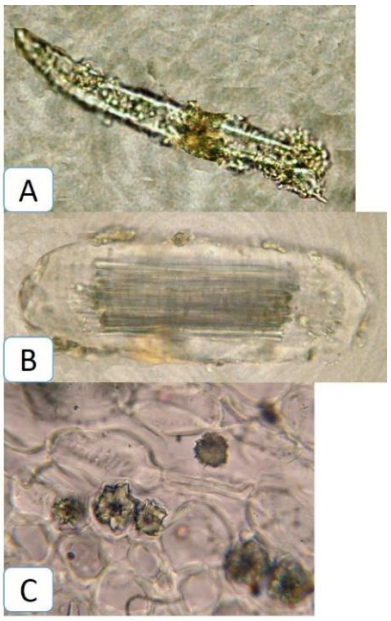


Figure 1: Characteristics of *Vitis vinifera L.* leaf powder, (A) Unicellular trichome, (B) Parenchymatous cells containing calcium oxalate raphides , (C) Calcium oxalate star-shaped druses

In this work, powder microscopic characteristics of red vine leaves, agree with the data related by French Pharmacopoeia 11th edition, which mentions the presence of unicellular, long, and tapered trichomes with a truncated base and numerous dispersed calcium oxalate raphides or located in cells. French Pharmacopoeia 11th edition also mentions fragments of parenchyma containing calcium oxalate star-shaped druses like found in this study (French Pharmacopoeia 2014).

3.2. Total phenolic, flavonoid, and condensed tannin contents

Table 3, compares total phenolic, flavonoid, and condensed tannin contents for the different samples.

Table 3: Total polyphenol, flavonoids, and condensed tannins content of grapevine leaves

Sample area	Total polyphenol content *	Flavonoïd content**	Condensed tannins content **
Aïn Temouchent	391±40	10,62±0.23	5,30±0.04
Oran	923±88	9,87 ±0.15	5,14±0.16
Boumerdes	842±76	7,93±0.43	4,15±0.03

Bordj-Bou-Arreridj	246±18	4,36±0.13	3.45±0.13
Mostaganem	299±14	7,02±0.04	5,00±0.05
El-Bayadh	316±13	5,74±0.25	1,81±0.04
Medea	423±22	4,10±0.07	2,32±0.04
Mascara	491±72	7,20±0.14	4,19±0.04
Annaba	499±82	2.07±0.08	0,85±0.03
Blida	558±88	4,15±0.12	1,82 ± 0.11

* : mg Eq in Gallic acid / g dry plant
** mg Eq in Catechin / g Dry plant
Means ± SD (n=3)

The highest contents of total phenolic were found in Oran and Boumerdes samples, which were respectively 923±88.06 and 842±76.02 mg Eq in gallic acid / g dry plant material. Aïn Temouchent and Oran samples contained also the highest flavonoid contents, which were 10.62-±0.23 and 9.87±0.15 mg Eq in catechin/g Dry plant material, respectively.

The highest condensed tannin contents were found in Ain Temouchent and Oran samples with values of 5.30±0.04 and 5.14±0.16 mg Eq in catechin/g Dry plant material, respectively. All Samples contained more flavonoids than condensed tannins. Overall, Oran sample was the richest in total phenolic, flavonoid and condensed tannin compared to the others. Therefore, this extract was used as the active ingredient in the emulsion. A global comparison of polyphenols, flavonoid, and condensed tannin contents indicates that the highest flavonoid and condensed tannin concentrations do not necessarily correspond to the highest phenolic concentrations, so their distribution differs from one region to another. The obtained results confirmed high polyphenol contents of *Vitis vinifera L.* leaves. These results were in agreement with those found by Yu, Lim et al (2014) who determined the average polyphenol content of *Vitis labruscana L.H.Bailey.* leaves, which was in the order of 328.5±1.0 mg Eq in gallic acid / g dry plant material (Yu et al. 2014).

The work of Pastrana-Bonilla, et al (2003) is among the first studies on red grapevine leaves, from which they were able to determine an average polyphenol content of 351.6 mg Eq in gallic acid / g dry plant material, this content was obtained from ten cultivars of muscadine (*Vitis rotundifolia Michx*) from southern Georgia in the United States. The results of this study also agree with this present work (Pastrana-Bonilla et al. 2003) .

Other studies such as that of Güler and Candemir (2014), who worked on five *Vitis vinifera L.* leaves samples from the Manisa region in Turkey found lower polyphenol levels with an average of 14.25 6 mg Eq in gallic acid / g dry plant material (Güler and Candemir 2014). The work of Taware et al (2010) also showed very low levels of polyphenols in five red vine leaves samples extracts from India, their contents average did not exceed 5 mg Eq in gallic acid / g dry plant material (Taware et al. 2010). Flavonoid contents were also variable from one region to another, the levels found in this work are close to those found by Güler and Candemir (2014), who reported total flavonoid values ranging from 5.08 to 7.22 mg catechin equivalent / g dry matter (Güler and Candemir 2014).

The same observation was found about condensed tannins with their variable distribution depending on samples. It was also noticed that all leave samples contained more flavonoids than condensed tannins.

Generally, sub-humid and humid areas such as Oran, Mascara and, Boumerdes had high amounts of polyphenols, whereas semi-arid and Saharan areas (Ain Temouchent and El-Bayadh) had lower levels. Flavonoid and condensed tannin content variations do not necessarily depend on bioclimatic stage nature, since similar amounts for leave samples from humid and arid regions have been observed, so other factors besides the bioclimatic nature influence these variations.

It is important to remind that environmental factors have a major effect on polyphenol content, these factors can be pedoclimatic (soil nature, sun exposure, rainfall) or agronomic (cultivation in greenhouses, organic cultivation, hydroponic cultivation, fruit yield per tree, etc.)Light exposure has a considerable effect on flavonoid amounts. The degree of plant maturity significantly affects polyphenol concentrations. Generally, phenolic acid concentrations decrease during plant ripening, while anthocyanin concentrations increase. Many polyphenols, especially phenolic acids, are directly involved in plant response to different types of stress: they contribute to the healing process by the lignification of damaged areas, they possess antimicrobial properties and their concentration can increase after infection (Manach et al. 2004). Hydric stress is also one of the elements that can significantly influence polyphenolic composition as reported by (Król et al. 2014). With the current state of knowledge, it is extremely difficult to identify key factors causing the variability of phenolic type contents.

3.3. Emulsion control

- Organoleptic test

Both emulsions (control and active) presented a smooth and stringy consistency, a bright aspect with white color for the control and clear brown color for the active emulsion. The organoleptic evaluation was based on color change, odor, liquefaction, and phase separation during two months.
Color remained stable for both emulsions without any change during the whole 60 days period regardless of the storage temperature. Liquefaction was absent in both emulsions from D0 to D60 at 25°C and 40°C.
Phase separation was noticed after 40 days in the active emulsion stored at 40°C. There was no change in organoleptic characteristics of the control emulsion.

- Emulsions type

Both formulations were oil-in-water (O/W) emulsions, lipophilic dye (red Soudan III) colored only the oil phase giving a discontinuous aspect to the coloration whereas; hydrophilic dye (methylene blue) colored the continuous aqueous phase uniformly giving a homogeneous color, which was confirmed by emulsions water dilution (Figure 2).

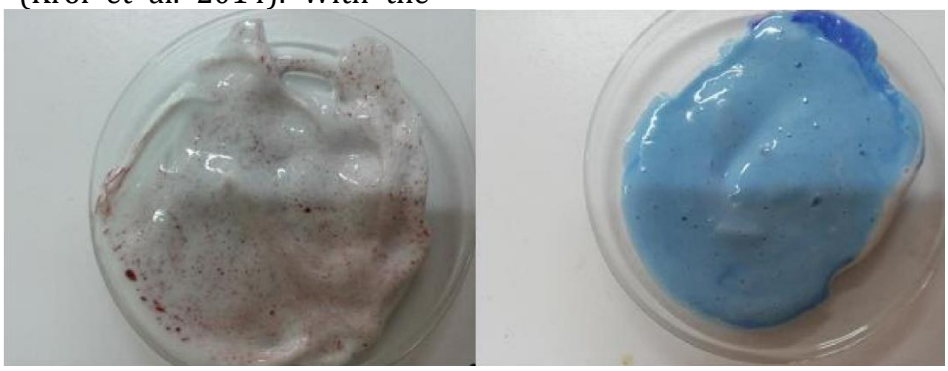


Figure 2: Dye solubility method

- Microscopic examination

Microscopic observation confirmed the emulsions type (O/w), the observed drops had a relatively homogeneous size, with no flocculation and no coalescence phenomena (Figure 3).

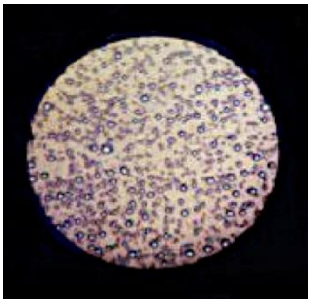


Figure 3: Microscopic observation of the emulsions colored by methylene-blue (magnification 100)

- Centrifugation stability

There was no phase separation after centrifugation for both emulsions. (Figure 4).

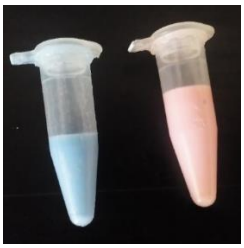


Figure 4: Emulsions appearance after centrifugation

Topical emulsions find their applications in various dermatological affections and many of them are plant extract-based, for example, Huma, et al (2020) reported that beet leaf extract- based emulsion possessed antioxidant and anti-UV properties (Huma et al. 2020). Usually, the emulsion type is determined by the ratio of the oil phase and the aqueous phase.

In this work, the emulsion was prepared with 40.0% oil, and 60.0% water (a weight phase ratio of 2: 3), so the emulsion is oil/water type (O/W). The Microscopic analysis confirmed that both emulsions were oil/water type. There was no color change in both emulsions during the two months of the study period at different storage temperatures; this indicates the stability of the two formulations at different storage conditions.

Color conservation can be explained by different factors contributing to the emulsion stability; red vine leaves extract may contain antibacterial substances that protect the emulsion from possible microbial multiplication that could cause coloration change (Khan et al. 2013). Centrifugation test is a very useful method for assessing and predicting emulsion shelf life. No phase separation after centrifugation was observed in both emulsions. This suggests that the good speed homogenization used during the preparation, avoided its breakdown during the stress test as reported by (Colucci et al. 2020). After emulsion preparation, time and temperature-related factors could produce viscosity-altering processes, which result in emulsion liquefaction. No liquefaction was observed for both emulsions during 60 days In this study, the results were better than those of Khan, et al (2013), who observed a liquefaction process in Cassia fistula extract-based emulsion on D 21 kept at 40°C (Khan et al. 2013). As well as those of Sharif, et al (2014), who observed a liquefaction process in the grape-seed extract- based emulsion on D 21 kept at 40°C (Sharif et al. 2014). Creaming phenomenon is due to a density difference between two phases under gravity effects, which leads to their separation (Salager 2000). In this study, phase separation was observed for the active emulsion on D40 kept at 40°C. For Sharif, et al (2014) study, phase separation occurred earlier on D21 at 40°C, in contrast to Khan, et al (2013) work, where no phase separation was noticed (Khan et al. 2013; Sharif et al. 2014).

This delayed phase separation at high temperature, indicates good emulsion

stability based on creaming criteria, for a conservation period no longer than 40 days.

- Electrical conductivity

The electrical conductivity was measured for all samples kept at 25 oC and 40 o C, immediately after preparation and after 1, 7, 14, 21, 30, 40, and 60 days. Percentage changes in the conductivity values are presented in figure 5. The conductivity test showed a non-significant change in conductivity after two months for both emulsions kept at 25°C.

A significant conductivity decrease was observed in the control emulsion kept at 40°C and a less significant decrease (5.40%) of the active emulsion conductivity kept at the same temperature. Conductivity differences occur if there is an emulsion creaming, with the oil fraction increasing in the upper part and the aqueous fraction increasing in the lower part (Salager 2000). In this study, a significant difference in conductivity change was observed for both emulsions kept at 40°C, which is a sign of instability at this temperature. These results agree with those of Khan, et al (2013) who also observed a significant difference in electrical conductivity change for all emulsions kept at 40°C (Khan et al. 2013).

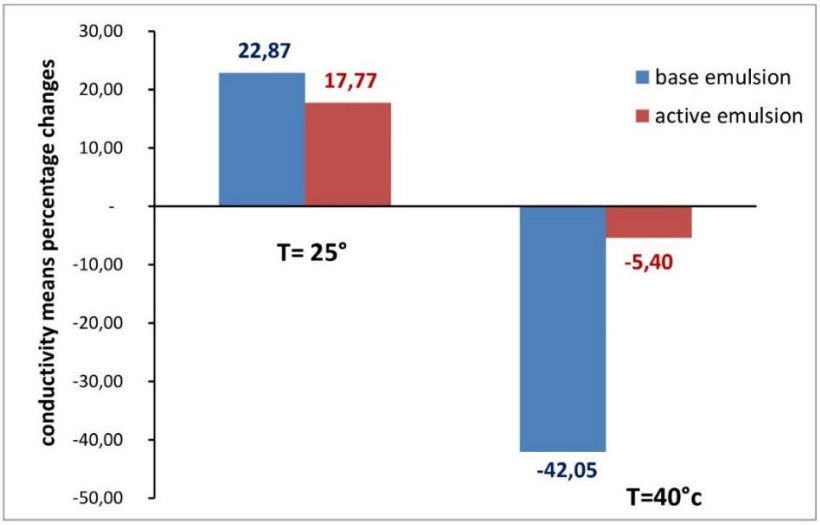


Figure 5: Means percentage changes in the conductivity of base emulsion and *Vitis vinifera L*. emulsion between day 0 and day 60 at 25°C and 40°C.

- pH test

Figure 6 showed that the pH of the control on D1 kept at 25°C and 40°C was 5.34 and 5.9 respectively, and that of the active emulsion was 4.77 and 4.74 respectively

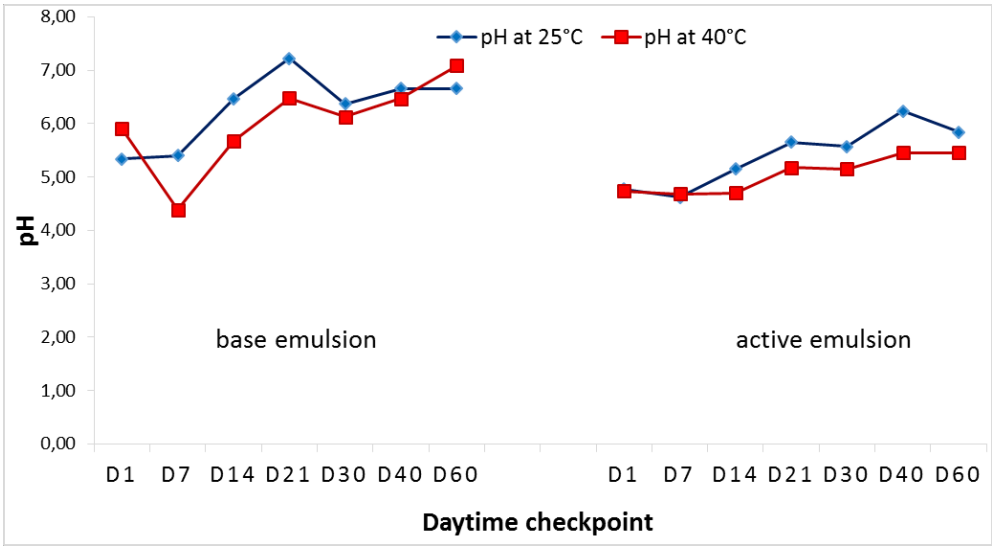


Figure 6: Evolution of pH as a time function in base emulsion and *Vitis vinifera L.* emulsion stored at 25°C and 40°C from D1 to D60

Control emulsion pH kept at 25°C increased gradually during the first three weeks then decreased progressively and stabilized on D40 at the value of 6.66 while for the control emulsion kept at 40°C, the pH decreased during the first week, then increased gradually exceeding its initial value and reaching 7.09 on D60.

pH evolution profiles for the active emulsion kept at 25°C and 40°C, were similar to those of the base emulsion with a slight decrease during the first week then a progressive increase reaching the value of 5.84 for the sample kept at 25°C and 5.05 for that kept at 40°C.

pH is an important indicator of topical emulsions safety and efficacy (Brossard et al. 2016), human body skin has a pH that varies from 4.5 to 6.0, a value of 5.5 is supposed to be the average skin pH, therefore, the formulations recommended for dermatological use should have a pH in this range (Lambers et al. 2006).

According to the results of this work, active emulsion pH was within dermatological pH standards, unlike to control emulsion pH, so it does not lead to any modification of factors regulating skin hydration (Lambers et al. 2006).

- DPPH radical-scavenging activity

Table 4 summarizes the percentage inhibition of DPPH radical for Oran extract sample and the emulsions as well as their IC 50.

Table 2. Extract and emulsions DPPH radical scavenging activity

sample	Total polyphenol content	IC 50	% inhibition
Grapevine leaves extract	923±88	2.9±0.04	48.80 ±9.24
Control emulsion	112±1.15	15.6±0.13	9,22 ±1.66
Active emulsion	208±14	9.37±0.21	18,20 ±2.29

IC50 : concentration of antioxidants needed to decrease the initial DPPH concentration by 50% expressed by µg/ml
Means ± SD (n=3)

The addition of grapevine leaves extracts to the emulsion increased its antioxidant activity twice, but it remained lower than that of the crude extract used in the formulation. The same observation can be made for the polyphenol content of the

emulsion, which was lower than that of the crude extract. The active emulsion showed a good antioxidant activity but lower than that of red vine leaf extract. This can be explained by the extract quantity (5%) used in the formulation. Active emulsion antioxidant activity was more important compared to that of the control emulsion, which indicates that the extract is effectively the factor responsible for inhibition degree change (from 9% for the control to more than 18% for the active emulsion). The comparison between polyphenol contents of the control and the active emulsion allowed us to think that the 18% DPPH free radical inhibition percentage is probably due to this phenolic fraction provided by the extract.

4. Conclusion

The main objective of this work was to valorize *Vitis vinifera L.* leaf extract by the development and the characterization of an emulsion containing this extract as an active ingredient, a work that has not been carried out to date. The quantitative analysis of the main polyphenolic classes (total polyphenols, flavonoids and, condensed tannins) gave us an overview for their variation contents, we could establish that bioclimatic stages have a relative influence on phenolic fraction composition; the samples from regions characterized by a humid climate are richer in polyphenols than those from regions with a dry climate. Variations in flavonoid and condensed tannin contents are obviously influenced by other factors that have to be identified.

Following the results of emulsion formulation and characterization part of this work, it can be concluded that the oil/water emulsion *Vitis vinifera L.* leaf extract based showed good physical properties, satisfying stability, and good antioxidant activity. All these properties offer a new delivery system potentially applicable in dermo-cosmetology.

Acknowledgements

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

Author Contribution

Concept and Design, Resources, Analysis, Interpretation and Writing were performed by Mohammed Adil SELKA and Amel CHENAFA –Supervision, Materials and Critical Reviews were directed by Mohammed Yacine ACHOURI, Data Collection and Literature Search were conducted by Nazim BELLIFA

Conflicts of Interest

The author declares no conflict of interest.

References

1. Al-Achi A. (2020). A brief review of pharmaceutics. Journal of Drug Discovery, Development and Delivery. 6(1):1036. https://doi.org/10.33513/PPPS/1901-10

2. Baydar NG, Özkan G, Sağdiç O. (2004). Total phenolic contents and antibacterial activities of grape (*Vitis vinifera L.*) extracts. Food control. 15(5):335-339. https://doi.org/10.1016/S0956-7135(03)00083-5

3. Benmeziane F, Djamai R, Cadot Y, Seridi R. (2014). Optimization of extraction parameters of phenolic compounds from Algerian fresh table grapes,(*Vitis Vinifera L.*). International Food Research Journal. 21(3).

4. Brossard D, Charrueau C, Chaumeil JC, Crauste-Manciet S, Hir AL. (2016). Pharmacie galénique: Bonnes pratiques de fabrication des médicaments [Galenic Pharmacy: Good drug manufacturing practices]. Elsevier Health Sciences, Paris. p 265-269.

5. Colucci G, Santamaria-Echart A, Silva SC, Fernandes IP, Sipoli CC, Barreiro MF. (2020). Development of water-in-oil emulsions as delivery vehicles and testing with a natural antimicrobial extract. Molecules. 25(9):2105. https://doi.org/10.3390/molecules25092105

6. French Pharmacopoeia, 10th edition. 2014. [accessed 2020 Feb 12].http://dev4-afssaps-marche2017.integra.fr/var/ansm_site/storage/original/application/aeaa1b4d72f30799f1206dd00f925c07.pdf

7. González S, Fernández-Lorente M, Gilaberte-Calzada Y. (2008). The latest on skin photoprotection. Clinics in dermatology. 26(6):614-626. https://doi.org/10.1016/j.clindermatol.2007.09.010

8. Güler A, Candemir A. (2014). Total Phenolic and Flavonoid Contents, Phenolic Compositions and Color Properties of Fresh Grape Leaves. Turkish Journal of Agriculture and Natural Sciences. 1:778-82. https://dergipark.org.tr/tr/download/article-file/142176.

9. Huma S, Khan HMS, Sohail M, Akhtar N, Rasool F, Majeed F, Daniyal M. (2020). Development, in-vitro characterization and assessment of cosmetic potential of Beta vulgaris extract emulsion. Journal of Herbal Medicine. 23:100372. https://doi.org/10.1016/j.hermed.2020.100372

10. International Organisation of Vine and Wine.2017 [accessed 2017 OCT 22]. https://www.oiv.int/js/lib/pdfjs/web/viewer.html?file=/public/medias/5479/oiv-en-bilan-2017.pdf,2017

11. Julkunen-Tiitto R. (1985). Phenolic constituents in the leaves of northern willows: methods for the analysis of certain phenolics. Journal of agricultural and food chemistry. 33(2):213-217. https://doi.org/10.1021/jf00062a013

12. Katalinic V, Mozina SS, Generalic I, Skroza D, Ljubenkov I, Klancnik A. (2013). Phenolic profile, antioxidant capacity, and antimicrobial activity of leaf extracts from six *Vitis vinifera L.* varieties. International journal of food properties. 16(1):45-60.https://doi.org/10.1080/10942912.2010.526274

13. Khan BA, Akhtar N, Khan H, Braga VdA. (2013). Development, characterization and antioxidant activity of polysorbate based O/W emulsion containing polyphenols derived from Hippophae rhamnoides and Cassia fistula. Brazilian Journal of Pharmaceutical Sciences. 49(4):763-773. https://doi.org/10.1590/S1984-82502013000400016

14. Król A, Amarowicz R, Weidner S. (2014). Changes in the composition of phenolic compounds and antioxidant properties of grapevine roots and leaves (*Vitis vinifera L.*) under continuous of long-term drought stress. Acta Physiologiae Plantarum. 36:1491-1499. https://doi.org/10.1007/s11738-014-1526-8

15. Lambers H, Piessens S, Bloem A, Pronk H, Finkel P. (2006). Natural skin surface pH is on average below 5, which is beneficial for its resident flora. International Journal of Cosmetic Science. 28. https://doi.org/10.1111/j.1467-2494.2006.00344.

16. Lim H, Jo M, Ban C, Choi YJ. (2020). Interfacial and colloidal characterization of oil-in-water emulsions stabilized by interface-tunable solid lipid nanoparticles. Food chemistry. 306:125619. https://doi.org/10.1016/j.foodchem.2019.125619

17. Lu GW, Gao P. (2010). Emulsions and microemulsions for topical and transdermal drug delivery. Handbook of non-invasive drug delivery systems. Elsevier; p. 59-94. https://doi.org/10.1016/B978-0-8155-2025-2.10003-4

18. Manach C, Scalbert A, Morand C, Rémésy C, Jiménez LV. (2004). Polyphenols: food sources and bioavailability. The American journal of clinical nutrition. 79 5:727-747. https://doi.org/10.1093/ajcn/79.5.727

19. Noon J, Mills TB, Norton IT. (2020). The use of antioxidant rutin hydrate Pickering particles to combat lipid oxidation in O/W emulsions. Journal of Food Engineering. 274:109830. https://doi.org/10.1016/j.jfoodeng.2019.109830

20. Orhan DD, Orhan N, Ozcelik B, Ergun F. (2009). Biological activities of *Vitis vinifera L.* leaves. Turk J Biol. 33:341-348. https://doi.org/10.3906/biy-0806-17

21. Pandey KB, Rizvi SI. (2009). Plant polyphenols as dietary antioxidants in human health and disease. Oxidative medicine and cellular longevity. 2(5):270-278. https://doi.org/10.4161/oxim.2.5.9498

22. Pastrana-Bonilla E, Akoh CC, Sellappan S, Krewer G. (2003). Phenolic content and antioxidant capacity of muscadine grapes. J Agric Food Chem. 51(18):5497-5503. https://doi.org/10.1021/jf030113c

23. Ralla T, Salminen H, Braun K, Edelmann M, Dawid C, Hofmann T, Weiss J. (2020). Investigations into the structure-function relationship of the naturally-derived surfactant glycyrrhizin: Emulsion stability. Food Biophysics. 15(3):288-296. https://doi.org/10.1007/s11483-020-09624-0

24. Rasul A, Akhtar N. (2012). Anti-aging potential of a cream containing milk thistle extract: Formulation and in vivo evaluation. African Journal of Biotechnology. 11(6):1509-1515. https://doi.org/10.5897/AJB11.2678

25. Salager JL.(2000). Emulsion properties and related know-how to attain them. Pharmaceutical emulsions and suspensions, 105: 73-125. https://doi.org/10.1201/b14005-4

26. Sánchez-Moreno C. (2002). Methods used to evaluate the free radical scavenging activity in foods and biological systems. Food science and technology international. 8(3):121-137. https://doi.org/10.1106/108201302026770

27. Seiller M, Martini MC. (1996). Formes pharmaceutiques pour application locale. Tec & Doc Lavoisier,Paris. pp 125-131.

28. Selka M, Achouri M, Chenafa A, Toumi H. (2019). The study of polyphenolic compounds profile and antioxidant activity of *Vitis vinifera L.* leaves from western regions of Algeria. IJP Algeria. 6(1):20-29. https://doi.org 10.13040/IJPSR.0975-8232.IJP.

29. Selka M, Chenafa A, Achouri M, Aoued L, Tareb S, Nourredine M, Toumi H. (2016). Activité antimicrobienne et antioxydante des feuilles de *Vitis vinifera L*. Phytothérapie. 14(6):363-369. https://doi.org/10.1007/s10298-016-1036-5

30. Sharif A, Akhtar N, Khan MS, Menaa B, Khan BA, Menaa F. (2014). Development and Optimization of Dimethicone-based Cream Containing Muscat Hamburg Grape Extract: In-vitro Evaluation. Journal of Pharmaceutical Care & Health Systems. 1:1-6. https://doi.org/10.4172/2376-0419.1000107

31. Smaoui S, Hlima HB, Chobba IB, Kadri A. (2017). Development and stability studies of sunscreen cream formulations containing three photo-protective filters. Arabian Journal of Chemistry. 10:S1216-S1222. https://doi.org/10.1016/j.arabjc.2013.02.020

32. Taware PB, Dhumal K, Oulkar DP, Patil SH, Banerjee K. (2010). Phenolic alterations in grape leaves, berries and wines due to foliar and cluster powdery mildew infections. International journal of pharma and bio sciences. 1:1-14. https://doi.org/10.22376/ijpbs

33. Ushikubo F, Cunha R. (2014). Stability mechanisms of liquid water-in-oil emulsions. Food Hydrocolloids. 34:145-153. https://doi.org/10.1016/j.foodhyd.2012.11.016

34. Wehrlé P. (2012). Pharmacie galénique: formulation et technologie pharmaceutique [Galenic pharmacy: formulation and pharmaceutical technology]. Maloine,Paris, pp 245-262.

35. Xia E-Q, Deng G-F, Guo Y-J, Li H-B. (2010). Biological activities of polyphenols from grapes. International journal of molecular sciences. 11(2):622-646. https://doi.org 10.3390/ijms11020622

36. Ying C, Wan D. (2012). Quantitative determination of total and individual flavonoids in stems and leaves of Buddleja davidii and Buddleja albiflora. Pharmacognosy Magazine. 8(32):273. https://doi.org/10.4103/0973-1296.103651

37. Yu Q-M, Lim EJ, Choi S-K, Seo J-S. (2014). Antioxidant effect of grapevine leaf extract on the oxidative stress induced by a high-fat diet in rats. Food Science and Biotechnology. 23:849-857. https://doi.org/10.1007/s10068-014-0114-4

38. Zhao X, Debeli DK, Shan G. (2020). A novel drug loading and release from a thermoresponsive hydrogel formed in situ emulsion polymerization. Journal of Applied Polymer Science. 137(19):48669. https://doi.org/10.1002/app.48669

39. Zillich O, Schweiggert-Weisz U, Eisner P, Kerscher M. (2015). Polyphenols as active ingredients for cosmetic products. International journal of cosmetic science. 37(5):455-464. https://doi.org/10.1111/ics.12218.

Research article
Curr. Pers. MAPs, (2022); 5(1): 50-62

Current Perspectives on Medicinal and Aromatic Plants

An International Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

Antioxidant and Antibacterial Activities of Methanol Extracts from Various Plant Parts of Pomegranate and Anatolian Black Pine

Omar SAID[1], Yunus AKSUT[1], Remziye Eda YARDIMCI[2], Suheyla KARATAS[2], Hesna YIGIT[3], Ahmet Zafer TEL[3,4], Nazli ARDA[5,6*]

[1] Department of Molecular Biology and Genetics, Institute of Graduate Studies in Sciences, Istanbul University, 34116, Fatih, Istanbul, Turkey
[2] Department of Aquaculture and Fish Diseases, Faculty of Aquatic Sciences, Istanbul University, 34134, Fatih, Istanbul, Turkey
[3] Department of Biology, Faculty of Science and Letters, Adiyaman University, 02040, Merkez, Adiyaman, Turkey
[4] Department of Agricultural Biotechnology, Faculty of Agriculture, Igdir University, 76000, Igdir, Turkey
[5] Department of Molecular Biology and Genetics, Faculty of Science, Istanbul University, 34134, Fatih, Istanbul, Turkey
[6] Center for Research and Practice in Biotechnology and Genetic Engineering (BIYOGEM), Istanbul University, 34452, Vezneciler, Fatih, Istanbul, Turkey, E-mail: narda@istanbul.edu.tr

*Corresponding author: narda@istanbul.edu.tr

https://doi.org/10.38093/cupmap.1122429

Received: 27/05/2022
Accepted: 30/06/2022

Abstract

Oxidative stress and bacterial infections threaten human and animal health. Different parts of the plants have a great potential to be used as a source of antioxidant and antibacterial agents for human or animal welfare, because of their active metabolites. This study was conducted to assess the antioxidant and antibacterial activities of methanolic extracts from the leaves, flowers, whole fruits, and woods of pomegranate (*Punica granatum* L.), and the leaves, cones, and woods of Anatolian black pine [*Pinus nigra* Arn. subsp. *pallasiana* (Lamb.) Holmboe]. Antioxidant activity was screened by DPPH and CUPRAC assays. Antimicrobial activity was examined by disc diffusion test against fish pathogens, *Aeromonas hydrophila*, *Vibrio anguillarum*, *Yersinia ruckeri* and *Lactococcus garvieae*.

Pomegranate whole fruit extract possessed superior antioxidant activity even higher than ascorbic acid. All parts of pomegranate, except wood, also exhibited significant antibacterial activity against fish pathogens. Black pine cone extract slightly inhibited the growth of fish pathogens while other pine extracts were inactive. This study reveals that the whole fruit of pomegranate is a prominent source of antioxidant and antibacterial metabolites. Cones of Anatolian black pine also seem to be a source of antibacterial compounds against fish pathogens.

Key Words: *Punica granatum*, *Pinus nigra*, *Aeromonas hydrophila*, *Vibrio anguillarum*, *Yersinia ruckeri*, *Lactococcus garvieae*

1. Introduction

Plant kingdom is the most prominent source for bioactive substances, like antioxidant and/or antimicrobial compounds. For ages, even today plant-based traditional remedies are used by various societies in the World. Up to date, a number of ethno-pharmaceuticals, nutraceuticals and cosmeceuticals are approved by the authorities as well (EMA, 2020; Kakade et al., 2021; Kına et al., 2021). Thus, plants seem to be persistent center of interest of pharmaceutical, food/feed and cosmetic industries.

Natural antioxidants attract attention of scientists since reactive oxygen metabolites (ROMs) including free radicals resulted from oxidative stress are involved in many harmful conditions for health, especially during the aging and many diseases (Uysal et al., 2021). Aside from their uses for medicinal purposes, antioxidants have a great potential to be used in various fields, which also directly or indirectly correlate with wellness, as a constituent of animal feed, as a preventive agent against deterioration in some foods or as an ingredient in anti-aging cosmetics.

Bacterial infections lead or contribute to undesirable health problems that threaten human and animal life. Attempts for discovery of new antibiotics become more important today more than ever because of multidrug resistance developed by the microorganisms against known antibiotics (WHO, 2013).

Pomegranate (*Punica granatum* L.) (Punicaeae), a special fruit whose name is commemorated in mythological records and sacred books since ancient times (Bhandari, 2019), is one of the most prominent natural product used in human nutrition concerning its health benefits. In Turkey, Mediterranean, Aegean and Southeast Anatolia are the regions producing the most amount of pomegranate (Kurt and Şahin, 2013). Its fruits are consumed in all over the world, and all parts (fruit, seed, root, leaf, flower and peel) are used as traditional remedies by various cultures since ancient times (Shaygannia et al., 2016; Bhandari, 2019). Therefore, its functional and medicinal activities such as antioxidant, antimicrobial, anti-inflammatory, antidiabetic, anti-aging, antimutagenic, anticarcinogenic, lipid-lowering, antiatherogenic, hepato-, dermo- and nephroprotective effects have been investigated intensely, especially in the last decade (Sevindik et al., 2017; Bhandari, 2019; Mohammed et al., 2020; Akgül et al., 2022). There is a number of publications on the antioxidant activity of plant parts that are consumed as fruit (arils) (Sravanthi and Rao, 2015; Yan et al., 2017; Amir et al., 2019) or juice (Les et al., 2015) as well as other plant parts (peels, barks, rinds, seeds, leaf) (Tozetto et al., 2017; Yan et al., 2017). *In vivo* antioxidant efficacy of pomegranate juice has been exerted on brine shrimp, *Artemia salina* (Les et al., 2015) and on human (Noori et al., 2016). Both antioxidant and antimicrobial activities of peels, rinds or seeds (Wendy et al., 2017; Ali Redha et al., 2018; Demir et al., 2019) and juice or arils (Ali Redha et al., 2018) have been investigated together in some studies.

Black pine (*Pinus nigra*) (Pinaceae) is a conifer, widely but fragmentally distributed across Europa and Asia (Enescu et al., 2016). *P. nigra* subsp. *pallasiana* grows naturally in Turkey, west side of cross Anatolia (Akkemik, 2018). Kızılarslan and Sevgi (2013) reviewed ethnobotanical uses of the *Pinus* L. genus in Turkey, and stated that *P. nigra* was the most preferred species used by local communities, especially in wood production or folk medicine. Various parts of *P. nigra*, such as branches, roots, tar or resin, are primarily used for medicinal purposes, mainly against respiratory system and skin problems, and usage of leaf and cone is in the second rank followed by other *Pinus* species, namely *P. sylsvestiris* and *P. brutia* (Kızılarslan and Sevgi, 2013). Antimicrobial and antioxidant activities of different parts and/or constituents from various *Pinus* species have

been investigated previously (Dığrak et al., 1999; Kilic et al., 2011; Eryilmaz et al., 2016; Sirakov et al., 2018; Fkiri et al., 2018). However, varieties of the *Pinus* species are undetermined or unspecified in some reports. Anatolian black pine [*P. nigra* Arn. subsp. *pallasiana* (Lamb.) Holmboe var. *pallasiana*] is one of the *Pinus* taxa naturally grown and widely distributed within pine flora of Turkey (Akkemik, 2018). It is mainly used in traditional tar production in central Western Turkey. Recently, potential use of cones of this variety as a natural dye material with antibacterial effect was investigated by Bahtiyari and Yilmaz (2018).

Metabolite content, thus bioactivity of the plant extracts depends on plant species, geographic conditions that the plant grown, plant part used in the extraction and the extraction solvent and method (Altemimi et al., 2017).

The present study aimed to determine the antioxidant and antibacterial activities of the methanolic extracts prepared from different parts of pomegranate and Anatolian black pine grown in the Southeastern Anatolia district of Turkey. Since polyphenolic compounds in plants are the basic constituents of natural antioxidants, and their radical scavenging activity is thought to have an important role in the prevention of numerous chronic diseases, total phenolic content of the extracts was also determined. The results were evaluated by the comparison within the parts of the same plant, as well as between the data reported in the literature.

2. Material and Methods

2.1. Plant Materials

Pomegranate (*Punica granatum* L.) and black pine [*P. nigra* Arn. subsp. *pallasiana* (Lamb.) Holmboe var. *pallasiana*] were collected from Adıyaman district (Turkey) and surroundings, and authenticated by Dr. Ahmet Zafer TEL at Adıyaman University, Faculty of Science, Department of Biology. Voucher specimens were deposited at the same department. After plucking the plant parts, all materials were washed with first tap water, then distilled water, and dried in the shade at room temperature. The dried materials were ground into powder, and stored in opaque containers with lid at refrigerator in desiccator (+4ºC) to avoid deterioration effect of light and high temperatures. Whole fruit of pomegranate included peels and carpelar membranes surrounding the edible parts (Machado et al., 2019). Plant materials were summarized in Table 1.

Table 1. Plant materials used in the study

Common Name	Systematical Name	Plant Parts	Herbarium No.	Collection Region and Habitat
Anatolian Black Pine	*Pinus nigra* Arn. subsp. *pallasiana* (Lamb.) Holmboe	Leaf Cone Wood	1377	Ulubaba Mountain, forest
Pomegranate	*Punica granatum* L.	Leaf Flower Whole fruit Wood	1378	Nemrut Mountain, forest-shrub

2.2. Preparation of Methanolic Extracts

Certain amount of each powdered plant material (5 g of all plant parts of pomegranate; 2 g of pine leaves, 10 g of pine cones and 5 g of pine woods) was weighed and extracted with 80 mL methanol using solvent extractor at 210 ºC for total 1 h 45 min (1 h immersion-30 min washing-15 min recovery). Methanolic extracts were then evaporated to dryness under reduced pressure using rotary evaporator, and the extraction yield was calculated. Each crude extract was then dissolved in a certain volume of methanol and centrifuged at 12.000xg at 4 ºC for 5 minutes, to eliminate insoluble materials. Supernatants were used as test materials, and kept in dark bottles at +4ºC until use.

2.3. Free Radical Scavenging Activity (DPPH Test)

DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical scavenging activity was measured by the determination of absorbance of the sample mixtures containing 40 µL of each extract (in a concentration of 0.065 - 1 mg/mL) and 160 µL of DPPH solution in methanol (0.2 mM) at 520 nm (Erol-Dayi et al., 2011). Blank sample was prepared mixing of DPPH solution with methanol instead of extract. All mixtures were kept in dark at RT for 10 min prior to measurement. Percentage inhibition of DPPH for each concentration was estimated by using following formula.

$$\% \text{ Inhibition of DPPH} = 1-(A_{sample} / A_{blank}) \times 100$$

where A_{sample} = absorbance of the sample mixture (extract + DPPH) and A_{blank} = absorbance of DPPH solution containing methanol instead of extract.

Ascorbic acid solutions in a concentration range of 0.005–0.1 mg/mL were used as reference antioxidants. The results were expressed as IC_{50}, which is defined as the concentration of sample required to inhibit DPPH by 50%.

2.4. Cupric Reducing Antioxidant Capacity (CUPRAC Test)

Cupric ion reducing powers of the extracts were determined using spectrophometric method described by Apak et al. (2006), with a slight modification. In this method, 250 µL of each, 10^{-2} M $CuCl_2$, 7.5×10^{-2} M ethanolic Neocuproine solution and 10^{-3} mM ammonium acetate buffer, pH 7.0, were added in each well of a 24-microwell flat bottom plate, respectively, and followed by 25 µL of each extract. The final volume of the reaction mixture was accomplished by adding 250 µL distilled water to each well. The mixtures were then kept in dark at RT for 1 h, and the absorbance was measured at 450 nm. Trolox was used as reference antioxidant, and results were expressed as one mmole trolox equivalent antioxidant capacity per gram dry weight of extract (mmole TEAC/g DW).

2.5. Total Phenolic Content

Total phenolic contents of the extracts were determined using Folin-Ciocalteu method (Singh et al., 2016), with slight modification. In this method, a stock solution of gallic acid as reference was prepared in a concentration of 125 µg/mL and serially diluted to 62.5 µg/mL, 31.25 µg/mL, 15.62 µg/mL, 7.8 µg/mL and 3.9 µg /mL. Into one milliliter of each crude extract or gallic acid solution, 0.5 mL of Folin's reagent, followed by 3 mL of Na_2CO_3 (Merck) solution (200 g/L) and 5.5 mL of distilled water were added. The mixtures were centrifuged at 1250×*g* at RT for 5 min. Two hundred microliter from each supernatant was transferred into the wells of a 96 microwell plate and the absorbances were recorded at 725 nm against blank containing of methanol instead of sample. The results were expressed as milligram gallic acid equivalent per gram of dry weight of extract (mg GAE/g DW), using standard gallic acid calibration curve.

Spectrophotometric data represented the mean ± SD of at least three independent experiments.

2.6. Antibacterial Activity Test against Fish Pathogens

Isolated strains of fish pathogens, *Aeromonas hydrophila*, *Vibrio anguillarum*, *Yersinia ruckeri* and *Lactococcus garvieae*, kept in the culture collection of the Department of Aquaculture and Fish Diseases, Faculty of Aquatic Sciences, Istanbul University (Table 2) were used in antibacterial activity tests. In addition, as a human, fish and aquatic bird pathogen (Miniero Davies et al., 2018), commercial strain of *Edwardsiella tarda* Ewing and McWhorter (ATCC® 15947) from human feces was employed.

Cultures were maintained in Nutrient Broth (NB) medium, and disc diffusion assay was employed for antimicrobial activity tests (Jorgensen and Turnidge, 2015). Two hundred microliter of stock cultures adjusted to 0.5 McFarland standard were added to 9800 µL of NB medium and incubated at 21ºC for 24 h. One hundred microliter from each fresh culture was transferred onto the solid medium of Mueller Hinton Agar (MHA) using "Drigalski" spatula, and spread with sterile cotton. Methanol extracts were sterilized by passing through the filters with 0.22 µm pore size. Certain volumes of the extracts in known concentrations were applied onto the aseptic filter paper discs with the diameter of 6 mm. Methanol in a volume equivalent to one in applied sample was used as blank. Following the methanol evaporated from the discs under the aseptic conditions, the discs were embedded onto the cultures in MHA media and incubated at 21ºC for 24 h. Diameters of inhibition zones as millimeter were measured and used as antimicrobial activity criteria. Ampicillin, oxytetracycline, kanamycin, trimethoprim sulfamethoxazole, flumequine, enrofloxacine and ciprofloxacin were used as reference antibiotics. Values were given as the mean ± SD of at least three independent experiments.

2.7. Statistical Analyses

The quantitative data were presented as mean ± standard deviation (SD) based on at least three independent experiments. Statistical analysis and graph generation were performed using the GraphPad Prism Software version 7.01. The statistical evaluation was performed with one-way analysis of variance (ANOVA) followed Tukey's multiple comparisons. The *P* value of<0.05 was taken as the criterion of statistically significance.

3. Results and Discussion

3.1. Antioxidant Activity

Antioxidant potential of the samples varied depending on the plant species, and different plant parts for the same plant (Table 3). DPPH scavenging and cupric ion reducing activities and total phenolic contents of the samples were compared in Figure 1.

Pomegranate extracts were the most active ones, as expected. Among the pomegranate samples, the whole fruit extract exhibited superior DPPH scavenging activity with the IC_{50} value of 0.030±0.007 mg/mL, even better than the reference antioxidant, ascorbic acid (0.048 mg/mL) (Table 3). Similar superiority of pomegranate extracts was also detected in CUPRAC assay. Cupric reducing activity of these extracts ranged between 1.409-2.323 mmole TEAC/g DW, with the priority of whole fruit extract. Surprisingly, the antioxidant activity of pomegranate wood extract was also prominent. There was no statistically significant difference between the antioxidant activities of wood and whole fruit extracts ($P>0.05$) in both DPPH and CUPRAC tests, although total phenolic content of wood extract was very low ($P<0.001$). This result indicated that wood extract might have bioactive constituents other than phenolic compounds.

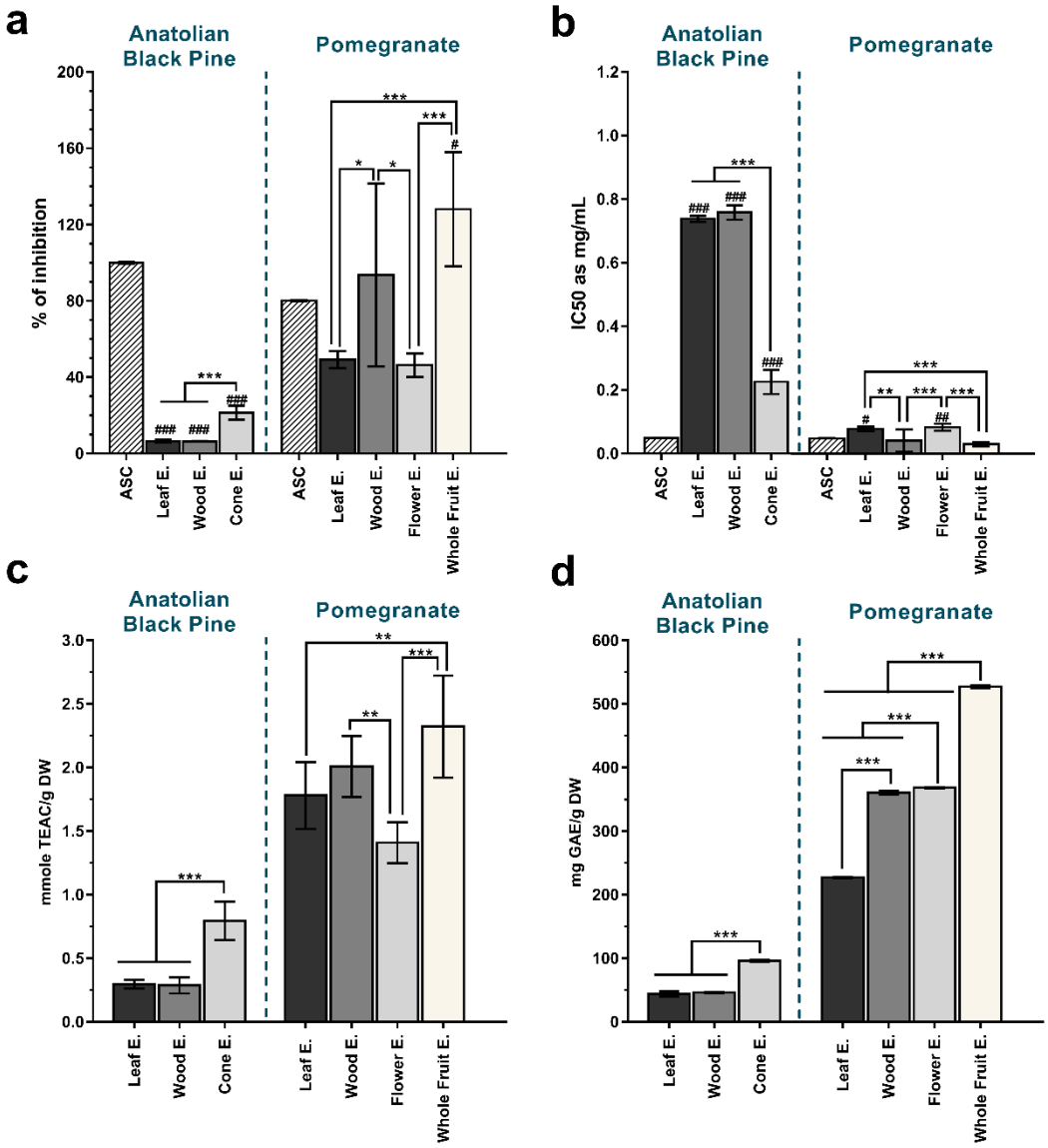


Fig 1. Comparison of **a)** percentage inhibition of DPPH, **b)** the half maximal DPPH inhibitory concentration (IC_{50}), **c)** CUPRAC value, and **d)** total phenolic content of the samples. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus ascorbic acid. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, which show multiple comparisons between different groups. *P* values were determined by one-way ANOVA using Tukey's multiple comparison test. (ASC: ascorbic acid, E.: extract).

Table 2. Fish pathogens and their origins

Isolate Name	Culture Collection Code	Collected Region of Fish	Isolated Fish	Isolated Organ	Isolation Media
Aeromonas hydrophila	PŞF	Sapanca, Marmara region, Turkey	Rainbow trout	Liver	TSA*
Vibrio anguillarum	HVA	Bodrum, Aegean district, Turkey	Sea bass	Liver	MA*
Yersinia ruckeri	YR38	Fethiye, Mediterranean district, Turkey	Rainbow trout	Liver	TSA
Lactococcus garvieae	EYJK-B1	Fethiye, Mediterranean district, Turkey	Rainbow trout	Spleen	TSA

*TSA: Tryptone Soy Agar; MA: Marine Agar

Table 3. Antioxidant activity and total phenolic content of the extracts prepared from different parts of the plants

Plant/parts	DPPH Scavenging Activity (IC_{50} as mg/mL)	Cupric Reducing Activity (mmole TEAC/g DW)	Total Phenolic Content (mg GAE/g DW)
Pomegranate/leaves	0.078±0.007	1.781±0.263	227.08± 0.960
Pomegranate/flowers	0.083±0.011	1.409±0.161	368.24±0.868
Pomegranate/ whole fruits*	0.030±0.007	2.323±0.401	526.85±2.128
Pomegranate/woods	0.041±0.035	2.008±0.240	360.30±3.290
Anatolian Black Pine/leaves	0.738±0.009	0.297±0.034	44.02± 4.032
Anatolian Black Pine/cones	0.225±0.038	0.793±0.151	96.14± 1.701
Anatolian Black Pine/woods	0.758±0.023	0.288±0.064	46.26± 0.790
Ascorbic acid	0.048±0.0002	-	-

*Whole fruit: Entire fruit with the rind [calyx, mesocarp, seeds (arils)] and a small portion of the stem peels and carpelar membranes surrounding the edible parts.

Pomegranate fruits are known to have several phenolic compounds, such as gallic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, caffeic acid, p-coumaric acid and ferulic acid (Singh et al., 2018) that contributes the antioxidant activity, and many other active metabolites, including flavonoids, anthocyanins, alkaloids, fatty acids and vitamins (Shaygannia et al., 2016).

In fact, promising antioxidant and antimicrobial activities of pomegranate have been manifested by a number of studies, previously. Although plant parts seem to be ambiguous in some of the publications, peels (barks, rinds) are the most studied parts in antioxidant activity tests, probably due to the attempts for utilization of large quantities of waste materials produced by food industry (Wendy et al., 2017; Yan et al., 2017; Tozetto et al., 2017; Ali Redha et al., 2018; Demir et al., 2019). Arils, seeds and fruits are in the second rank in research, since they are the plant parts consumed by humans or used for juice production (Sravanthi and Rao, 2015; Les et al., 2015; Yan et al., 2017; Ali Redha et al., 2018; Amir et al., 2019). Some researchers have investigated antioxidant activity of commercial or hand-made juices and some by-products (bagasse) (Yan et al., 2017). However, no study on the antioxidant activity of woods appeared in the literature. Our results demonstrate that not only the solvent (MeOH) but also the method (continuous Soxhlet extraction) used here seems to be highly effective for extracting total phenolics from pomegranate, since TPC of the extracts ranged from 227.08 to 526.85 mg GAE/g DW. Especially, TPC of whole fruit extract (526.85) was at least 40-fold higher than that of fruits from 5 varieties (4-11.8 mg GAE/g DW) grown in India (Sravanthi and Rao, 2015).

In this study, IC_{50} values of MeOH extracts from different parts of Anatolian black pine in DPPH test were ranged between 0.225 mg/mL and 0.758 mg/mL (Table 3). Cone extract having at least two fold phenolic compounds than the other parts (leaf and wood) showed the highest radical scavenging activity. Phenolic content of cones from *P. nigra* grown in Northwest Turkey (Bartın) have been previously investigated by Kilic et al. (2011), and catechin, a known flavonoid was detected as major phenolic, in addition to much lower of vanillin and 3,4-dihydroxybenzoic acid. Very recently, Topal (2020) reported

antioxidant activity and secondary metabolites of ethanol extracts of cones from Scots pine (*P. sylvestris*) trees grown in three different provinces in Eastern Anatolia (Gumushane, Erzurum and Sarikamis). Ethanolic extract of *P. sylvestris* grown in Gumushane seems to contain more total phenolic compounds (131.82 µg GAE/mg) than Sarikamis sample (99.09 µg GAE/mg) as well as than the Anatolian black pine cone extract studied here (96.14 µg GAE/mg DW). Although phenolic contents of the cone extracts from Scots pine grown in Sarikamis and Anatolian black pine grown in Adiyaman were very close, Scots pine cone extract with IC_{50} value of 30.13 µg/mL seems to be more effective scavenger than Anatolian black pine cone extract with IC_{50} value of 225±0.038 µg/mL obtained in this study. More than seven-fold difference between these samples may be dependent on the lack of some bioactive compounds because of species-specific genetic factors and environmental/climatic conditions, and/or partially loss of them during the extraction.

3.2. Antibacterial Activity

Although there are numerous reports on *in vitro* or *in vivo* antibacterial activity of various extracts of pomegranate and/or its constituents on various bacteria (Jalali et al., 2021), including *Aeromonas hyrophila* (Belal et al., 2009; Wendy et al., 2017), *Yersinia ruckerii* (Acar et al., 2018), *Lactobacillus gravieae* (Goudarzi et al., 2011) and *Edwardsiella tarda* (Wendy et al., 2017), there is no publication appeared in the literature concerning its activity on *Vibrio anguillarum* up to date. Methanolic extracts from all parts of pomegranate, except wood, inhibited most of the bacteria studied here (Table 4). Antibacterial activity in pomegranate extracts against *V. anguillarum* was reported for the first time. Whole fruit extract was the most active one, and *Y. ruckeri* and *L. garvieae* were the most sensitive fish pathogens to this extract. Pomegranate leaf and flower extracts also inhibited growth of 4 out of 5 bacteria species, except *A. hydrophila*, while wood extract was inactive against the test bacteria. No activity was detected in leaf extract against *A. hydrophila*. Within a number of reports indicating antibacterial activity of pomegranate, those on fish pathogens appeared in the literature in the last decade. Plant materials were mostly commercial products or waste materials, such as peels, seeds, fruits, rinds, flowers (Goudarzi et al., 2011, Wendy et al., 2017) in the studies conducted with fish pathogens, and *A. hydrophila* was the most studied organism. Besides, *in vivo* activity of seed oil (Acar et al., 2018) and fruits (Belal et al., 2009) were shown against *Y. ruckeri* in rainbow trout and *A. hydrophila* in mice, respectively.

The data obtained in this study are substantially in agreement with the results of previous reports. It is well known that phenolic compounds act as effective antimicrobials by leading to irreversible changes in the structure and properties of the bacterial membrane (Borges et al., 2013). Hence, methanolic extract of whole fruit consisting of peels and carpelar membranes surrounding the arils, which contain the highest total phenolic content, is expected to have antibacterial activity.

Although antioxidant and antimicrobial activities of needles and/or their essential oils from Anatolian black pine have been

investigated previously (Fkiri et al., 2018), no study was reported on the antibacterial activity of this variety against bacteria tested here. We demonstrated that only the methanolic cone extract of Anatolian black pine was able to inhibit the growth of all bacteria tested here moderately, with the inhibition zones ranging between 8.7±0.6 mm to 11.0±1.2 mm. Pine leaf and wood extracts were inactive against the bacteria tested here, probably due to their insufficient amount on the disc (Table 4).

Table 4. Inhibition zone diameters caused by methanolic extracts and reference antibiotics in disc diffusion assay where all discs used were 6 mm (Conc.: Concentration)

		Inhibition Zone Diameter (mm±SD)				
Plant/parts	**~Amount (mg)**	**Ah***	**Va***	**Yr***	**Lg***	**Et***
Pomegranate/leaves	10	–	11.0±2.0	12.0±0.6	11.0± 0.6	11.0±1.2
Pomegranate/ flowers	10	–	12.0±0.6	13.0±0.6	13.0±1.0	12.0±0.6
Pomegranate/whole fruits**	7	13.0±1.0	12.0±0.6	15.0±0.6	16.0±1.0	14.0±1.5
Pomegranate/ woods	2	–	–	–	–	–
Anatolian Black Pine/leaves	2	–	–	–	–	–
Anatolian Black Pine/cones	10	8.7±0.6	9.3±1.5	9.0±1.0	11.0±2.1	11.0±1.2
Anatolian Black Pine/woods	5	–	–	–	–	–
Reference antibiotics	~Amount (µg)	Ah*	Va*	Yr*	Lg*	Et*
Amp**	10	–	–	–	–	–
Oxytet**	30	18.0±2.5	11.0±2.0	24.0±1.0	13.0±0.5	17.0±1.0
Kan**	30	16.0±0.8	0	23.0±2.0	0	–
Trimet/ Sulfamet**	25	–	–	–	–	–
Flum**	30	13.0±1.0	23.0±2.0	12.0±0.5	14.0±1.5	–
Enrof**	5	15.0±1.0	30.0±2.5	21.0±1.5	16.0±1.0	15.0±1.0
Cipro**	1	15.0±1.0	12.0±2.0	19.0±1.0	14.0±1.5	11.0±1.0

*Ah, Va, Yr, Lg and Et are *Aeromonas hydrophila*, *Vibrio anguillarum*, *Yersinia ruckeri*, *Lactococcus garvieae* and *Edwardsiella tarda*, respectively.
**Amp, Oxytet, Kan, Trimet/ Sulfamet, Flum, Enrof and Cipro are abbreviations for Ampicillin, Oxytetracycline, Kanamycin, Trimethoprim/ Sulfamethoxazole, Flumequine, Enroflaxacine and Ciprofloxacin, respectively.
– means no inhibition zone was observed.

Antimicrobial activity of various part and/or constituents from *Pinus* species has been studied intensively (Sharma et al., 2015, Ramos et al., 2022). In a previous study having a similar concept with this study, antimicrobial activities of chloroform, acetone and methanol extracts from different parts of *P. nigra* in the same habitat have been compared by disc diffusion test (Dığrak et al., 1999). Methanolic extracts from leaves, cones and bark inhibited the growth of most of the bacteria, including *Bacillus megaterium*, *B. subtilis*, *B. cereus*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Mycobacterium smagmatis*, *Proteus vulgaris* and *Pseudomonas aeruginosa* at different levels. Methanolic cone extract was detected as the most active one against these bacteria, producing the inhibition zone of 17 mm on *P. vulgaris*, even higher than reference antibiotic ampicillin (10 µg). Ethereal extracts of cones from *P. nigra*, *P. brutia* and *P. halepensis* collected from various provinces in Turkey (*P. nigra* from Bartın at Western Black Sea Region, two others from Izmir and Mugla, respectively, at Aegean Region) have been investigated for their antimicrobial activities (Eryilmaz et al., 2016). Only *P. nigra* cone extract was found to be moderately active against *S. aureus* (MRSA), and *P. halepensis* cone

extract against *P. aeruginosa* (Eryilmaz et al., 2016).

Yersinia ruckeri, *Vibrio anguillarum*, *Lactococcus garvieae* and *Aeromonas hydrophila* lead to fish diseases namely yersiniosis, vibriosis, lactococcosis, motile Aeromonas septicemia, respectively, and they are of the bacteria cause great losses in aquaculture economics (Candan et al., 2007; Öztürk and Altınok, 2014). Importance of development and use of herbal drugs against harmful pathogens in aquaculture was discussed by Ramudu and Dash (2013). Our results demonstrated that the methanolic extracts of the pomegranate whole fruits and pine cones could be useful for the development of new natural and economical antibiotics, against the fish pathogens used in this study.

4. Conclusion

Aerial parts of pomegranate, mainly the whole fruits and woods are the most promising plant materials for obtaining antioxidant and antibacterial metabolites. For example, they might be used as a feed additive, especially for fish, or take part in packaging materials to prolong the shelf life in the future.

Moderate antioxidant and antibacterial activities detected in the cone extract of Anatolian black pine in this study make the pine cones an attractive plant source, although they have been regarded as an insignificant source for commercial production of antioxidants (Kilic et al., 2011). However, pine cones, which are produced in large quantities annually around the world (Bahtiyari and Yilmaz, 2018), can be used as a source for both antioxidant and antibacterial compounds and recycled. Plant habitats, especially pine forests have a great potential with not only their known plant sources but also undiscovered or by/waste products and residues, as stated by Ferreira-Santos et al. (2020).

Understanding the biological activities of different parts of these plants may arise their use as a source for active metabolites against oxidative stress and fish pathogens, directly or indirectly.

Further studies on these plant extracts, such as optimization of the extraction method, detection of active constituents and measurement of *in vivo* activities are in progress, in order to evaluate their uses in feed/food, cosmetic and pharmaceutical industries and to obtain standardized products.

Acknowledgements

This study was funded by Scientific Research Projects Coordination Unit of Istanbul University (Project number: 58297).

Author Contribution

NA, SK and HY designed the research and responsible for the conceptualization. OHS conducted the experiments and contributed to outline. AZT collected and identified voucher specimens of the plant materials. HY and AZT prepared the plant materials. OHS and NA prepared the extracts. YA and NA helped in collecting the data on antioxidant activity. SK, REY and HY helped in collecting the data on antibacterial activity. NA drafted and HY revised the paper. All authors read and approved the final manuscript.

Conflicts of Interest

The authors have no conflicts of interest to declare and disclose any financial field.

References

1. Acar, Ü., Parrino, V., Kesbiç, O.S., Paro, G.L., Saoca, C., *et al.* (2018). Effects of different levels of pomegranate seed oil on some blood parameters and disease resistance against *Yersinia ruckeri* in rainbow trout. *Frontiers in Physiology*, *9*, 1-7. https://doi.org/10.3389/fphys.2018.00596
2. Akgül, H., Mohammed, F. S., Kına, E., Uysal, İ., Sevindik, M. and Doğan, M. (2022). Total Antioxidant and Oxidant Status and DPPH Free radical activity of *Euphorbia eriophora*. *Turkish Journal of Agriculture-Food Science and*

Technology, 10(2), 272-275. http://dx.doi.org/10.24925/turjaf.v10i2.272-275.4685
3. Akkemik, Ü., (2018). *Pinus* L. (Çamlar) In: Akkemik, Ü., Editor. Türkiye'nin Doğal-Egzotik Ağaç ve Çalıları. Ankara: T.C. Orman ve Su İşleri Bakanlığı Orman Genel Müdürlüğü, pp. 118-127.
4. Ali Redha, A.A., Hasan, A.M. and Mandeel, Q., (2018). Phytochemical investigations of pomegranate (*Punica granatum*) rind and aril extracts and their antioxidant, antidiabetic and antibacterial activity. *Natural Products Chemistry & Research*, *6*(4), 2-10. http://doi.org/10.4172/2329-6836.1000332
5. Altemimi, A., Lakhssassi, N., Baharlouei, A., Watson, D.G., and Lightfoot, D.A. (2017). Phytochemicals: Extraction, isolation, and identification of bioactive compounds from plant extracts. *Plants (Basel, Switzerland)*, *6*(4), 42. https://doi.org/10.3390/plants6040042
6. Amir, M., Ahmad, N., Sarfaroz, M.D., Ahmad, W. and Mujeeb, M., (2019). Pharmacognostical, physicochemical standardization and *in vitro* antioxidant activity of *Punica granatum* Linn fruit. *Pharmacogn J. 11*(2), 272-277. https://doi.org/10.5530/pj.2019.11.42
7. Apak, R., Guclu, K., Ozyurek, M., Karademir, S.E. and Ercag, E., (2006). The cupric ion reducing antioxidant capacity and polyphenolic content of some herbal teas. *International Journal of Food Sciences and Nutrition*, *57*, 292-304. https://doi.org/10.1080/09637480600798l3
8. Bahtiyari, M.I. and Yilmaz, F., (2018). Investigation of antibacterial properties of wool fabrics dyed with pine cones. *Industria Textila*, *69*(3), 369-374. https://doi.org/10.35530/IT.069.05.1516
9. Belal, S.K.M., Rahman, A.H.A., Mohamed, D.S., Osman, H.E.H. and Hassan, N.A., (2009). Protective effect of pomegranate fruit juice against *Aeromonas hydrophila*-induced intestinal histopathological changes in mice. *World Applied Science Journal*, *7*(2): 245-254.
10. Bhandari, P.R., (2019). Pomegranate (*Punica granatum* L). Ancient seeds for modern cure? Review of potential therapeutic applications. *International Journal of Nutrition, Pharmacology, Neurological Diseases*, *2*(3), 171-184. http://doi.org/10.4103/2231-0738.99469
11. Borges, A., Ferreira, C., Saavedra, M.J. and Simões, M., (2013). Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. *Microbial Drug Resistance (Larchmont, N.Y.)*, 19(4), 256-65. https://doi.org/10.1089/mdr.2012.0244
12. Candan, A., Karataş, S., Küçüktaş, H. and Okumuş, İ., (2007). Marine Aquaculture in Turkey, Turkish Marine Research Foundation, Publication No.27, Istanbul TURKEY.
13. Demir, T., Akpınar, O., Kara, H. and Gungor, H., (2019). Nar (*Punica granatum* L.) kabuğunun *in vitro* antidiyabetik, sitotoksik, antioksidan ve antimikrobiyal aktivitesi. *Akademik Gıda*, *17*(1), 61-71. https://doi.org/10.24323/akademik-gida.544647
14. Dığrak, M., İlçim, A. and Alma, H., (1999). Antimicrobial activities of several parts of *Pinus brutia*, *Juniperus oxycedrus*, *Abies cilicia*, *Cedrus libani* and *Pinus nigra*. Phytotherapy Research, *13*, 584-587. https://doi.org/10.1002/(SICI)1099-1573(199911)13:7<584::AID-PTR508>3.0.CO;2-S
15. EMA (European Medicine Agency) (2020) Annual Report-The European Medicines Agency's contribution to science, medicines and health in 2020, Herbal Medicines, Annex 15 – HMPC European Union herbal monographs in 2020, pp. 55-56. https://www.ema.europa.eu/en/annual-report-2020/index.html
16. Enescu, C.M., de Rigo, D., Caudullo, G., Mauri, A. and Houston Durrant, T., (2016). *Pinus nigra* in Europe: distribution, habitat, usage and threats. In: San-Miguel-Ayanz J, de Rigo D, Caudullo G, Houston Durrant T, Mauri A, editors. European Atlas of Tree Species. Publication Office of the European Union, Luxembourg, pp.126-127.
17. Erol-Dayi, Ö., Pekmez, M., Bona, M., Aras-Perk, A., and Arda, N., (2011). Total phenolic contents, antioxidant activities and cytotoxicity of three *Centaurea* species: *C. calcitrapa* subsp. *calcitrapa*, *C. ptosimopappa* and *C. spicata*. *Free Radicals and Antioxidants*, *1*(2), 31-36. https://doi.org/10.5530/ax.2011.2.7
18. Eryilmaz, M., Tosun, A. and Tümen, I., (2016). Antimicrobial activity of some species from Pinaceae and Cupressaceae. *Turkish Journal of Pharmaceutical Sciences*, 13(1), 35-40. http://doi.org/10.5505/tjps.2016.43534
19. Ferreira-Santos, P., Zanuso, E., Genisheva, Z., Rocha, C.M.R. and Teixeira, J.A., (2020). Green and sustainable valorization of bioactive phenolic compounds from *Pinus* by-products. *Molecules*, *25*(12), 2931. https://doi.org/10.3390/molecules25122931
20. Fkiri, S., Mezni, F., Quarghi, A., Ghazghazi, A., Larbi Khouja, M., *et al.* (2018). Variability of phenolic compounds and antioxidant efficacy in needles extracts of *Pinus nigra* Arn. *Journal of New Sciences, Agriculture and Biotechnology*, *53*(1), 3528-3535.
21. Goudarzi, M.A., Hamedi, B., Malekpoor, F., Abdizadeh, R., Pirbalouti, A.G. and Raissy, M., (2011). Sensitivity of *Lactococcus garvieae* isolated from rainbow trout to some Iranian medicinal herbs. *Journal of Medicinal Plants Research*, *5*(14), 3067-3073. https://doi.org/10.5897/JMPR.9001125

22. Jalali, A., Kiafar, M., Seddigh, M. and Zarshenas, M.M., (2021). *Punica granatum* as a source of natural antioxidant and antimicrobial agent: A comprehensive review on related investigations. *Current Drug Discovery Technologies*, *18*(2), 207-224. https://doi.org/10.2174/1570163817666200430001822
23. Jorgensen, J.H. and Turnidge, J.D., (2015). Susceptibility test methods: dilution and disk diffusion methods, Chapter 71 In: Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA, editors, Manual of Clinical Microbiology, 11th ed., ASM Press, Washington, pp.1253-1273.
24. Kakade, V.V., Nimbalkar, V.V., Nirmal, S.A. and Kardile, G.N., (2021). A review on: "International and local market size of herbal medicines". *International Journal of Emerging Technologies and Innovative Research*, *8*, 843-849. http://www.jetir.org/papers/JETIR2108593.pdf
25. Kilic, A., Hafizoglu, H., Tümen, L. and Dönmez, I.E., (2011). Phenolic extractives of cones and berries from Turkish coniferous species. *European Journal of Wood and Wood Products*, *69*, 63-66. https://doi.org/10.1007/s00107-010-0433-y
26. Kına, E., Uysal, İ., Mohammed, F. S., Doğan, M. and Sevindik, M. (2021). In-vitro antioxidant and oxidant properties of *Centaurea rigida*. *Turkish Journal of Agriculture-Food Science and Technology,* *9*(10), 1905-1907. http://dx.doi.org/10.24925/turjaf.v9i10.1905-1907.4603
27. Kızılarslan, Ç. and Sevgi, E., (2013). Ethnobotanical uses of genus *Pinus* L. (Pinaceae) in Turkey. *Indian Journal of Traditional Knowledge*, *12*(2), 209-220.
28. Kurt, H. and Şahin, G. (2013). Bir ziraat coğrafyası çalışması: Türkiye'de nar (*Punica granatum* L.) tarımı. Marmara Coğrafya Dergisi, 27, 551-574.
29. Les, F., Prieto, J.M., Arbones-Mainar, J.M., Valero, M.S. and Lopez, V., (2015). Bioactive properties of commercialized pomegranate (*Punica granatum*) juice: antioxidant, antiproliferative and enzyme inhibiting activities. *Food & Function*, *6*, 2049-2057. https://doi.org/10.1039/c5fo00426h
30. Machado, A.P.D.F., Sumere, B.R., Mekaru, C., Martinez, J., Bezerra, R.M.N. and Rostagno, M.A., (2019). Extraction of polyphenols and antioxidants from pomegranate peel using ultrasound: influence of temperature, frequency and operation mode. International *Journal of Food Science & Technology*, *54*(9), 2792–2801. https://doi.org/10.1111/ijfs.14194
31. Mohammed, F. S., Şabik, A. E., Sevindik, E., Pehlivan, M. and Sevindik, M. (2020). Determination of Antioxidant and Oxidant Potentials of *Thymbra spicata* Collected from Duhok-Iraq. *Turkish Journal of Agriculture-Food Science and Technology, 8*(5), 1171-1173.
32. Miniero Davies, Y., Xavier de Oliveira, M.G., M. aulo Vieira Cunha, M., Soares Franco, L., Pulecio Santos, S.L., *et al.* (2018). *Edwardsiella tarda* outbreak affecting fishes and aquatic birds in Brazil. *Veterinary Quarterly*, *38*(1), 99-105. https://doi.org/10.1080/01652176.2018.1540070
33. Noori, M., Jafari, B. and Hekmatdoost, A., (2016). Pomegranate juice prevents development of non-alcoholic fatty liver disease in rats by attenuating oxidative stress and inflammation. *Society of Chemical Industry*, *97*(8), 2327-2332. https://doi.org/10.1002/jsfa.8042
34. Öztürk, R.Ç. and Altınok, İ., (2014). Bacterial and viral fish diseases in Turkey. *Turkish Journal of Fisheries and Aquatic Sciences*, 14, 275-297.
35. Ramos, P.A.B., Pereira, C., Gomes, A.P., Neto, R.T., Almeida, A., *et al.* (2022). Chemical characterisation, antioxidant and antibacterial activities of *Pinus pinaster* Ait. and *Pinus pinea* L. bark polar extracts: prospecting forestry by-products as renewable sources of bioactive compounds. *Applied Sciences*, *12*(2), 784. https://doi.org/10.3390/app12020784
36. Ramudu, K.R. and Dash, G., (2013). A review on herbal drugs against harmful pathogens in aquaculture. *American Journal of Drug Discovery and Development*, *3*(4), 209-219. http://doi.org/10.3923/ajdd.2013.209.219
37. Sevindik, M., Akgul, H., Pehlivan, M., and Selamoglu, Z. (2017). Determination of therapeutic potential of *Mentha longifolia* ssp. *longifolia*. *Fresen Environ Bull, 26*(7), 4757-4763.
38. Sharma, A., Goyal, R. and Sharma, L., (2015). Potential biological efficacy of *Pinus* plant species against oxidative, inflammatory and microbial disorders. *BMC Complementary and Alternative Medicine*, *16*, 35. https://doi.org/10.1186/s12906-016-1011-6
39. Shaygannia, E., Bahmani, M., Zamanzad, B. and Rafieian-Kopaei, M., (2016). A review study on *Punica granatum* L. *Journal of Evidence-Based Complementary & Alternative Medicine*, *21*(3), 221-227. https://doi.org/10.1177/2156587215598039
40. Singh, B., Singh, J.P., Kaur, A. and Singh, N., (2018). Phenolic compounds as beneficial phytochemicals in pomegranate (*Punica granatum* L.) peel: A review. *Food Chemistry*, *261*, 75-86. https://doi.org/10.1016/j.foodchem.2018.04.039
41. Singh, G., Passsari, A.K., Leo, V.V., Mishra, V.K., Subbarayan, S., *et al.* (2016). Evaluation of phenolic content variability along with antioxidant, antimicrobial, and cytotoxic potential of selected traditional medicinal plants from India. *Frontiers in Plant Science*, *7*, 407. https://doi.org/10.3389/fpls.2016.00407

42. Sirakov, I., Velichkova, K. and Slavcheva-Sirakova, D., (2018). *In vitro* study of the use of some medicinal plants against the fish pathogen *Aeromonas hydrophila*. *Scientific Bulletin. Series F. Biotechnologies*, *XXII*, 168-171.
43. Sravanthi, J. and Rao, G.S. (2015). Evaluation of antioxidant-phytochemical compounds in *Punica granatum*. *International Journal of Pharmaceutical Sciences and Research*, *6*(12), 5295-5300. http://doi.org/10.13040/IJPSR.0975-8232.6(12).5295-00
44. Topal, M., (2020). Secondary metabolites of ethanol extracts of *Pinus sylvestris* cones from Eastern Anatolia and their antioxidant, cholinesterase and α-glucosidase activities. *Records of Natural Products*, *14*(2), 129-138. http://doi.org/10.25135/rnp.155.19.06.1326
45. Tozetto, J.T., Tozetto, A.T., Hoshino, B.T., Andrighetti, C.R., Ribeiro, E.B., *et al.* (2017). Extract of *Punica granatum* L.: An alternative to BHT as an antioxidant in semissolid emulsified systems. *Quimica Nova*, *40*(1), 97-104. https://doi.org/10.21577/0100-4042.20160160
46. Uysal, İ., Mohammed, F. S., Şabik, A. E., Kına, E. and Sevindik, M. (2021). Antioxidant and Oxidant status of medicinal plant *Echium italicum* collected from different regions. *Turkish Journal of Agriculture-Food Science and Technology,* *9*(10), 1902-1904. http://dx.doi.org/10.24925/turjaf.v9i10.1902-1904.4588
47. Wendy, W., Julius, Y.S.F., Desy, F.S., Zulhisyam, A.K. and Lee, S.W., (2017). Characterization of antimicrobial, antioxidant, cytotoxic activity and chemical composition of *Punica granatum* rind extract. *Journal of Tropical Resources and Sustainable Science*, *5*, 23-26. http://umkeprints.umk.edu.my/10694/
48. WHO Traditional Medicine Strategy 2014–2023. © World Health Organization 2013, ISBN 978 92 4 150609 0. http://www.who.int/publications/i/item/9789241506096
49. Yan, L., Zhou, X., Shi, L., Shalimu, D., Ma, C. and Liu, Y., (2017). Phenolic profiles and antioxidant activities of six Chinese pomegranate (*Punica granatum* L.) cultivars. International Journal of Food Properties, 20(Sup1), S94-S107. http://doi.org/10.1080/10942912.2017.1289960

Review
Curr. Pers. MAPs, (2022); 5(1): 63-73

Current Perspectives on Medicinal and Aromatic Plants

An International Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

A Review of Moroccan Medicinal Plants Used in the Treatment of Urolithiasis

Elhassan IDM'HAND*

Department of Biotechnology and Valorization of Natural Resources, Faculty of Sciences, University Ibn Zohr, 8106, Agadir, Morocco, E-mail: idmhand-h@hotmail.com, ORDID ID: 0000-0001-9548-6231

*Corresponding author : idmhand-h@hotmail.com

https://doi.org/10.38093/cupmap.1078416

Received: 24/02/2022
Accepted: 03/04/2022

Abstract

In Morocco, surveys on the medicinal plants used in the treatment of urolithiasis have been carried out by various researchers during ethnobotanical missions.

The main objective of this work is to contribute to the knowledge of medicinal plants used in the treatment of this disease in Morocco in order to help in the formulation of improved traditional medicines.

Data concerning the use of medicinal plants against urolithiasis are gather together from published documents concerning the various ethnomedicinal surveys conducted in Morocco for synthesis and analysis. In total, 82 species of plants belonging to 42 families are recorded to be used by the Moroccan population to treat urolithiasis. Apiaceae, Lamiaceae, Leguminosae and Poaceae are the most represented families. The most cited plant species are *Petroselinum crispum and Citrus limon*. Many parts of the plant are used, especially the use of the seeds and leaves are the most used parts. Decoction and infusion are the most common methods of preparation of the these plants for utilised.

Morocco has an important floristic biodiversity in terms of antilithiasic plants. These results form the basis of subsequent studies aimed at experimentally evaluating the potential of these plants.

Key Words: Medicinal plants, Urolithiasis, Ethnopharmacology, Antilithiasic effect, Morocco

1. Introduction

Medicinal plants have been used since antiquity to relieve and cure human illnesses. In fact, their therapeutic properties are due to the presence of hundreds, even thousands, of bioactive natural compounds called: secondary metabolites. These are then accumulated in different organs and sometimes in specialized cells of the plant. Despite the progress of pharmacology, in the absence of a modern medical system, the therapeutic use of medicinal plants is quite common in some countries of the world and especially in developing countries.

Urolithiasis is the third most common disorder of the urinary tract, after infections and pathological disorders of the prostate (Delfan et al., 2015). Urinary stone formation affects 10–12% of the population in industrialized countries (Atmani and Khan, 2000, Butterweck and Khan, 2009, Dayapule et al., 2021). Urolithiasis is the presence of hard crystals in the urinary tract (bladder,

urethra or ureters). They vary greatly in size, with diameters ranging from a few millimeters to several centimeters, but there are several calculation methods. The most common (80%) are calcium oxalate (CaOx) stones formed by deposits of calcium, phosphates and oxalates which eventually take the form of a stone which can lead to severe pain. Kidney stone formation is a complex process resulting from the succession of several physicochemical events, including supersaturation, nucleation, growth, aggregation and retention in the renal tubules, but the mechanisms of these processes are not exactly understood (Atmani et al., 2004a; Atmani and Khan, 2000, Atmani et al., 2004b). These stones can persist for an indefinite period and have serious consequences for the life of the patient (Kumari et al., 2016). Unfortunately, despite considerable advances in medical treatment, there are no satisfactory drugs to treat kidney stones (Atmani and Khan, 2000; Atmani et al., 2003; Butterweck and Khan, 2009; Fouada et al., 2006). In some cases, it is necessary to break the stone or resort to surgery to remove it. In addition to the high cost of the surgery, various side effects, such as urinary tract infections, are expected (Atmani et al., 2004a; Delfan et al., 2015). Because there is no suitable medical treatment for these disorders, it is imperative to search for new or lesser-known medicinal plants that could be a potential source for new bioactive compounds with a therapeutic value. Thus, in Morocco, as in many countries, most patients use medicinal plants as an alternative therapy for many diseases, including urolithiasis.

2. Ethnobotanical studies

In Morocco, several ethnobotanical surveys have been carried out to identify the medicinal plants used to treat urolithiasis. However, no systematic study has yet been conducted to introduce different herbs used to treat kidney stones in different regions of Morocco. This study aims to systematically introduce medicinal plants from different regions of Morocco that are reported to be effective against urolithiasis according to ethnobotanical documents. This was carried out by searching studies in Google, Google Scholar, PubMed, Medline, Science Direct, Researchgate, and other online databases.

The results obtained made it possible to identify 82 medicinal plants, used in Morocco to treat urolithiasis, the majority of which correspond to spontaneous plants. These are divided into 42 families. The most represented families are Apiaceae (10 species), Lamiaceae (7 species), Leguminosae (6 species), Poaceae (6 species), Compositae (4 species), Amaryllidaceae (3 species), Brassicaceae (3 species), Euphorbiaceae (3 species), Rosaceae (3 species), Anacardiaceae (2 species), Caryophyllaceae (2 species), Juncaceae (2 species) and Ranunculaceae (2 species). The other remaining families have only one species.

Analysis of the information collected shows that 17 medicinal plants are the most used in the Morocco (Table 1). The species *Petroselinum crispum* and *Citrus limon* were used in four different regions. *Atriplex halimus*, *Apium graveolens*, *Opuntia ficus-indica*, *Herniaria hirsuta*, *Euphorbia falcata*, *Crocus sativus*, *Glycyrrhiza glabra*, *Linum usitatissimum*, *Olea europaea*, *Cynodon dactylon*, *Hordeum vulgare*, *Zea mays*, *Ziziphus lotus*, *Urtica urens* and *Vitis vinifera* were used in three different regions. The rest of the medicinal plants reported in Table 1, were only used in one or two regions.

Several parts of the listed plants are utilized. The results obtained show that seed and leaf are the most used parts by the population.

Several methods of preparation are used by the Moroccan population to treat urolithiasis. The data analysis showed that the decoction is the most used method of preparation,

followed by the infusion and then by the powder.

Ethnobotanical surveys carried out with the aim of listing antilithiasic medicinal plants in different regions of Morocco underline the importance of this plant heritage in the traditional pharmacopoeia and in particular in the treatment of urolithiasis.

During an ethnobotanical survey in the province of Tan-Tan (southern Morocco), 50 plant species belonging to 29 families were recorded as remedies used by the local population to treat kidney stones. The most represented family is that of the Apiaceae. The results of the study showed that the leaf and the seed are the most used parts. The decoction is the most used method of preparation (Ghourri et al., 2013).

An ethnobotanical study of medicinal plants used in the treatment of genitourinary diseases was carried out between 2016 and 2018 in the Rif region (Northern Morocco). A total of 548 local traditional healers were interviewed. The survey identified 27 species of medicinal plants belonging to 18 botanical families. Medicinal plants are mostly used in the treatment of kidney stones (Chaachouay et al., 2020).

Another ethnobotanical study of medicinal plants traditionally used in the treatment of urolithiasis was conducted in 2013–2014 on the population of Rabat, Salé and Temara. This study showed 35 plant species used in the treatment of kidney stones. The most cited plant species are *Herniaria hirsuta*, *Petroselium crispum*, *Zizyphus lotus* and *Citrus limon* (Khouchlaa et al., 2017).

A study that we recently carried out in the province of Tarfaya identified 40 medicinal plants, divided into 27 families, used by the local population for the treatment of urolithiasis. Apiaceae, Lamiaceae, Leguminosae and Poaceae are the most represented families. As for the dominant species, there are essentially *Herniaria hirsuta*, *Anastatica hierochuntica*, *Apium graveolens*, *Ziziphus lotus*, *Allium sativum* and *Ranunculus muricatus* (Idm'hand et al., 2019).

Information on the use of these plants as reported by local people is given in Table 1.

3. Pharmacological studies

Several studies show that medicinal plants have a beneficial effect in case of urolithiasis. However, their mechanisms of action are not fully understood. Some of them help prevent the formation and especially recurrence of kidney stones, while others facilitate the excretion of wastes by the kidneys or inhibit the formation and aggregation of calcium oxalate crystals. (Aggarwal et al.; 2014, Atmani, 2003; Grases et al., 2009). In Morocco, 82 plants have been inventoried as antilithiasis, but only a few have been scientifically evaluated. Indeed, experimental work has been carried out in order to verify the antilithiasis activity of some of these plants, as well as the active compounds responsible for this activity. These are *Atriplex halimus*, *Pistacia lentiscus*, *Ammi visnaga* , *Ammodaucus leucotrichus*, *Coriandrum sativum*, *Daucus carota*, *Foeniculum vulgare*, *Petroselinum crispum* , *Phoenix dactylifera*, *Opuntia ficus-indica* , *Herniaria hirsuta*, *Citrullus lanatus*, *Crocus sativus*, *Cicer arietinum*, *Trigonella foenum-graecum*, *Punica granatum*, *Cynodon dactylon*, *Hordeum vulgare*, *Zea mays*, *Adiantum capillus-veneris*, *Nigella sativa* and *Malus pumila*.

The antilithiasic activity of some plants has also been proven experimentally. This is the case of the species mentioned below:

Herniaria hirsuta has a prophylactic effect against the formation of calcium oxalate-based stones (the most frequent stones) (Atmani et al., 2003). *In vitro*, an extract of *Herniaria hirsuta* promoted the nucleation of calcium oxalate crystals, increasing their number but reducing their size (Atmani and

Khan, 2000). In vivo, administration of *Herniaria hirsuta* extract to rats reduced the deposition of calcium oxalate crystals in the kidneys (Atmani et al., 2004).

Cynodon dactylon extracts showed a beneficial effect on the prevention and elimination of calcium oxalate deposits in the rat kidney (Atmani et al., 2009, Rad et al., 2011). Administration of hydroacholic extract of *Cynodon dactylon* reduced the growth of urolithiasis in rats (Khajavi Rad et al., 2011). These results provide scientific substantiation for the roles of *Cynodon dactylon* in the prevention and treatment of kidney stones in humans.

Treatment of rats with aqueous and ethanolic extracts of *Nigella sativa* significantly reduced the number and size of calcium oxalate deposits in the kidneys. It also reduced the concentration of calcium oxalate in urine. This beneficial action can be attributed to the antioxidant and anti-inflammatory activities of *Nigella sativa* extract (Hadjzadeh et al., 2011; Khoei et al., 2009).

Administration of *Hordeum vulgare* seed extract reduced the growth of kidney stones in rats. It seems that the treatment effect is more effective than preventive. The mechanism of action could be due to its diuretic effect, its antioxidant power, its nephroprotective property and its ability to decrease the concentration of kidney stone constituents (Shah et al., 2012).

In vivo experiments have shown that aqueous and n-butanol extracts of *Phoenix dactylifera* at a dose of 200 mg/kg possess antiurolithiatic activities (Reddy and Vardhaman, 2013). Therefore, it can be suggested that the aqueous extract or other products of *Phoenix dactylifera* can be used for the prevention and treatment of urolithiasis in humans; further studies are needed to clarify the mechanism.

Indeed, some plants that we have noted open up promising prospects in the search for new active ingredients, thus being able to provide new economically beneficial and socially important products by producing effective and low-cost drugs for the treatment of urolithiasis.

Indeed, additional research on the inventoried plants is necessary in order to better determine the active compounds responsible for their activities and to evaluate their effectiveness. After the positive effects of these plants are proven to be true, it is possible to produce useful drugs in the treatment and control of kidney stones.

Table 1. List of plants used in the treatment of urolithiasis in Morocco

Family	Scientific name	Local name	Used part	Preparation	RFC*	Previous ethnobotanical studies	Previous pharmacological studies
Amaranthaceae	*Atriplex halimus* L	Legtef	Leaf	Decoction	0.5	(Ghourri et al.; 2013; Idm'hand et al., 2019)	(Beghalia et al., 2009)
Amaryllidaceae	*Allium ampeloprasum* L.	Borro	Bulb	Decoction	0.25	(Khouchlaa et al., 2017)	-
	Allium cepa L.	Lbaesla	Bulb	Decoction	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	-
	Allium sativum L.	Touma	Bulb	Decoction	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	-
Anacardiaceae	*Pistacia atlantica* Desf.	Igg	Seed	Decoction	0.25	(Idm'hand et al., 2019)	-

	Pistacia lentiscus L.	Drou	Leaf	Infusion	0.25	(Chaachouay et al., 2020)	(Cheraft-Bahloul et al., 2017)
Apiaceae	*Ammi visnaga* (L.) Lam	Bûŝnîkha	Flower	Decoction	0.25	(Ghourri et al., 2013)	(Khan et al., 2001)
	Ammodaucus leucotrichus Coss. & Dur	Kmoun reg	Seed	Decoction	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	(Beghalia et al., 2009)
	Apium graveolens L.	Lkrafes	Seed	Decoction	0.75	(Ghourri et al., 2013; Idm'hand et al., 2019; Khouchlaa et al., 2017)	-
	Conium maculatum L.	Choukran	Leaf	Poultice	0.25	(Chaachouay et al., 2020)	-
	Coriandrum sativum L.	Lqezbor	Leaf	Decoction	0.25	(Khouchlaa et al., 2017)	(Chandrasekaran and Veerasamy, 2018)
	Daucus carota L.	Khizzou	Seed	Decoction	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	(Bawari et al., 2018)
	Eryngium triquetrum Vahl	Zreyga	Leafy stem	Decoction	0.25	(Ghourri et al., 2013)	-
	Foeniculum vulgare Mill.	Nafaa	Seed	Infusion	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	(Ibrahim and El-Khateeb, 2013)
	Petroselinum crispum (Mill.) Fuss	Maadnous	Leafy stem	Decoction	1	(Chaachouay et al., 2020; Ghourri et al., 2013, Idm'hand et al., 2019; Khouchlaa et al., 2017)	
	Pimpinella anisum L.	Habbat hlaoua	Seeds	-	0.25	(Chaachouay et al., 2020)	-
Apocynaceae	*Caralluma europaea* (Guss.) N.E.Br.	Daghmous	Latex	Raw	0.5	(Chaachouay et al., 2020; Khouchlaa et al., 2017)	-
Arecaceae	*Phoenix dactylifera* L.	Tmer	Fruit	Decoction	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	(Reddy and Vardhaman, 2013)
Aristolochiaceae	*Aristolochia baetica* L.	Berztam	Leaf	Poultice	0.25	(Chaachouay et al., 2020)	-
Boraginaceae	*Borago officinalis* L.	Lhamhem	Flower	Infusion	0.25	(Chaachouay et al., 2020)	-
Brassicaceae	*Anastatica hierochuntica* L	Lkemcha	Leafy stem	Decoction	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	-
	Lepidium sativum L	Hab rchad	Seed	Raw	0.5	(Ghourri et al., 2013;	-

						Idm'hand et al., 2019)	
	Raphanus raphanistrum subsp. sativus (L.) Domin	Lefjel	Seed	Raw	0.25	(Khouchlaa et al., 2017)	-
Burseraceae	*Commiphora africana* (A.Rich.) Endl.	Oumm ennas	Gum	Powder	0.25	(Ghourri et al., 2013)	-
Cactaceae	*Opuntia ficus-indica* (L.) Mill.	Aknari	Flowers	Powder	0.75	(Ghourri et al., 2013; Idm'hand et al., 2019; Khouchlaa et al., 2017)	(Touiti et al., 2020)
Capparaceae	*Capparis spinosa* L.	Lkbbar	Fruit	Powder	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	-
Caryophyllaceae	*Herniaria hirsuta* L.	Harasst lhjar	Whole plant	Decoction	0.75	(Ghourri et al., 2013; Idm'hand et al., 2019; Khouchlaa et al., 2017)	(Atmani et al., 2004a)
	Spergularia rubra (L.) J.Presl & C.Presl	Harasst lhjar	Whole plant	Decoction	0.25	(Chaachouay et al., 2020)	-
Cistaceae	*Cistus populifolius* L.	Irgel	Seed	Decoction	0.25	(Idm'hand et al., 2019)	-
Compositae	*Artemisia herba-alba* Asso	Chih	Seed	Decoction	0.25	(Khouchlaa et al., 2017)	-
	Asteriscus graveolens (Forssk.) Less	Tafsa	Flowers	Decoction	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	-
	Chamaemelum nobile (L.) All.	Babounj,	Stem	Decoction	0.25	(Khouchlaa et al., 2017)	-
	Taraxacum campylodes G.E.Haglund	Oudjem	Leaf	Infusion	0.25	(Khouchlaa et al., 2017)	-
Cucurbitaceae	*Citrullus lanatus (Thunb.)* Matsum. & Nakai	Dellah	Fruit	Raw	0.25	(Khouchlaa et al., 2017)	(Siddiqui et al., 2018)
Euphorbiaceae	*Euphorbia falcata* L	Hayyat noufous	Whole plant	Decoction	0.75	(Ghourri et al., 2013; Idm'hand et al., 2019; Khouchlaa et al., 2017)	-
	Euphorbia granulata Forssk.	Kbidet eddobb	Root	Powder	0.25	(Ghourri et al., 2013)	-
	Mercurialis annua L.	Harrigua melsa	Leafy stem	Decoction	0.25	(Khouchlaa et al., 2017)	-
Iridaceae	*Crocus sativus* L.	Zaafran	Stigmat	Decoction	0.75	(Ghourri et al., 2013; Idm'hand et al., 2019; Khouchlaa et al., 2017)	(Amin et al., 2015)
Juncaceae	*Juncus acutus L.*	Smaar	Seed	Decoction	0.25	(Khouchlaa et al., 2017)	-
	Juncus maritimus Lam.	Smaar	Seed	Decoction	0.25	(Ghourri et al., 2013)	-

Lamiaceae	*Lavandula angustifolia* Mill.	Lkhzama	Flower	Infusion	0.25	(Chaachouay et al., 2020)	-
	Lavandula dentata L.	Lkhozama lbeldiya	Leafy stem	Decoction	0.25	(Ghourri et al., 2013)	-
	Lavandula multifida L.	Khilt lkheyl	Leafy stem	Decoction	0.25	(Ghourri et al., 2013)	-
	Origanum compactum Benth.	Azokenni	Leaf	Decoction	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	-
	Ocimum basilicum L.	Lahbak	Aerial part	Decoction	0.25	(Idm'hand et al., 2019)	-
	Rosmarinus officinalis L	Lyazir	Leaf	Decoction	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	-
	Thymus broussonetii Boiss.	Tazoukennit	Leaf	Decoction	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	-
Lauraceae	*Cinnamomum verum* J.Presl	Lqerfa	Bark	Powder	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	-
Leguminosae	*Cassia fistula L*	Lkharoub lhindi	Fruit	Decoction	0.25	(Ghourri et al., 2013)	-
	Cicer arietinum L.	Lhemees	Fruit	Decoction	0.5	(Chaachouay et al., 2020; Khouchlaa et al., 2017)	(Biglarkhani et al., 2019)
	Glycyrrhiza glabra L.	Arq souss	Root	Decoction	0.75	(Ghourri et al., 2013; Idm'hand et al., 2019; Khouchlaa et al., 2017)	-
	Medicago sativa L.	Lfessa	Seed	Decoction	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	-
	Ononis natrix L.	Hannet reg	Leafy stem	Decoction	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	-
	Trigonella foenum-graecum L.	Lhelba	Seed	Maceration	0.25	(Khouchlaa et al., 2017)	(Laroubi et al., 2007)
Linaceae	*Linum usitatissimum* L.	Zariit lkettan	Seed	Decoction	0.75	(Ghourri et al., 2013; Idm'hand et al., 2019; Khouchlaa et al., 2017)	-
Lythraceae	*Punica granatum* L.	Remman	Bark	Infusion	0.25	(Khouchlaa et al., 2017)	(Rathod et al., 2012)
Molluginaceae	*Corrigiola litoralis subsp. telephiifolia* (Pourr.) Briq.	Sarghina	Whole plant	Decoction	0.25	(Chaachouay et al., 2020)	-
Myristicaceae	*Myristica fragrans* Houtt	Lgouza	Fruit	Decoction	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	-

Myrtaceae	*Syzygium aromatiqueum* (L.) Merr. & L.M.Perry	Lqronfel	Cloves	Decoction	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	-
Oleaceae	*Olea europaea* L	Zaytoune	Fruit	Raw	0.75	(Ghourri et al., 2013; Idm'hand et al., 2019; Khouchlaa et al., 2017)	-
Papaveraceae	*Papaver rhoeas* L.	Belaaman	Stem	Decoction	0.25	(Khouchlaa et al., 2017)	-
Pedaliaceae	*Sesamum indicum* L.	Jenjlane	Seed	Infusion	0.25	(Chaachouay et al., 2020)	-
Poaceae	*Cynodon dactylon* L. Pers	Njem	Rhizome	Decoction	0.75	(Ghourri et al., 2013; Idm'hand et al., 2019; Khouchlaa et al., 2017)	(Rad et al., 2011)
	Festuca glauca Vill.	Agouzmir	Seed	Infusion	0.25	(Chaachouay et al., 2020)	-
	Hordeum vulgare L.	Zraa	Seeds	Decoction	0.75	(Ghourri et al., 2013; Idm'hand et al., 2019; Khouchlaa et al., 2017)	(Shah et al., 2012)
	Saccharum officinarum L	Kasab sokkar	Stem	Juice	0.25	(Khouchlaa et al., 2017)	-
	Stipagrostis pungens (Desf.) De Winter	Ssbet	Leaf	Decoction	0.25	(Ghourri et al., 2013)	-
	Zea mays L.	Dra	Stigmat	Decoction	0.75	(Ghourri et al., 2013; Idm'hand et al., 2019; Khouchlaa et al., 2017)	(Grases et al., 1993)
Pteridaceae	*Adiantum capillus-veneris* L.	Qzibra	Whole plant	Powder	0.25	(Ghourri et al., 2013)	(Ahmed et al., 2013)
Ranunculaceae	*Nigella sativa* L.	Sanouj	Seeds	Powder	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	(Khoei et al., 2009)
	Ranunculus muricatus L	Wden lhalouf	Root	Decoction	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	-
Rhamnaceae	*Ziziphus lotus* (L.) Lam.	Seder	Leaf	Powder	0.75	(Ghourri et al., 2013; Idm'hand et al., 2019, Khouchlaa et al., 2017)	-
Rosaceae	*Malus pumila* Mill.	Teffah	Bark	Decoction	0.25	(Khouchlaa et al., 2017)	(Sinha and Tagore, 2010)
	Prunus cerasus L.	Hab lmlouk	Fruit	Decoction	0.25	(Khouchlaa et al., 2017)	-
	Prunus domestica L.	Lberqouq	Gum	Infusion	0.25	(Ghourri et al., 2013)	-

Rutaceae	*Citrus limon* (L.) Osbeck	Lhamed	Fruit	Juice	1	(Chaachouay et al., 2020; Ghourri et al., 2013; Idm'hand et al., 2019; Khouchlaa et al., 2017)	-
Urticaceae	*Urtica urens* L.	Lhorriyga	Whole plant	Decoction	0.75	(Chaachouay et al., 2020; Ghourri et al., 2013; Idm'hand et al., 2019)	-
Theaceae	*Camellia sinensis* (L.) Kuntze	Atay	Leaf	Decoction	0.5	(Ghourri et al., 2013; Idm'hand et al., 2019)	-
Thymelaeaceae	*Thymelaea lythroides* Barratte & Murb.	Metnan	Leaf	Decoction	0.25	(Khouchlaa et al., 2017)	-
Vitaceae	*Vitis vinifera* L.	Zbib	Fruit	Decoction	0.75	(Ghourri et al., 2013; Idm'hand et al., 2019; Khouchlaa et al., 2017)	-
Xanthorrhoeaceae	*Aloe vera* (L.) Burm.f.	Aloe vera	Gel	Juice	0.25	(Khouchlaa et al., 2017)	-
Zingiberaceae	*Zingiber officinale* Roscoe	Skenjbir	Root	Maceration	0.25	(Khouchlaa et al., 2017)	-

*RFC : Relative Frequency of Citation

4. Conclusion

In conclusion, the review of the literature of medicinal plants of Morocco reported in various research documents showed that 82 species of plants are used against urolithiasis of which 17 of them are more used, therefore more important. Nevertheless, several plants have not been the subject of a scientific investigation in the laboratory to finally justify their biological activities against urolithiasis. Therefore, studies are needed to better promote the use of medicinal plants in Morocco.

This study constitutes a useful documentation, which can contribute to preserve the knowledge on the use of the medicinal plants and to valorize them in order to discover new natural active compound usable in pharmacology for the treatment of the urinary urolithiasis.

Acknowledgements

-

Author Contribution

Elhassan IDM'HAND wrote the paper.

Conflicts of Interest

No conflict of interest was reported by the author.

References

1. Aggarwal, A., Singla, S., and Tandon, C., 2014. Urolithiasis: Phytotherapy as an adjunct therapy. Indian Journal of Experimental Biology, 53, 103-111.
2. Ahmed, A., Wadud, A., Jahan, N., Bilal, A., and Hajera, S., 2013. Efficacy of *Adiantum capillus veneris* Linn in chemically induced urolithiasis in rats. Journal of ethnopharmacology, 146(1), 411-416.

3. Amin, B., Feriz, H.M., Hariri, A.T., Meybodi, N.T., and Hosseinzadeh, H., 2015. Protective effects of the aqueous extract of *Crocus sativus* against ethylene glycol induced nephrolithiasis in rats. EXCLI journal, 14, 411.
4. Atmani, F., 2003. Medical management of urolithiasis, what opportunity for phytotherapy. Front Biosci, 8(6), 507-514.
5. Atmani, F., Farell, G., and Lieske, J.C., 2004a. Extract from *Herniaria hirsuta* coats calcium oxalate monohydrate crystals and blocks their adhesion to renal epithelial cells. The Journal of urology, 172(4), 1510-1514.
6. Atmani, F., and Khan, S., 2000. Effects of an extract from *Herniaria hirsuta* on calcium oxalate crystallization in vitro. Bju International, 85(6), 621-625.
7. Atmani, F., Sadki, C., Aziz, M., Mimouni, M., and Hacht, B., 2009. *Cynodon dactylon* extract as a preventive and curative agent in experimentally induced nephrolithiasis. Urological research, 37(2), 75-82.
8. Atmani, F., Slimani, Y., Mimouni, M., Aziz, M., Hacht, B., and Ziyyat, A., 2004b. Effect of aqueous extract from *Herniaria hirsuta* L. on experimentally nephrolithiasic rats. Journal of ethnopharmacology, 95(1), 87-93.
9. Atmani, F., Slimani, Y., Mimouni, M., and Hacht, B., 2003. Prophylaxis of calcium oxalate stones by *Herniaria hirsuta* on experimentally induced nephrolithiasis in rats. BJU international, 92(1), 137-140.
10. Bawari, S., Sah, A.N., and Tewari, D., 2018. Antiurolithiatic Activity of *Daucus carota*: An in vitro Study. Pharmacognosy Journal, 10(5).
11. Beghalia, M., Ghalem, S., Allalia, H., Belouatek, A., and Marouf, A., 2009. Effects of *Atriplex halimus* and *Ajuga iva* L. Schreb on Calcium Oxalate Urolithiasis Risk in vitro. Asian Journal of Chemistry, 21(2), 1119.
12. Biglarkhani, M., Amir Zargar, M.A., Hashem-Dabaghian, F., Amini Behbahani, F., and Meyari, A., 2019. *Cicer arietinum* in the treatment of small renal stones: a double-blind, randomized and placebo-controlled trial. Research Journal of Pharmacognosy, 6(1), 35-42.
13. Butterweck, V., and Khan, S.R., 2009. Herbal medicines in the management of urolithiasis: alternative or complementary? Planta medica, 75(10), 1095-1103.
14. Chaachouay, N., Benkhnigue, O., Khamar, H., and Zidane, L., 2020. Ethnobotanical study of medicinal and aromatic plants used in the treatment of genito-urinary diseases in the Moroccan Rif.
15. Chandrasekaran, S., and Veerasamy, V., 2018. Anti-urolithiatic activity of whole plant aqueous and ethanolic extract of *Coriandrum sativum* L.-an in vitro approach. Malaya Journal of Biosciences (MJB), 5(1), 28-36.
16. Cheraft-Bahloul, N., Husson, C., Ourtioualous, M., Sinaeve, S., Atmani, D., Stévigny, C., Nortier, J.L., and Antoine, M.-H., 2017. Protective Effects of *Pistacia lentiscus* L. fruit extract against calcium oxalate monohydrate induced proximal tubular injury. Journal of ethnopharmacology, 209, 248-254.
17. Dayapule, S., Yamajala, S.P., Anand, A., Patankar, R., Krishna, B.M., and Chadalawada, N.R., 2021. Role of Osteoprotegerin as Novel Marker in Urinary Stone Formers: A Pilot Study. The journal of nephro-urology monthly, 13(2), 1-5.
18. Delfan, B., Baharvand-Ahmadi, B., Bahmani, M., Mohseni, N., Saki, K., Rafieian-Kopaei, M., Shahsavari, S., Naghdi, N., Taherikalani, M., and Ghafourian, S., 2015. An ethnobotanical study of medicinal plants used in treatment of kidney stones and kidney pain in Lorestan province, Iran. Journal of Chemical and Pharmaceutical Sciences, 8(4), 693-699.
19. Fouada, A., Yamina, S., Nait, M.A., Mohammed, B., and Abdlekrim, R., 2006. In vitro and in vivo antilithiasic effect of saponin rich fraction isolated from *Herniaria hirsuta*. J Bras Nefrol, 28(4), 199-203.
20. Ghourri, M., Zidane, L., and Douira, A., 2013. Catalogue des plantes médicinales utilisées dans le traitement de la lithiase rénale dans la province de Tan-Tan (Maroc saharien). International Journal of Biological and Chemical Sciences, 7(4), 1688-1700.
21. Grases, F., March, J., Ramis, M., and Costa-Bauzá, A., 1993. The influence of *Zea mays* on urinary risk factors for kidney stones in rats. Phytotherapy research, 7(2), 146-149.
22. Grases, F., Prieto, R.M., Gomila, I., Sanchis, P., and Costa-Bauzá, A., 2009. Phytotherapy and renal stones: the role of antioxidants. A pilot study in Wistar rats. Urological research, 37(1), 35.
23. Hadjzadeh, M., Rad, A.K., Rajaei, Z., Tehranipour, M., and Monavar, N., 2011. The preventive effect of N-butanol fraction of *Nigella sativa* on ethylene glycol-induced kidney calculi in rats. Pharmacognosy magazine, 7(28), 338-343.
24. Ibrahim, F.Y., and El-Khateeb, A., 2013. Effect of herbal beverages of *Foeniculum vulgare* and *Cymbopogon proximus* on inhibition of calcium oxalate renal crystals formation in rats. Annals of Agricultural Sciences, 58(2), 221-229.
25. Idm'hand, E., Msanda, F., and Cherifi, K., 2019. Étude ethnobotanique des plantes médicinales utilisées dans le traitement de la lithiase urinaire dans la province de Tarfaya (Maroc). International Journal of Innovation and Applied Studies, 26(3), 711-719.
26. Khajavi Rad, A., Hajzadeh, M.-A.-R., Rajaei, Z., Sadeghian, M.-H., Hashemi, N., and Keshavarzi, Z., 2011. Preventive effect of *Cynodon dactylon* against ethylene glycol-induced nephrolithiasis in male rats. Avicenna Journal of Phytomedicine, 1(1), 14-23.

27. Khan, Z.A., Assiri, A.M., Al-Afghani, H.M., and Maghrabi, T.M., 2001. Inhibition of oxalate nephrolithiasis with *Ammi visnaga* (AI-Khillah). International urology and nephrology, 33(4), 605-608.
28. Khoei, A., Hadjzadeh, Z., and Parizady, M., 2009. Ethanolic extract of *Nigella sativa* L seeds on ethylene glycol-induced kidney calculi in rats. Urology journal, 4(2), 86-90.
29. Khouchlaa, A., Tijane, M., Chebat, A., Hseini, S., and Kahouadji, A., 2017. Ethnopharmacology study of medicinal plants used in the treatment of urolithiasis (Morocco).
30. Kumari, P., Singh, T., and Singh, P., 2016. Medicinal plants of buxar district of bihar used in treatment of urinary tract and kidney stones. Indian Journal of Life Sciences, 5(2), 37-42.
31. Laroubi, A., Touhami, M., Farouk, L., Zrara, I., Aboufatima, R., Benharref, A., and Chait, A., 2007. Prophylaxis effect of *Trigonella foenum* graecum L. seeds on renal stone formation in rats. Phytotherapy Research, 21(10), 921-925.
32. Rad, A.K., Rajaei, Z., Mohammadian, N., Valiollahi, S., and Sonei, M., 2011. The beneficial effect of *Cynodon dactylon* fractions on ethylene glycol-induced kidney calculi in rats. Urology journal, 8(3), 179-184.
33. Rathod, N., Biswas, D., Chitme, H., Ratna, S., Muchandi, I., and Chandra, R., 2012. Anti-urolithiatic effects of *Punica granatum* in male rats. Journal of ethnopharmacology, 140(2), 234-238.
34. Reddy, C.S., and Vardhaman, P., 2013. Evaluation of *Phoenix dactylifera* fruits for Antiurolithiatic activity. Hygeia. JD Med, 5(1), 135-140.
35. Reddy, C.S., and Vardhaman, P., 2013. Evaluation of *Phoenix dactylifera* fruits for Antiurolithiatic activity. Hygeia. JD Med, 5(1), 135-140.2013.
36. Shah, J.G., Patel, B.G., Patel, S.B., and Patel, R.K., 2012. Antiurolithiatic and antioxidant activity of *Hordeum vulgare* seeds on ethylene glycol-induced urolithiasis in rats. Indian journal of pharmacology, 44(6), 672.
37. Siddiqui, W.A., Shahzad, M., Shabbir, A., and Ahmad, A., 2018. Evaluation of anti-urolithiatic and diuretic activities of watermelon (*Citrullus lanatus*) using in vivo and in vitro experiments. Biomedicine & Pharmacotherapy, 97, 1212-1221.
38. Sinha, M.R., and Tagore, R., 2010. Effect of acid-hydrolyzation of fruits juice of apple (*Malus domestica*), Moushmi (*Citrus medica*) and Amla (*Emblica officinalis*) on solubility of urinary stones. Asian Journal of Chemistry, 22(6), 4840-4846.
39. Touiti, N., Bousta, D., Boukhira, S., Chebaibi, M., and Achour, S., 2020. Phytochemical screening, acute and sub-acute toxicity of aqueous extract from a mixture of some recipe of *Herniaria glabra* L., *Opuntia ficus-indica*, *Zea mays* L. and *Zizyphus lotus* L. used traditionally against renal lithiases. Pharmacognosy Research, 12(1), 60.

Review
Curr. Pers. MAPs, (2022); 5(1): 74-85

Current Perspectives on Medicinal and Aromatic Plants

An International Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

An Overview and Renewed Emphasis on Ethnopharmacology of Rosemary (*Salvia rosmarinus*)

Flavien SHIMIRA[1*], Ghassan ZAHID[2] Fildaus NYIRAHABIMANA[2]

[1] Department of Horticulture, Faculty of Agriculture, Çukurova University, 01330, Adana, Türkiye, E-mail: flavien.shimira@outlook.com, ORCID ID: 0000-0003-3382-4068
[2]Department of Biotechnology, Institute of Natural and Applied Sciences, Çukurova University, 01330 Adana, Türkiye, E-mail: ghassanzahid@gmail.com, ORCID ID: 0000-0003-3516-362X
[2]Department of Biotechnology, Institute of Natural and Applied Sciences, Çukurova University, 01330 Adana, Türkiye, E-mail: f.nyirahabimana@outlook.com, ORCID ID: 0000-0002-8964-4835

*Corresponding author : flavien.shimira@outlook.com

https://doi.org/10.38093/cupmap.1098392

Received: 04/04/2022
Accepted: 31/05/2022

Abstract

Medicinal herbs have been employed to treat a wide range of illnesses and as an approach to maintain excellent health since ancient times. Substantially, their efficacies have been methodically investigated to guarantee the high standards of medicinal plant-derived products. This review focuses on Rosemary (*Salvia rosmarinus*), formerly referred as *Rosmarinus officinalis*, an aromatic evergreen shrub in the Lamiaceae family. Our goal is to compile all ethnobotanical applications of the herb, as well as relevant phytoconstituent, *in vivo*, and *in vitro* studies, and to thoroughly outline its potential use in contemporary herbal-based medicine. Using popular search engines, multiple sources were successfully identified. Moreover, the search for published materials such as books, articles, and conference proceedings from today to the previous eras was prioritized. The most notable findings about this herb's ethnopharmacological actions include the inhibition of Dipeptidyl Peptidase (DPP-4), the most pertinent enzyme in Type 2 Diabetes Mellitus (T2DM), as well as its anticancer activity, particularly the overall inhibition actions of its active compounds on endothelium and cancer cell proliferation. In a broad sense, it is a good medicinal plant with a lot of potential for improving human health.

Keywords: Anticancer, anti-inflammatory, antimicrobial, antioxidant, hepatoprotective, phenolic compounds

1. Introduction

Generally, plants have always played an essential role in the history of medicine. As a result, they have made a significant contribution to the development of contemporary medicine (Labiad et al., 2020). *Salvia rosmarinus*, originally *Rosmarinus officinalis* and popularly identified as rosemary, is a perennial evergreen herb with aromatic, needle-shaped leaves and blue, white, purple, or pink flowers that is endemic to the Mediterranean region. *Salvia rosmarinus* has been demonstrated to have a lot of therapeutic properties, which are mostly attributable to its aromatic essential

oils (Berdahl and Mckeague, 2015; Miraj, 2016). Various studies have revealed that *Salvia rosmarinus* has anti-Alzheimer, anti-androgenic, anti-anxiety, antibacterial, anticancer, anti-dermatophytic, antifungal, anti-inflammatory, antimicrobial, anti-obesity, antioxidant, antiplatelet, anti-tumor, hepatoprotective, memory-improving, and radioprotective properties (Miraj, 2016). Furthermore, *Salvia rosmarinus* essential oil (SREO) has been found to have anticancer and antibacterial properties, as well as being a brain and nerve tonic and a treatment for mental weariness (Sadeh et al., 2019). *Salvia rosmarinus* has a lengthy culinary history and its intense aroma has restricted its culinary application to substantially flavored foods like meats, where its strong flavor is partially concealed (Berdahl and Mckeague, 2015).

Salvia rosmarinus extracts (SREs) contain a wide spectrum of phenolic compounds, including abietane diterpenes, carnosol, and ursolic acid, which may have antimicrobial effects in addition to their antioxidant activity. Antioxidants in spices, particularly rosemary, are well known. From time immemorial, spices and shrubs have been used for seasoning, to serve as preservatives, to enhance the sensory characteristics of food, and for their nutritious and healthy properties. Additionally, *Salvia rosmarinus* is acknowledged as a source of various dietary compounds, particularly diterpenes, with carnosol as the leading polyphenol utilized to standardize SREs accepted as a food preservative, but currently, SREs are not approved as a food preservative (Vemu et al., 2021).

SREs and SREOs from leaves and flowers are employed in Indian traditional medicine to treat several disorders, including diseases of the cardio-vascular system, the central nervous system, and some genito-urinary conditions (Fernández et al., 2014). Moreover, essential oils are combinations of volatile substances extracted from medicinal and aromatic plants, primarily through steam distillation. The *Lamiaceae* family is one of the most significant in the production of antioxidant and antibacterial essential oils (Nieto, 2017). SREOs have thoroughly investigated in terms of their chemical composition as well as their biological activities (Satyal et al., 2017).

The current review highlights and evaluates available studies on the use of *Salvia rosmarinus* in the cure of numerous illnesses in all regions of the world. Because it contains several active compounds, acknowledging its medicinal advantages and curative actions based on methodically documented reports will greatly lead to the creation of innovative and suitable herbal-based medicinal products. The desire to keep up with new information on the ethnomedicinal use of *Salvia rosmarinus* was also a primary motivation for writing this review. We helped to fill a gap in the literature engendered by the recent change in genus membership from *Rosmarinus* to *Salvia*.

2. Methodology

For the bibliographic search, several eminent search engines were used (The search period was from January 1, 2005, through December 31, 2021.), amongst others; PudMed (https://pubmed.ncbi.nlm.nih.gov), SciFinder (https://sso.cas.org), Web of Science (https://www.webofknowledge.com), Embase (https://www.otago.ac.nz), Google Scholar (https://scholar.google.com) and Scopus (https://www.scopus.com). This review prioritized recent published materials in high-quality peer-reviewed journals as well as by reputed academic publishers. A total of 53 reference sources were compiled in this review.

3. Botanical description

Salvia rosmarinus is a Mediterranean woody evergreen shrub that is now used as an ornamental plant all over the world. It can flourish up to an altitude of 1500 m above the sea level. The plant grows to a height of 60–

200 cm and is a pointed, dense, evergreen perennial herb. The leaf's upper surface is dark green or blue, while the underside is white; the leaves are resinous. Its needle-shaped leaves are waxy and somewhat curved (Figure 1) (Elamrani et al., 2000; Begum et al., 2013; Berdahl and Mckeague, 2015). The trunks are brown, square, and woody, with tight branches and scratched bark. It is characterized by cymose inflorescences with whitish, blue (Figure 2), or purple flowers (Elamrani et al., 2000; Sasikumar, 2012; Begum et al., 2013).

Figure 1. Rosemary (*Salvia rosmarinus*) leaves.

It got its name from the fact that it is very common on the seashore and flourishes in the sea climate. It is one of the Mediterranean region's distinctive plants (Sasikumar, 2012; Begum et al., 2013). Although it grows naturally along Turkey's western and southern coasts, it is widely cultivated in countries such as France, Italy, Spain, Portugal, and Greece (Malayoğlu, 2010). It can averagely withstand salt and drought stress. Cultivated *Salvia rosmarinus* is produced from transplanted cuttings, which means it is costly than productions employing direct seeding. *Salvia rosmarinus* can be harvested three to four times a year, and *Salvia rosmarinus* stands can last for up to seven years. Carnosic acid is mostly found in *Salvia rosmarinus* chloroplasts, where it assists in the protection of the photosynthetic machinery and chloroplast membrane against oxidative stress (Berdahl and Mckeague, 2015). *Salvia rosmarinus* belongs to xeromorphic species which thrives naturally on cliffs, sand, and stony zones close to the sea across the globe, including Africa, Europe, and Asia (Santos et al., 2015).

Figure 2. Rosemary (*Salvia rosmarinus*) flowers.

4. Chemical compounds

Later, in the 1960's, researchers advanced the extraction process for the antioxidant compounds from *Salvia rosmarinus*, as well as removing a part of the essential oils to make the extracts taste less harsh. The following is a list of some of the antioxidant compounds found in *Salvia rosmarinus*; carnosic acid, carnosol, methyl carnosate, rosmanol, epirosmanol, isorosmanol, 12-*O*-methyl carnosic acid, rosmanol-7-ethyl ether, dimethoxy-rasmanol, rosmadial,

rosmariquinone (miltirone), rosmaridiphenol, rosmarinic acid, luteolin, luteolin-7-O-glucoside and homoplantaginin (Berdahl and Mckeague, 2015). It was reported by Kontogianni et al. (2013) that SREs contain about twice the amount of diterpenoid phenolics, essentially carnosic acid plus carnosol. Thus, many studies consider carnosic acid to be the most compelling antioxidant in *Salvia rosmarinus*. It should be mentioned that there has been confirmation of compelling variations in the chemical makeup of SREOs. Several factors have been highlighted to influence the makeup of essential oils, such as geographic origin, parts of plants, harvesting season, and stage of maturity, as well as the isolation method (Teixeira et al., 2013). According to Rašković et al. (2014), there are three main chemotypes. The main chemotype, which includes Tunisian, Turkish, Moroccan, and Italian oils, has more than 40% 1,8-cineole, whereas the second chemotype, which includes French, Greek, and Spanish oils, contains the same amount of α-pinene, camphor, and 1,8-cineole (20-30%). Finally, South America has a myrcene-rich rosemary oil chemotype.

Hussain et al. (2010) reported that different biological activities might result due to the country of origin of collected essential oils. According to Rašković et al. (2014), essential oils distilled from *Salvia rosmarinus* had a variable yellow tint color and a strong smell, and the acquired yield of the essential oil had approximately 1.03% (v/w) of dry matter. In terms of chemical components, the *Salvia rosmarinus* essential oils are primarily made up of oxygenated monoterpenes (approximately 63-67%), followed by monoterpene hydrocarbons (approximately 26-31%). Table 1 shows details of all chemical constituents of SREOs, with three distinctive chemical compound groupings (monoterpene hydrocarbons, oxygenated monoterpenes, and sesquiterpene hydrocarbons) standing out (Hussain et al., 2010; Rašković et al., 2014). Furthermore, from the identified chemical constituents distinct chemotypes (infraspecific chemical variations) of essential oils were discovered, including the 1,8-cineole chemotype, the myrcene chemotype, the α-pinene/verbenone/bornyl acetate chemotype, the 1,8-cineole/α-pinene/camphor chemotype, and the 1,8-cineole/borneol/p-cymene chemotype (Santos et al., 2015).

It's imperative to understand the distribution of bioactive compounds in *Salvia rosmarinus* plants that are responsible for a whole range of biological effects (Moreno et al., 2006). The polyphenol composition of *Salvia rosmarinus* flowers, fresh leaves, and branches following a methanol extraction was investigated using HPLC chromatography by the same authors. Leaf extract contained carnosol, rosmarinic acid, and carnosic acid in its chromatographic profile. In comparison to flowers, leaves had a larger amount of carnosic acid and rosmarinic acid, whereas branches had no notable presence of these polyphenol compounds. The main active polyphenols were found in the highest concentrations in leaves and flowers. Furthermore, they conducted several extraction processes using organic solvents and discovered that acetone and methanol extracts displayed major peaks matching to rosmarinic acid, carnosol, and carnosic acid, with 52.2 and 36.5 percent ratios, respectively. Extraction of phytochemical components by supercritical fluid and extraction by hydro-distillation utilizing liquid solvents such as hexane and ethanol are the most popular methods. For instance, supercritical fluid technology is used to recover a number of antioxidants from *Salvia rosmarinus* (Vicente et al., 2013). SREOs can be extracted using hydro and steam distillation processes, according to Santos et al. (2015). Hydro-distillation was employed by Hussain et al. (2010) to extract essential oil from air-dried and finely chopped leaves. Although bioactive compounds have a broad spectrum of polarity and their solubility in extraction

media varies, they can pose a few challenges during extraction (Berdahl and Mckeague, 2015).

5. Antioxidant activity

Antioxidants extracts are beneficial to human health because they suppress or delay the oxidation mechanism by limiting the beginning or spread of oxidative sequences. They play an essential task in preventing a range of diseases, like aging, atherosclerosis, cancer, and ischemia (Moreno et al., 2006; Nieto et al., 2018). Several researchers have developed and employed distinct in vitro chemical-based approaches to evaluate the antioxidant activity (AOA) of SREOs and their individual compounds. And ones the most used is DPPH (2, 2-diphenyl-1-picrylhydrazyl and/or 1, 1-diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging assay. Thus, DPPH is a steady free radical that has been utilized to replicate the antioxidant action of active compounds found in essential oils, extracts, and other natural products. (Hussain et al., 2010; Karadağ et al., 2019; do Nascimento et al., 2020).

Table 1. Major volatile constituents of *Salvia rosmarinus* essential oils (SREOs).

Identification Method	Potency Level - Identified compounds	Oxygenated Monoterpenes	Monoterpene Hydrocarbons	Sesquiterpene Hydrocarbons	References
GC-MS analysis	Potency Level	(67.0%)	(26.0%)	(N/A)	(Hussain et al., 2010)
	Identified compounds	• (+)-Camphor, • 1,8-Cineol, • Borneol, • Isoborneol, • Linalool, • α-Terpineol	• Camphene, • Limonene, • α-Phellandren, • α-Pinene, • β-Myrcene, • β-Pinene, • γ-Terpinene	• Verbenone, • β-Caryophyllene, • β-Farnesene, • γ-Muurolene	
GC-FID and GC-MS analysis	Potency Level	(63.88%),	(31.22%)	(4.77%)	(Rašković et al., 2014)
	Identified compounds	• 1,8-Cineole, • Borneol, • Bornyl acetate • Camphor, • Isoborneol, • Linalool, • Terpinen-4-ol, • α-Terpineol, • γ-Terpineol,	• Camphene, • Limonene, • p-Cymene, • Sabinene, • Tricyclene, • α-Phellandrene, • α-Pinene, • α-Terpinene, • α-Terpinolene • α-Thujene, • β-Myrcene, • β-Pinene, • γ-Terpinene, • δ3-Carene,	• Germacrene D, • Longifolene, • α-Copaene, • α-Humulene, • β-Caryophyllene, • δ-Cadinene	

GC-MS: Gas Chromatography associated with Mass Spectrometric detection
GC-FID: Gas Chromatography associated with Flame Ionization detection

Through this DPPH assay and for medicinal research, a strong AOA of *Salvia rosmarinus* and its potential as an anti-inflammatory agent and cell protector in heart illness, cancer, pathological liver conditions, neurodegenerative diseases, and other diseases were confirmed (Vicente et al., 2013; Rašković et al., 2014; Habtemariam, 2016; Yimam et al., 2017). While in food industry, antioxidants are often employed in the food industry to prevent food degradation, and the high applicability of rosemary extracts arises from their ability to control oxidation in foods and beverages, which is facilitated by a diverse variety of antioxidant phenolic compounds with distinct chemical properties (Moreno et al., 2006; Berdahl and Mckeague, 2015).

Salvia rosmarinus contains numerous compounds with AOA, the majority of which are polyphenols. Carnosic acid and carnosol are likely accountable for the majority of the activity (Moreno et al., 2006; Jiang, 2019). Other phenolic diterpenes (methyl carnosate and rosmanol) and phenolic acids (caffeic acids and rosmarinic) are the two most abundant antioxidant phenolic substances in *Salvia rosmarinus* (Vicente et al., 2013). Most of extracts are obtained from wild *Salvia rosmarinus* in country like Morocco, as well as cultivated *Salvia rosmarinus* in France, Romania, Spain, and the United States (Berdahl and Mckeague, 2015). When assessing a fresh SRE, the most important factor to consider is the process of extraction and the type of solvent employed, as these will impact the antioxidant attributes (Nieto et al., 2018). The mode of action of these compounds has been described in countless ways in the literature. The antioxidant capabilities of *Salvia rosmarinus* were found to be due to its high content of isoprenoid quinones, which function as free radical chain terminators and chelators of reactive oxygen species (ROS). Thus, the phenolic compounds found in commercial SREs work as main antioxidants when they react with hydroxyl and lipid radicals to convert them to stable molecules. Furthermore, it was proposed that these compounds could operate as metal ion (Fe^{+2}) chelators, limiting the production ratio of reactive species from oxygen (Nieto et al., 2018). According to Habtemariam (2016), carnosic acid and carnosol's antioxidant mechanisms are based on the eprivation of hydrogen from their phenolic hydroxyl groups, resulting in the production of quinone derivatives. In vitro and in vivo, this antioxidant system protects brain cells from oxidative injury.

For instance, Hussain et al. (2010) used a DPPH radical scavenging assay to validate the AOA of SREOs. They measured the percent inhibition of peroxidation in the linoleic acid system and found that SROEs had the highest inhibition compared to the individual and main components of the oil. This demonstrates a synergistic impact of certain minor components found in SREO. The scavenging activities in both DPPH· and ·OH were evaluated and only rosmarinic acid had the highest AOA among the twelve compounds isolated from *Salvia rosmarinus* ethanol extract (SREE), according to Nei et al. (2019). Similarly, Moreno et al. (2006) used the DPPH method to assess AOA in aliquots of extracts from rosemary branches, leaves and flowers. It was discovered that leaves and flowers had a high AOA, whereas branches had a low activity. Furthermore, they utilised HPLC chromatography fractions following partition of the methanol extract of leaves to evaluate AOA and discovered that fractions comprising rosmarinic acid, carnosol, and carnosic acid had a high AOA. Antioxidant activity was also tested in acetone, methanol, and water SREs using a micro titer model system. AOAs were found to be higher in methanol and acetone extracts. Water extract, on the other hand, demonstrated lower AOA. *Salvia rosmarinus* diterpenes have also been shown to have protective effects in neurodegenerative diseases like Alzheimer, comprising lipid peroxidation and cell protection from oxidative cell death. For instance, carnosic acid has been found to

protect neuronal cells from ischemic injury by scavenging reactive oxygen species (ROS) (Habtemariam, 2016).

Rašković et al. (2014) investigated at rosemary's AOA and its role in treatment of various pathological liver disorders. In vivo tests in rats were conducted to examine the preventive impact of SREO against carbon tetrachloride-induced liver injury, and they confirmed that SREO prevented the rise in lipid peroxidation caused by carbon tetrachloride in liver homogenates. Additionally, pre-treatment with the tested essential oil for a few days dramatically reduced the activity of antioxidant enzymes such as catalase, glutathione peroxidase, peroxidase, and glutathione reductase in liver homogenates, particularly at 10 mg/kg dosage. Bakırel et al. (2008) revealed that SREs had antidiabetic properties, including the ability to suppress lipid peroxidation and activate antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT), which are involved in the direct removal of ROS. Recently, DPPH and ABTS methods were employed to assess the antioxidant potential of the SRE fractions. According to Karadağ (2019), the ethyl acetate fraction of *Salvia rosmarinus* has a stronger AOA than the n-hexane fraction. Taking everything into account, more research on Rosemary's antioxidant activity is needed strong reliance on a range of factors, including the plant maturity, presence of an inhibitor, presence of a synergistic effect among several constituents, and the concentration of active constituents in the extract (Nieto et al., 2018).

6. Antimicrobial activity

The most well-known species is *Salvia rosmarinus*, sometimes known as "rosemary" (Drew et al., 2017). As the aerial part of these plants has been widely utilized as natural preservatives since ancient times. The essential oils derived from *Salvia rosmarinus* have been reported in various studies for their antibacterial and antifungal characteristics (Borges et al., 2019; Pieracci et al., 2021). It was reported that the combination of SREE with cefuroxime demonstrated synergistic antibacterial effect against all methicillin-resistant Staphylococcus aureus (MRSA) (Jarrar et al., 2010). Other research has found that rosemary oil has antibacterial activity against *Aeromonas hydrophila, Bacillus cereus, Clostridium perfringens, E. coli, Staphylococcus aureus, and Salmonella choleraesuis* (Sirocchi et al., 2013; Nieto et al., 2018). In a previous study, rosemary was identified as a good broad-spectrum antibacterial agent (Abers et al., 2021). Another study investigated the antimicrobial activity of five *Salvia rosmarinus* species and found that they had a mild antibacterial effect on the tested bacteria strains (Pieracci et al., 2021).

A recent study observed the antibacterial activity of rosemary extracted in ethanol using an agar well diffusion assay against 10 multidrug-resistant (MDR) clinical isolates, human type culture pathogens, and meat-borne bacterial isolates. The findings suggested that *Salvia rosmarinus* could be a good source as an antimicrobial agent for treating drug-resistant bacteria and meat-borne pathogens (Manilal et al., 2021). *Salvia rosmarinus* has also been recommended for use as a natural antibacterial in food processing (Saricaoglu and Turhan, 2018). The antibacterial and antifungal properties of rosemary's principal components, such as 1,8-cineol, α-pinene, camphor, carnosol, and carnosic acid, make it efficient against a wide range of infections (Nieto et al., 2018).

Antibiotic use has increased dramatically in medicine, agriculture, and livestock, resulting in an increase in multidrug-resistant bacteria (Qabaha, 2013). Antimicrobial resistance is a global public health concern, and researchers have been focusing more on this field in order to find new antimicrobial effective bioactives (Petrolini et al., 2013). SREOs include insecticidal, antiparasitic, and antifungal characteristics in addition to antibacterial properties, which are useful for the control of microbial illnesses in humans (Andrade et al., 2018). *Rosemary* spp. has a bright future in medicine and food science. As a result, additional reliable trials are needed in the

future to assess *Salvia rosmarinus*'s antibacterial capabilities and undiscovered phytocompound activity, as well as its safety and efficacy.

7. Antidiabetic activity

Type 2 Diabetes Mellitus (T2DM) is a metabolic condition that results in hyperglycemia due to pathophysiological reasons and may induce various health problems (Galicia-Garcia et al., 2020). The first step in managing this illness is to keep blood glucose levels under control. As reported by the World Health Organization and the International Diabetes Federation (IDF), T2DM is expected to impact over 439 million persons by 2030 (Naimi et al., 2017). Dipeptidyl Peptidase (DPP-4) is the most significant enzyme in mellitus T2DM (Röhrborn et al., 2015). *Salvia rosmarinus* has been found to inhibit the DPP-4 enzyme, resulting in a diabetic impact without any negative health implications (Salim et al., 2017).

A Turkish study looked at the effects of an ethanolic extract of *Salvia rosmarinus* leaves on glucose homeostasis and antioxidant defense in rabbits and found that SRE has a significant anti-diabetic impact (Bakırel et al., 2008). Rosemary extract, as well as the rosemary extract polyphenols carnosic acid and rosmarinic acid, have been demonstrated to have insulin-like effects in insulin target cells in vitro, as well as strong anti-diabetic benefits in various animal models of T2DM in vivo (Naimi et al., 2017). These positive results from in vivo animal experiments show that *Salvia rosmarinus* and its polyphenolic ingredients could be useful in the treatment of diabetes and management of blood sugar levels. It is also shown that the SRE has outstanding hypoglycemic and antihyperglycemic action as a result of its various effects involving both pancreatic and extra-pancreatic mechanisms (Bakırel et al., 2008). However, more research is needed to fill in the gaps in the literature and to assess the efficacy and safety of *Salvia rosmarinus*' antidiabetic activities.

8. Anticancer activity

Herbs and plants contain chemicals that may have anticancer properties and can halt the progression of carcinogenesis at various levels (Allegra et al., 2020). Because of its antioxidant potential, *Salvia rosmarinus* has been considered as a significant anticancer medication. In fact, It can function on free radicals and safeguard DNA, lipids, and proteins from oxidative deterioration (Xiang et al., 2013). Some angiogenic properties of endothelial cells, such as differentiation, proliferation, migration, and differentiation capacity, are blocked by carnosic acid and carnosol. Numerous studies have found that their effects on endothelium and cancer cell proliferation might be connected to programmed cell death stimulation. In vivo studies verified the inhibition of angiogenesis in vitro by *Salvia rosmarinus* compounds (López-Jiménez et al., 2013).

According to the systematic review, SRE includes several polyphenols, with carnosic acid and rosmarinic acid having the largest amounts, and they have diverse powerful and effective anticancer activities (Moore et al., 2016). *Salvia rosmarinus* was found to have antitumoral activity, which could be linked to an antioxidant mechanism (Choukairi et al., 2020). In recent years, the focus has switched to developing new targeted cancer medicines that can modify specific cancer pathways that are frequently altered. There have been few studies on the signaling molecules and pathways that SRE targets. Animal studies must be conducted in a more methodical manner before human trials can begin (Moore et al., 2016). As a result, the use of *Salvia rosmarinus* and its derivatives in cancer therapy is a fascinating topic of research. Large, well-designed studies are needed to conclusively determine the true influence of this substance in clinical practice in the future.

9. Anti-inflammatory properties

Inflammation is presently thought to have a key role in the pathophysiology of depression (Miller and Raison, 2016). SREs, the key active ingredients isolated from *Salvia rosmarinus*, have sparked widespread interest due to their anti-inflammatory properties. *Salvia rosmarinus* demonstrated a potent anti-inflammatory effect, particularly in vivo, where SREO and SRE were demonstrated to significantly suppress leukocyte migration (de Melo et al., 2011). This lowered the amount of leukocytes (white blood cells) at the inflammatory site, giving rise in an anti-inflammatory reaction (Benincá et al., 2011; de Melo et al., 2011; Mengoni et al., 2011). Other pro-inflammatory components, like nitric oxide and inflammation-related genes, were similarly hindered by SRE (Benincá et al., 2011; Mengoni et al., 2011; Yu et al., 2013). Although carnosol and carnosic acid tend to be essential, rosemary's anti-inflammatory effect is most probably due to a synergistic process involving a number of its constituents (de Melo et al. 2011; Mengoni et al., 2011; Yu et al., 2013). These investigations indicate that *Salvia rosmarinus* has a considerable anti-inflammatory impact; in particular, the anti-inflammatory activities of pure carnosol and carnosic acid were shown to be nine times greater than those of indomethacin, a popular anti-inflammatory medicine (Mengoni et al., 2011). SREs inhibited gut microbiota dysbiosis, depressive-like behaviors, and inflammatory reaction activation in the hippocampus and serum of CRS mice, as well as inflammatory reaction activation in BV-2 microglia cells caused by lipopolysaccharide (LPS). Likewise, another study conducted by (Guo et al., 2018) revealed that SRE may reduce the variety of gut microbiota, reduce the sequencing percentage of both Proteobacteria and Bacteroidetes, and increase the affluence of Lactobacillus and Firmicutes, suggesting that the anti-depressant effects of RE arise from gut microbiota rebalancing. (Borges et al., 2019) reviewed and highlighted the role of SREO. The studies confirmed the application of SREOs in diseases associated with inflammation and their future potential as a muscle relaxant and anti-inflamatory oils. This anti-inflammatory action of SREO can be attributable mostly to 1,8-cineole and -pinene, which are frequently found in higher concentrations and whose mechanisms are well characterized (Borges et al., 2019). However, more research and clinical trials are required to determine the direct impacts of RE and its polyphenolic contents in certain cells and tissue types as well as their mode of action particularly in humans.

10. Conclusion

This review article corroborates that the plant's extracts contain high antioxidant, antimicrobial, and antibacterial properties, making *Salvia rosmarinus* a perfect replacement for more hazardous artificial food supplements. Furthermore, it has shown great promise as a natural food preservative as well as a medicinal agent. Many of its significant biological properties, including anti-diabetic and anticancer processes, are attributed to the powerful antioxidant chemicals contained in its extract and essential oil. If it persists to exhibit such potent anti-diabetic and anticancer properties with minimal side effects, *Salvia rosmarinus* could potentially provide a unique therapy addressing these two critical diseases. *Salvia rosmarinus* has also been highly beneficial in the treatment of depression, neurological diseases, obesity, and inflammation. The findings pave the way for a prospective expansion in the use of supercritical SREs in food formulations for inflammatory disease mitigation or prevention. Although the preliminary findings are promising, more study and research are needed to validate its safety and efficacy as a therapeutic and preservative agent. Although preliminary findings are encouraging, more research and study are required to corroborate its safety and potency as a medicinal and preservative agent.

Acknowledgements

We extend our gratitude to anonymous reviewer for his invaluable comments on earlier versions of the manuscript. We are also thankful to Senem Uğur and İlker Kaya for technical support.

Author Contribution

The study and design were conceptualized and designed by FS and GZ. The first draft of the manuscript was written collaboratively by FS, GZ, and FN. The work was revised critically by FS. All authors read and approved the final manuscript.

Conflicts of Interest

The authors have no relevant financial or non-financial interests to disclose. The authors also declare that no funds, grants, or other support were received during the preparation of this manuscript.

References

1. Abers, M., Schroeder, S., Goelz, L., Sulser, A., St. Rose, T., Puchalski, K., & Langland, J., (2021). Antimicrobial activity of the volatile substances from essential oils. BMC Complementary Medicine and Therapies, 21, 124. https://doi.org/10.1186/s12906-021-03285-3
2. Allegra, A., Tonacci, A., Pioggia, G., Musolino, C., & Gangemi, S., (2020). Anticancer activity of Rosmarinus officinalis L.: Mechanisms of action and therapeutic potentials. Nutrients, 12(6), 1739. https://doi.org/10.3390/nu12061739
3. Andrade, J.M., Faustino, C., Garcia, C., Ladeiras, D., Reis, C.P., & Rijo, P., (2018). Rosmarinus officinalis L.: An update review of its phytochemistry and biological activity. Future Science OA, 4(4), FSO283. https://doi.org/10.4155/fsoa-2017-0124
4. Bakırel, T., Bakırel, U., Keleş, O.Ü., Ülgen, S.G., & Yardibi, H., (2008). In vivo assessment of antidiabetic and antioxidant activities of rosemary (Rosmarinus officinalis) in alloxan-diabetic rabbits. Journal of Ethnopharmacology, 116(1), 64–73. https://doi.org/10.1016/j.jep.2007.10.039
5. Begum, A., Sandhya, S., Ali, S.S., Vinod, K.R., Reddy, S., & Banji, D., (2013). An in-depth review on the medicinal flora Rosmarinus officinalis (Lamiaceae). Acta scientiarum polonorum Technologia alimentaria, 12(1), 61-73.
6. Benincá, J.P., Dalmarco, J.B., Pizzolatti, M.G., & Fröde, T.S., (2011). Analysis of the anti-inflammatory properties of Rosmarinus officinalis L. in mice. Food Chemistry, 124(2), 468–475. https://doi.org/10.1016/j.foodchem.2010.06.056
7. Berdahl, D.R., & Mckeague, J., (2015). Rosemary and sage extracts as antioxidants for food preservation. In: Shahidi, F. (Ed.), Woodhead Publishing Series in Food Science, Technology and Nutrition, Handbook of Antioxidants for Food Preservation (1st edition). Woodhead Publishing, Cambridge, pp. 177–217.
8. Borges, R.S., Ortiz, B.L.S., Pereira, A.C.M., Keita, H., & Carvalho, J.C.T., (2019). Rosmarinus officinalis essential oil: A review of its phytochemistry, anti-inflammatory activity, and mechanisms of action involved. Journal of Ethnopharmacology, 229, 29-45. https://doi.org/10.1016/j.jep.2018.09.038
9. Choukairi, Z., Hazzaz, T., José, M.F., & Fechtali, T., (2020). The cytotoxic activity of Salvia officinalis L. and Rosmarinus officinalis L. leaves extracts on human glioblastoma cell line and their antioxidant effect. Journal of Complementary and Integrative Medicine, https://doi.org/10.1515/jcim-2018-0189
10. de Melo, G.A.N., Grespan, R., Fonseca, J.P., Farinha, T.O., Silva, E.L., Romero, A.L., Bersani-Amado, C.A., & Cuman, R.K.N., (2011). Rosmarinus officinalis L. essential oil inhibits In vivo and In vitro leukocyte migration. Journal of Medicinal Food, 14(9), 944–946. https://doi.org/10.1089/jmf.2010.0159
11. do Nascimento, L.D., de Moraes, A.A.B., da Costa, K.S., Galúcio, J.M.P., Taube, P.S., Costa, C.M.L., Cruz, J.N., Andrade, E.H.dA., & de Faria, L.J.G., (2020). Bioactive Natural Compounds and Antioxidant Activity of Essential Oils from Spice Plants: New Findings and Potential Applications. Biomolecules, 10(7), 988. https://doi.org/10.3390/biom10070988
12. Drew, B.T., González-Gallegos, J.G., Xiang, C., Kriebel, R., Drummond, C.P., Walker, J.B., & Sytsma, K.J., (2017). Salvia united: The greatest good for the greatest number. Taxon, 66, 133-145. https://doi.org/10.12705/661.7
13. Elamrani, A., Zrira, S., Benjilali, B., & Berrada, M., (2000). A study of Moroccan rosemary oils. Journal of Essential Oil Research, 12(4), 487–495. https://doi.org/10.1080/10412905.2000.9699572
14. Fernández, L.F., Palomino, O.M., & Frutos, G., (2014). Effectiveness of Rosmarinus officinalis essential oil as antihypotensive agent in primary hypotensive patients and its influence on health-related quality of life. Journal of

Ethnopharmacology, 151(1), 509–516. https://doi.org/10.1016/j.jep.2013.11.006

15. Galicia-Garcia, U., Benito-Vicente, A., Jebari, S., Larrea-Sebal, A., Siddiqi, H., Uribe, K.B., Ostolaza, H., & Martín, C., (2020). Pathophysiology of Type 2 diabetes Mellitus. International Journal of Molecular Sciences, 21(17), 6275. https://doi.org/10.3390/ijms21176275

16. Guo, Y., Xie, J., Li, X., Yuan, Y., Zhang, L., Hu, W., Luo, H., Yu, H., & Zhang, R., (2018). Antidepressant effects of Rosemary extracts associate with anti-inflammatory effect and rebalance of gut microbiota. Frontiers in Pharmacology, 9, 1126. https://doi.org/10.3389/fphar.2018.01126

17. Habtemariam, S., (2016). The therapeutic potential of Rosemary (Rosmarinus officinalis) diterpenes for Alzheimer's disease. Evidence-Based Complementary and Alternative Medicine, 2016, 1–14. https://doi.org/10.1155/2016/2680409

18. Hussain, A.I., Anwar, F., Chatha, S.A.S., Jabbar, A., Mahboob, S., & Nigam, P.S., (2010). Rosmarinus officinalis essential oil: antiproliferative, antioxidant and antibacterial activities. Brazilian Journal of Microbiology, 41(4), 1070–1078. https://doi.org/10.1590/S1517-838220100004000027

19. Jarrar, N., Abu-Hijleh, A., & Adwan, K., (2010). Antibacterial activity of Rosmarinus officinalis L. alone and in combination with cefuroxime against methicillin–resistant Staphylococcus aureus. Asian Pacific Journal of Tropical Medicine, 3, 121–123. https://doi.org/10.1016/S1995-7645(10)60049-1

20. Jiang, T.A., (2019). Health benefits of culinary herbs and spices. Journal of AOAC International, 102(2), 395–411. https://doi.org/10.5740/jaoacint.18-0418

21. Karadağ, A.E., Demirci, B., Çaşkurlu, A., Demirci, F., Okur, M.E., Orak, D., Sipahi, H., & Başer, K.H.C., (2019). In vitro antibacterial, antioxidant, anti-inflammatory and analgesic evaluation of Rosmarinus officinalis L. flower extract fractions. South African Journal of Botany, 125, 214–220. https://doi.org/10.1016/j.sajb.2019.07.039

22. Kontogianni, V.G., Tomic, G., Nikolic, I., Nerantzaki, A.A., Sayyad, N., Stosic-Grujicic, S., Stojanovic, I., Gerothanassis, I.P., & Tzakos, A.G. (2013). Phytochemical profile of Rosmarinus officinalis and Salvia officinalis extracts and correlation to their antioxidant and anti-proliferative activity. Food Chemistry, 136, 120–129. https://doi.org/10.1016/j.sajb.2019.07.039

23. Labiad, H., Et-tahir, A., Ghanmi, M., Satrani, B., Aljaiyash, A., Chaouch, A., & Fadli, M., (2020). Ethnopharmacological survey of aromatic and medicinal plants of the pharmacopoeia of northern Morocco. Ethnobotany Research and Applications, 19,1–16.

24. López-Jiménez, A., García-Caballero, M., Medina, M.Á., & Quesada, A.R., (2011). Anti-angiogenic properties of carnosol and carnosic acid, two major dietary compounds from rosemary. European Journal of Nutrition, 52(1), 85–95. https://doi.org/10.1007/s00394-011-0289-x

25. Malayoğlu, H.B., (2010). The Antioxidant Effect of Rosemary (Rosmarinus officinalis L.) (in Turkish). Hayvansal Üretim, 51(2), 59-67.

26. Manilal, A., Sabu, K.R., Woldemariam, M., Aklilu, A., Biresaw, G., Yohanes, T., Seid, M., & Merdekios, B., (2021). Antibacterial activity of Rosmarinus officinalis against Multidrug-resistant clinical isolates and meat-borne pathogens. Evidence-Based Complementary *and* Alternative Medicine, 2021, 6677420. https://doi.org/10.1155/2021/6677420

27. Mengoni, E.S., Vichera, G., Rigano, L.A., Rodriguez-Puebla, M.L., Galliano, S.R., Cafferata, E.E., Pivetta, O.H., Moreno, S., & Vojnov, A.A., (2011). Suppression of COX-2, IL-1β and TNF-α expression and leukocyte infiltration in inflamed skin by bioactive compounds from Rosmarinus officinalis L. Fitoterapia. 82(3), 414–421. https://doi.org/10.1016/j.fitote.2010.11.023

28. Miller, A.H., & Raison, C.L., (2016). The role of inflammation in depression: from evolutionary imperative to modern treatment target. Nature Reviews Immunology, 16(1), 22–34. https://doi.org/10.1038/nri.2015.5

29. Miraj, S., (2016). An evidence-based review on herbal remedies of Rosmarinus officinalis. Der Pharmacia Lettre, 8(19), 426-436.

30. Moore, J., Yousef, M., & Tsiani, E., (2016). Anticancer Effects of Rosemary (Rosmarinus officinalis L.) Extract and Rosemary Extract Polyphenols. Nutrients, 8(11), 731. https://doi.org/10.3390/nu8110731

31. Moreno, S., Scheyer, T., Romano, C.S., & Vojnov, A.A., (2006). Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. Free Radical Research, 40(2), 223–231. https://doi.org/10.1080/10715760500473834

32. Naimi, M., Vlavcheski, F., Shamshoum, H., & Tsiani, E. (2017). Rosemary extract as a potential anti-hyperglycemic agent: Current evidence and future perspectives. Nutrients, 9(9), 968. https://doi.org/10.3390/nu9090968

33. Nie, J., Li, R., Wang, Y., Tan, J., Tang, S., & Jiang, Z.-T., (2019). Antioxidant activity evaluation of rosemary ethanol extract and their cellular antioxidant activity toward HeLa cells. Journal of Food Biochemistry, 43(7), e12851. https://doi.org/10.1111/jfbc.12851

34. Nieto, G., (2017). Biological activities of three essential oils of the Lamiaceae family. Medicines, 4(3), 63. https://doi.org/10.3390/medicines4030063

35. Nieto, G., Ros, G., & Castillo, J. (2018). Antioxidant and antimicrobial properties of Rosemary (Rosmarinus officinalis, L.): A Review. Medicines, 5(3), 98. https://doi.org/10.3390/medicines5030098

36. Petrolini, F.V.B., Lucarini, R., de Souza, M.G.M., Pires, R.H., Cunha, W.R., & Martins, C.H.G., (2013). Evaluation of the antibacterial potential of Petroselinum crispum and Rosmarinus officinalis against bacteria that cause urinary tract infections. Brazilian Journal of Microbiology, 44(3), 829–834. https://doi.org/10.1590/S1517-83822013005000061

37. Pieracci, Y., Ciccarelli, D., Giovanelli, S., Pistelli, L., Flamini, G., Cervelli, C., Mancianti, F., Nardoni, S., Bertelloni, F., & Ebani, V.V. (2021). Antimicrobial activity and composition of five Rosmarinus (now Salvia spp. and varieties) essential oils. Antibiotics, 10(9), 1090. https://doi.org/10.3390/antibiotics10091090

38. Qabaha, K., (2013). Antimicrobial and free radical scavenging activities of five Palestinian medicinal plants. African Journal of Traditional, Complementary and Alternative Medicines, 10(4), 101–108. https://doi.org/10.4314/ajtcam.v10i4.17

39. Rašković, A., Milanović, I., Pavlović, N., Ćebović, T., Vukmirović, S., & Mikov, M. (2014). Antioxidant activity of rosemary (Rosmarinus officinalis L.) essential oil and its hepatoprotective potential. BMC Complementary and Alternative Medicine, 14, 225. https://doi.org/10.1186/1472-6882-14-225

40. Röhrborn, D., Wronkowitz, N., & Eckel, J., (2015). DPP4 in diabetes. Frontiers in Immunology, 6,386. https://doi.org/10.3389/fimmu.2015.00386

41. Sadeh, D., Nitzan, N., Chaimovitsh, D., Shachter, A., Ghanim, M., & Dudai, N., (2019). Interactive effects of genotype, seasonality and extraction method on chemical compositions and yield of essential oil from rosemary (Rosmarinus officinalis L.). Industrial Crops and Products, 138, 111419. https://doi.org/10.1016/j.indcrop.2019.05.068

42. Salim, B., Hocine, A., & Said, G., (2017). First Study on Anti-diabetic Effect of Rosemary and Salvia by Using Molecular Docking. Journal of Pharmaceutical Research International, 19(4), 1-12. https://doi.org/10.48347/IMIST.PRSM/ajmap-v4i1.11380

43. Santos, R.R., Costa, D.C., Cavaleiro, C., Costa, H.S., Albuquerque, T.G., Castilho, M.C., Ramos, F., Melo, N.R., & Sanches-Silva, A. (2015). A novel insight on an ancient aromatic plant: the rosemary (Rosmarinus officinalis L.). Trends in Food Science & Technology, 45(2), 355–368. https://doi.org/10.1016/j.tifs.2015.07.015

44. Saricaoglu, F.T., & Turhan, S., (2018). Antimicrobial Activity and Antioxidant Capacity of Thyme, Rosemary and Clove Essential Oils and Their Mixtures. Journal of Innovative Science and Engineering, 2(1), 25-33.

45. Sasikumar, B., (2012). Rosemary. In: Woodhead Publishing Series in Food Science, Technology and Nutrition: Hand book of Herbs and Spices. 1st edition (Woodhead Publishing, Cambridge), pp. 452–468.

46. Satyal, P., Jones, T., Lopez, E.M., McFeeters, R., Ali, N.A.A., Mansi, I., Al-kaf, A.G., & Setzer, W.N. (2017). Chemotypic characterization and biological activity of Rosmarinus officinalis. Foods, 6(3), 20. https://doi.org/10.3390/foods6030020

47. Sirocchi, V., Caprioli, G., Cecchini, C., Coman, M.M., Cresci, A., Maggi, F., Papa, F., Ricciutelli, M., Vittori, S., & Sagratini, G., (2013). Biogenic amines as freshness index of meat wrapped in a new active packaging system formulated with essential oils of Rosmarinus officinalis. . International Journal of Food Sciences and Nutrition, 64(8), 921–928. https://doi.org/10.3109/09637486.2013.809706

48. Teixeira, B., Marques, A., Ramos, C., Neng, N.R., Nogueira, J.M.F., Saraiva, J.A., & Nunes, M.L., (2013). Chemical composition and antibacterial and antioxidant properties of commercial essential oils. Industrial Crops and Products, 43, 587–595. https://doi.org/10.1016/j.indcrop.2012.07.069

49. Vemu, B., Tocmo, R., Nauman, M.C., Flowers, S.A., Veenstra, J.P., & Johnson, J.J., (2021). Pharmacokinetic characterization of carnosol from rosemary (Salvia Rosmarinus) in male C57BL/6 mice and inhibition profile in human cytochrome P450 enzymes. Toxicology and Applied Pharmacology, 431, 115729. https://doi.org/10.1016/j.taap.2021.115729

50. Vicente, G., Molina, S., González-vallinas, M., García-risco, M.R., Fornari, T., Regleroa, G., & de Molina, A.R., (2013). Supercritical rosemary extracts, their antioxidant activity and effect on hepatic tumor progression. The Journal of Supercritical Fluids, 79, 101–108. https://doi.org/10.1016/j.supflu.2012.07.006

51. Xiang, Q., Liu, Q., Xu, L., Qiao, Y., Wang, Y., & Liu, X., (2013). Carnosic acid protects biomolecules from free radical-mediated oxidative damage in vitro. Food Science and Biotechnology, 22(5),1–8. https://doi.org/10.1007/s10068-013-0226-2

52. Yimam, M., Lee, Y.C., Jiao, P., Hong, M., Brownell, L., & Jia, Q., (2017). A standardized composition comprised of extracts from Rosmarinus officinalis, Annona squamosa and Zanthoxylum clava-herculis for cellulite. Pharmacognosy Research, 9(4), 319-324. https://doi.org/10.4103/pr.pr_70_17

53. Yu, M.-H., Choi, J.H., Chae, I.G., Im, H.-G., Yang, S.-A., More, K., Lee, I.-S., & Lee, J., (2013). Suppression of LPS-induced inflammatory activities by Rosmarinus officinalis L. Food Chemistry, 136(2), 1047–1054. https://doi.org/10.1016/j.foodchem.2012.08.085

Review article
Curr. Pers. MAPs, (2022); 5(1): 86-105

Current Perspectives on Medicinal and Aromatic Plants

An International Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

Bee Venom and its Biological Effects

Nurten ABACI[1*], Ilkay ERDOGAN ORHAN[1]

[1]Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, 06330 Ankara, Türkiye
E-mail: nurtenabaci@gazi.edu.tr ORCID ID: 0000-0002-4144-7074
E-mail: iorhan@gazi.edu.tr ORCID ID: 0000-0002-7379-5436

*Corresponding author: nurtenabaci@gazi.edu.tr

https://doi.org/10.38093/cupmap.1127949
Received: 08/06/2022
Accepted: 28/06/2022

Abstract

Apitherapy is defined as "the use of *Apis mellifera* L. products such as royal jelly, pollen, honey, propolis, beeswax, and bee venom (BV) in the treatment of ailments". Although honey is the primary product acquired, other bee products are also obtained in Turkey. These commodities, in addition to being utilized as nutrition, have been employed to promote human health since ancient times owing to the biologically active compounds they contain. BV is increasingly commonly used in apitherapy and has a wide range of biological effects including antiviral, antidiabetic, anticancer, antirheumatic, anticoagulant, antibacterial, anti-cancer, anti-aging, neuroprotective, analgesic, antioxidant, hepatoprotective, and anti-asthmatic properties. According to the literature, BV has promising biological implications for human health, which constitutes the topic of this review.

Key Words: Bee venom, apitoxin, apitherapy, bee venom acupuncture, melittin.

1. Introduction

Apitherapy is a complementary therapy that utilizes products of *Apis mellifera* L., also known as the honey bee, such as honey, pollen, propolis, royal jelly, beeswax, and bee venom (BV) to prevent or treat some disorders. Since the time of Ancient Egypt and the Romans, bee-based products have been utilized to cure ailments, maintain well-being and prevent diseases (Cherbuliez, 2013). Although all of the aforementioned bee components are employed in apitherapy, BV is the product that seems most widely used today to treat various diseases. BV, also known as apitoxin, is a light-colored, odorless, acidic, and poisonous animal secretion with a pungent and bitter taste, which is stored in the venom sacs of female worker bees and aids in their defense against the predators. BV contains a wide range of active compounds such as enzymes, peptides, non-peptide components, and physiologically active amines, where it consists of 88% water and just 0.1 µg of dry venom. Dry venom made up complex peptides (particularly melittin, apamin, adolapin, mast cell degranulating peptide, minimine, secarpine, melittin F, cardiopep etc.), biologically active amines (histamine, epinephrine, dopamine, noradrenaline etc.), enzymes (phospholipase A2, hyaluronidase,

phosphomonoesterase, lysophospholipase, phospholipase B, α-D-glucosidase etc.), lipids, carbohydrates (glucose, fructose etc.), free amino acids (glycine etc.), minerals (potassium, calcium, magnesium), volatile substances and organic acids (citric acid, malic acid, malonic acid etc.). Changes in the chemical composition of BV can be seen as a result of bee species, location, and dietary changes. For this reason, it is critical to standardize BV and evaluate its toxicity before using it for medical purposes (Son et al., 2007; Sung et al., 2021; Wehbe et al., 2019). The composition of dry BV is detailed in Table 1.

BV therapy has been used in the treatment of various ailments such as rheumatism and arthritis since ancient times. There are numerous BV treatments available including bee sting therapy, BV acupuncture (BVA), directly injection of BV into human body and the use of BV products externally. Bee sting treatment is injecting BV into human skin *via* live bee stings. However, this treatment technique has a significant chance of causing undesirable side effects. BVA is a pharmacopuncture treatment derived from bee sting treatment that is effective in the treatment of diseases such as rheumatoid arthritis, chronic low back pain, acne, diabetes, Parkinson's disease, Alzheimer's disease, and asthma by injecting diluted BV into acupuncture points. Both the pharmacological effects of BV and the stimulation of acupuncture points elicit a response. On the other hand, BVA includes injecting BV diluted with saline into a specific acupoint, has been used to treat many forms of pain in the clinical domains of traditional, complementary, and alternative medicine and does not have the same hazards as bee sting therapy (Seo et al., 2017; Sung & Lee, 2021; Wehbe et al., 2019).

The idea of using BV in medicine originated, when beekeepers suffered from ailments such as rheumatism and joint pain (Wehbe et al., 2019). The effects of BV on a range of ailments were then studied using various treatment techniques. BV has been shown to have analgesic, anti-cancer, anti-asthmatic, antioxidant, anti-aging, anti-atherosclerotic, anti-diabetic, hepatoprotective, antivira, neuroprotective and anti-rheumatoid arthritis biological activities as a result of scientific research, and its use in the cosmetic industry has recently increased.

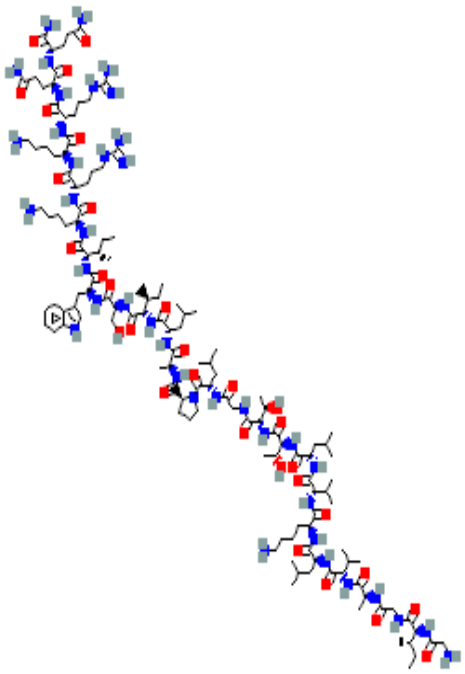
2. Method

A literature search was undertaken to evaluate the biological activity of BV by using various filters to search English papers in Scopus, Web of Science, Google Scholar Library, and PubMed. The following search phrases were used: bee venom, bee venom acupuncture, melittin, and bee venom treatment.

3. BV composition

BV has a very complex structure. The main components have been mentioned below.

3.1. Melittin



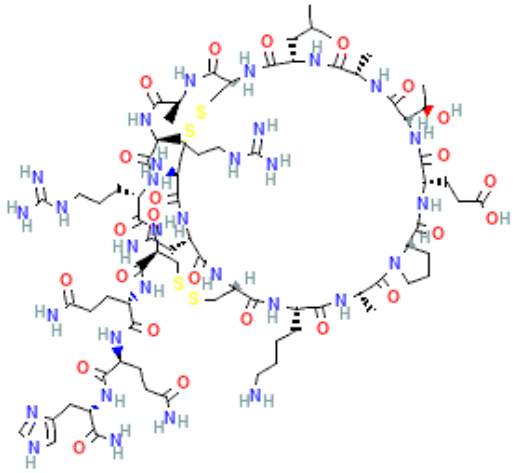
The 2D structure of melittin (PubChem CID: 16133648 was downloaded from the PubChem databank)

Table 1: BV components

Molecule type	Components	References
Polypeptides	Melittin (50%), apamin (2%), adolapin (1%), mast cell degranulating peptide (2-3%), minimine (2-3%), procamine A-B, secarpine, melittin F, cardiopep	(Azam et al., 2018; Bellik, 2015; Fenard et al., 1999; H. Y. Kim et al., 2020; Lee & Bae, 2016; Moreno & Giralt, 2015; Shimpi et al., 2016; Sığ et al., 2019; Son et al., 2007; Wehbe et al., 2019; Yong et al., 1990)
Biologically active amines	Histamine, epinephrine, dopamine, noradrenaline, serotonin	(Bellik, 2015; Han et al., 2000; Hossen et al., 2017; Nielsen, 2020; Rakha et al., 2018; Sığ et al., 2019; Son et al., 2007; Yasin et al., 2000; Yong et al., 1990)
Enzymes	Phospholipase A2 (12-15%), hyaluronidase (1-2%), phosphomonoesterase, lysophospholipase, α-D-glucosidase (0.6%), phospholipase B (1%)	(Bellik, 2015; Han et al., 2000; Hossen et al., 2017; Kemeny et al., 1984; Lee & Bae, 2016; Pattabhiramaiah et al., 2020; Shimpi et al., 2016; Sığ et al., 2019; Wehbe et al., 2019)
Carbohydrates	Glucose (2-4%), fructose	(Bellik, 2015; Han et al., 2000; Shimpi et al., 2016; Sığ et al., 2019; Wehbe et al., 2019; Ye et al., 2016)
Free amino acids	α-amino acids, β-aminoisobutyric acid	(Bellik, 2015; Han et al., 2000; Rady et al., 2017; Shimpi et al., 2016; Wehbe et al., 2019)
Minerals	Potassium, calcium, magnesium	(Bellik, 2015; Moreno & Giralt, 2015; Sığ et al., 2019; Wehbe et al., 2019)
Volatile substances	Complex ethers	(Bellik, 2015; Sarhan et al., 2020; Wehbe et al., 2019)
Organic acids (constitute 7-8% of the total chemical structure)	Citric acid, malic acid, succinic acid, fumaric acid, malonic acid, glutaric acid, and kynurenic acid	(Bellik, 2015; Han et al., 2000; Pawlak et al., 2020; Rady et al., 2017)

Melittin is a poisonous and allergic peptide with a molecular weight of 3850 Da and 26 amino acid residues that makes up half of the dry BV. It is in charge of cell lysis, determining the toxicity of BV, and inducing itching and irritation. The amino terminal section (residues 1-20) is hydrophobic and has no lytic activity, whereas the carboxy terminal section (residues 21-26) is hydrophilic and has lytic action. Melittin's amphoteric feature renders it soluble in water as a monomer. Melittin peptide accumulation causes cell lysis by altering the phospholipid structure in the cell membrane. Melittin possesses anti-cancer, anti-inflammatory, antiviral, antibacterial, and neuroprotective properties (Lee & Bae, 2016; Son et al., 2007; Yong et al., 1990). Some viruses are coated in a membrane that is similar to the host's cell membrane and includes specific viral proteins. Enveloped viruses easily evade the host's immune system and that makes their treatment more difficult. Melittin was shown to have an antiviral impact on enveloped viruses such as retroviruses and herpesviruses in a research by causing membrane lysis surrounding encapsulated viruses (Fenard et al., 1999; Yong et al., 1990). Several studies are available in the literature that examine anticancer activity of melittin. Anticancer activity is the most notable effect of melittin as well as BV, since several researches pointed out to its substantial anticancer activity in the majority of the investigations. According to the findings, melittin shows its anticancer activity through suppressing TLR2, TLR4, CD14, NEMO, and PDGFRβ signaling pathways and activating p38, ERK1/2, AKT, and PLCγ1 pathways, increasing calcium channel activation, activating death receptors (DR4, DR5), and indirectly stimulating caspase 3 and caspase 9 enzymes that play a role in apoptosis (Lee & Bae, 2016; Wehbe et al., 2019).

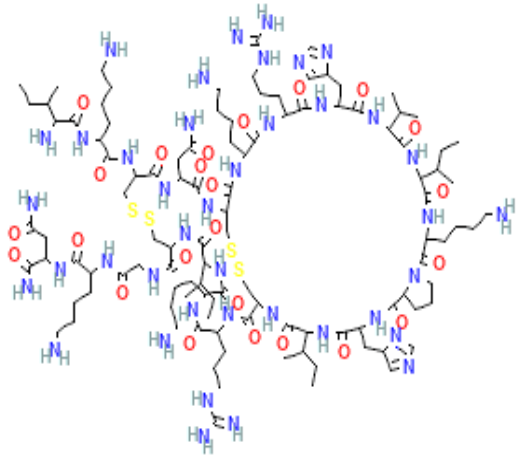
3.2. Apamin



The 2D structure of apamine (PubChem CID: 16133797 was downloaded from the PubChem databank)

Apamin is a blood-brain barrier-crossing polypeptide made up of 18 amino acids that accounts for less than 2% of the dry venom's weight and contains two disulfide bridges (Azam et al., 2018; H. Y. Kim et al., 2020). It is also an antioxidant and anti-inflammatory substance. In a study in which a lipopolysaccharide-induced acute kidney injury model was generated in male C57BL/6N mice, it was revealed that the kidney dysfunction and histological damage, particularly tubular damage, of the mice in the treatment group improved significantly compared to the control group after being injected with apamin at a dose of 10 mg/kg. This effect has been linked to a considerable decrease in tubular cell apoptosis caused by apamine-induced caspase-3 activation and TLR-4-mediated regulation of cytokine production. Apamin was also shown to reduce LPS-induced oxidative stress *via* altering the expression of nicotinamide adenine dinucleotide phosphate oxidase 4 and heme oxygenase-1 in the same research (J. Y. Kim et al., 2020).

3.3. MCD (Mast cell degranulating) peptide



The 2D structure of mast cell degranulating peptide (PubChem CID: 16132290 was downloaded from the PubChem databank)

It is the neurotoxic component of BV and accounts for 2-3% of dry BV. It has a molecular structure that is similar to apamine and comprises of 22 amino acid residues. MCD was proven to cause a remarkable decrease in blood pressure in rat experiment. In this context, it is the component considered to be responsible for the hypotension seen in BV intoxication (Wehbe et al., 2019).

3.4. Adolapin

It is a polypeptide of 103 amino acids that accounts for around 1% of dried BV. Adolapin has anti-inflammatory, anti-nociceptive, and antipyretic activities by reducing cyclooxygenase activity and blocking prostaglandin synthesis (Bellik, 2015; Cherniack & Govorushko, 2018).

3.5. Phospholipase A2

BV phospholipase A2 (BV-PLA2) is the alkaline component that accounts for 12-15% of dry BV, includes 128 amino acid residues, and 4 disulfide bridges, while it possesses the maximum lethality. During the

erythrocyte lysis process, it interacts synergistically with melittin, creating gaps in the cell membrane and allowing melittin to flow through, killing the cells by breaking down the phospholipid layers, which are the major components of the cell membrane. It has antiparasitic, anticancer, and anti-inflammatory properties (Dudler et al., 1992; Hossen et al., 2017; Wehbe et al., 2019).

3.6. Phospholipase B

It is detected in a 1% concentration in dried BV, which stimulates PLA2 activity (Hossen et al., 2017).

3.7. Hyaluronidase

It is a polypeptide with 350 amino acid residues that makes up 1-2% of BV and selectively breaks down hyaluronic acid in the extracellular matrix of the skin, allowing other venom components to enter the body. It is known that it stimulates blood vessel dilation, increasing permeability, and so boosting blood circulation, which therefore enhances BV circulation (Hossen et al., 2017).

4. Safety of BV for human

BV causes allergic reactions after application. These reactions might emerge in the skin, respiratory tract, cardiovascular system or gastrointestinal tract. Severe anaphylactic shock can then result in cerebral or myocardial ischemia. The proteic components in the venom are considered to trigger these allergic reactions. PLA2, melittin, and hyaluronidase are considered to be major allergens and inducers of immunoglobulin E (IgE) in BV. When BV venom is administered to a human body, symptoms such as dyspnea, limb paralysis, loss of consciousness, nausea, and exhaustion might occur. High amounts of BV (100 µg/mL) can induce human lymphocyte instability, although smaller concentrations are not genotoxic and do not cause oxidative damage. The skin sensitivity level of BV (10 mg/mL) was classified as Grade 1-poor in a skin sensitization study done on 50 guinea pigs using the Buehler test. As a result, it is advised that an allergy test and controlled application be performed prior to treatment with BV (W. R. Lee et al., 2011; Zhang et al., 1995). When BV is applied to the human body, mild side effects such as fatigue, localized edema, pruritus, skin rash nausea, and vomiting may occur, as can severe side effects such as limb paralysis, dyspnea, and loss of consciousness (Cheng & Ren, 2004; Ko et al., 2022; Zhang et al., 1995). According to a retrospective study conducted at the hospital between January 2010 and April 2019, only 0.175% of 8580 patients admitted to the hospital reported type 1 hypersensitivity and 0.047% anaphylactic shock (Lee et al., 2020). The incidence of anaphylactic shock seen during treatment with penicillin, an antibiotic that has been used for a long time in the treatment of many infections, has been reported to be between 0.02-0.04% (Patterson & Stankewicz, 2021). BVA therapy is currently limited due to the risk of anaphylactic shock and other allergic responses during BV treatment. However, as compared to penicillin therapy, the rate of anaphylactic shock reported with BVA treatment is substantially lower.

5. The process of obtaining BV

BV can be obtained in three different ways. First, a device known as a BV collector is placed at the hive's entrance and then collected by scraping after enough BV has accumulated. This instrument uses low voltage electricity to acquire and extract a greater amount of BV from the bee. Another technique is to set an appliance for the bee's sting. The poison left by the bee is collected on the hive machinery. The third way is to use tweezers to mechanically retrieve the sacs in which the honey bee stores its venom (De Graaf et al., 2021). The collected BVs are purified and powdered using the lyophilization procedure in a specific pre-sterile environment. The gathered BV is

treated in a pre-sterile special environment before being refined. Lyophilized BV is powdered and diluted with distilled water at a certain quantity. BV is put into appropriate packaging, packed, sanitized, and packaged for clinical usage as pharmacopuncture injections (Sung et al., 2021).

6. Biological effects of BV

6.1. Anti-acne activity

Acne vulgaris is an inflammatory dermatological disorder characterized by excessive sebum production, aberrant follicular keratinization, and proliferation of *Propionibacterium acnes*, which is an anaerobic and gram positive bacteria on skin microbiota. *P. acnes* causes an increase in the production of proinflammatory cytokines such as IL-1ß, IL-8, and TNF-α (Han et al., 2016).

In a clinical study conducted on 30 participants aged 12-33 years, 77% of which are being Caucasian with acne problems. The facial serum containing 0.7-0.9 g of purified BV was applied on entire clean face of all participants twice a day, morning and evening, for 6 weeks. The Modified Cook's Acne Grading Scale was used to assess the effects of BV serum on acne. Changes in visible acne lesion counts following therapy with pure BV (PBV™) serum were recorded at 3 and 6 weeks, as well as changes in acne lesion numbers by image analysis. Acne is classified into 5 types: open comedones, closed comedones, papules, pustules, and nodules. According to the findings of this study, while all forms of acne improved after 3 and 6 weeks of treatment, open comedones had the most benefit. The administration of pure BV serum for 6 weeks resulted in a 92% in open comedones compared to the first day. The absence of allergic reactions or irritation in the participants throughout the research gives credence to the application's safety. After 6 weeks of applying pure BV serum to the skin, the reduction in open comedones, closed comedones, and papules was 92 %, 72.2 % and 43.4%, respectively. Interestingly, although all of the pustules vanished after 3 weeks, they reappeared after 6 weeks (Han et al., 2016).

A research on 30 eight-week-old ICR mice examined the effect of purified BV on *P. acnes*-induced dermatological illness. Mice were divided into 6 groups. While the control group received no intervention, the mice in the second group had their left ears injected intradermally with $1.0x10^7$ CFU of *P. acnes* in 20 µL of phosphate buffer solution (PBS) and an equal volume of PBS alone in their right ears, followed by 0.05 g vaseline. Following injections of *P. acnes* into both the right and left ears of the mice in the other groups, vaseline containing 1 µg, 10 µg, and 100 µg pure BV was administered topically to only the right ear of each mouse. At the end of 24 hour-period, histological examinations were performed. Following *P. acnes* injection, swelling, erythema, and redness, were observed to develope in mice ears resulting in significant production of pro-inflammatory cytokines such as TNF-α and IL-1ß. Although BV treatment decreased edema, erythema, and redness induced by *P. acnes*, the group treated with 1 µg BV exerted the greatest improvement. As a result of this observation, it can be stated that the side effects of using high concentrations of BV appear. The administration of BV effectively inhibited the secretion of pro-inflammatory cytokinins such as TNF-α and IL-1ß, according to western blot analysis (An et al., 2014).

Previous research has revealed that the increase in pro-inflammatory cells following *P. acnes* treatment was mediated by TLR2 (Kim et al., 2002). The double immunofluorescence investigation found that BV suppressed TLR2 expression and reduced the release of proinflammatory cytokines. Similarly, BV has been demonstrated to suppress the activation of transcription factors involved in the secretion and regulation of pro-inflammatory

cytokines such as AP-1 and NF-κB (An et al., 2014).

In vitro and *in vivo* experiments were conducted to explore the effect of mellitin, the primary component of BV, on *P. acnes*-induced inflammatory skin disease. In a research on *P. acnes*-induced HaCaT cells, 1 µg/mL mellitin dramatically reduced the levels of pro-inflammatory cytokines TNF-α, IL-1ß, IL-8, and *IFN*-γ compared to the control group. It has been observed that mellitin regulates TLR activation on the same cell line and that mellitin reduces the p38 MAPK signal, which in turn reduces the production of pro-inflammatory cytokines. The same group of researchers investigated the effect of mellitin on *acne vulgaris* in an animal model. Mellitin was demonstrated to suppress the production of TNF-α and IL-1ß cytokines in 8-week-old ICR mice infected with *P. acnes* through regulating the AP-1 and NF-κB transcription factors (Lee et al., 2014). As a result of all these studies, it sheds light on the fact that BV applied in the right dose can be effective in skin infection caused by *P. acnes*, in which the effective substance may be mellitin. Oral and topical antibiotic therapy is used for a long time in the treatment of *acne vulgaris*. This treatment method not only causes the development of antibiotic resistance, but also causes side effects such as diarrhea, nausea, and skin rashes. *In vitro* studies have been supported by *in vivo* studies and the effect of BV on the treatment of *acne vulgaris* has also been demonstrated by clinical studies. These developments can be an alternative to the use of corticosteroids, retionoids, and antibiotics in the treatment of *acne vulgaris* and can be a new perspective for both pharmaceutical and the cosmetic industries. The pathways through which anti-acne effect of BV takes place should be supported by more detailed studies.

6.2. Antiviral activity

Although infectious illnesses have always played an important role in human history, the new coronavirus SARS-CoV-2 virus that emerged in the Wuhan, Hubei Province in December, 2019 affected the entire world. Although treating viral infections such as COVID-19, hepatitis, AIDS, and influenza is challenging, there is sometimes no particular medicine to cure. The contagiousness of viral infections is also extremely dangerous to public health. The virus infects the host replicates its genetic material in the host cell. As a result, in the treatment of viral illnesses, a selective impact is essential; otherwise, antiviral medications may potentially possesses a mutagenic effect on the host cell. Today, the chronic and acute toxicity of antiviral medications used in the clinic, their inadequacy in therapy, the rapid evolution of virus resistance to these treatments, and the high cost of the drugs all contribute to the hunt for novel antiviral compounds (Chan et al., 2020; Musarra Pizzo et al., 2021).

According to the results of a survey of 5115 beekeepers, 723 of whom were in Wuhan, the epicenter of the COVID-19 outbreak, by the local beekeepers association in China, the beekeepers did not show any symptoms of COVID-19 and their general health was quite good. It was determined that 3 out of 121 patients treated with apitherapy between October 2019 and December 2019 were not infected with SARS-CoV-2 despite being in close contact with patients infected with SARS-CoV-2 (Yang et al., 2020). The researchers, who conducted the mentioned study, believed that the common thread across all of these patients was that they developed a tolerance to BV, which triggers a severe allergic reaction. They emphasized that this research should be carried out on monkeys. It was hypothesized that the regulatory T cells were differentiated because immunization against BV developed in people exposed to BV or because it works as an ACE receptor blocker or as an ACE2 inhibitor against the ACE receptors utilized by the novel coronavirus (SARS-CoV-2) to enter cells. Melittin, a component of BV, exhibits antiviral properties by puncturing the protective membrane envelopes

surrounding viruses such as SARS-CoV-2 (Kasozi et al., 2020; Yang et al., 2020). In contrast to this study, a study conducted in Germany with 234 participants who are members of the German Beekeepers Association found that 2 beekeepers died as a result of COVID-19, while 42 beekeepers were infected with the SARS-CoV-2. Furthermore, the same study discovered that beekeepers' age, the number of bee stings in 2020, the total number of bee stings so far, gender, and whether or not they had any comorbidities had no effect on their immunity. The hypothesis which was conducted in China that beekeepers would be somehow immune to the illnesses caused by SARS-CoV-2 was disproved as a consequence of this investigation (Männle et al., 2020).

Antiviral effect of BV on the blood-borne hepatitis C (HCV) virus, which causes liver damage, was examined in the human hepatoma-derived Huh7it-1 cell line in a study. BV at various dilutions (0.01, 0.1, 1, 10, 100, and 1000 ng/mL) was found to have dose-dependent anti-HCV efficacy in this cell line. The IC_{50} value of BV was determined to be 0.05 ng/mL, and it was discovered to have very significant anti-HCV activity.
To ascertain which component for antiviral activity was responsible, efficacy of BV to replicate hepatitis C virus was considerably low following proteinase K protein removal. Phospholipase A2 is one of the enzymes found in BV (Sarhan et al., 2020). Previously, it was considered that the compound responsible for anti-HCV activity investigation of snake venom was due to phospholipase A2, which destroys the virus' envelope and coated with the envelope having a phospholipid composition (Chen et al., 2017). To evaluate if BV's anti-HCV action includes enzymatic activity, BV (100 ng/mL) was heated at 95 °C for 30 minutes, and at the conclusion of the treatment, it was figured out that the active compound was a material resistant to 95 °C, with no impact attributable to enzymatic activity. It is hypothesized that the molecule responsible for the action has a proteic structure, whereas it has a very low molecular weight. Melittin, apamin, and mast cell-degranulating peptide, which are among the primary peptides in BV, were treated and incubated separately with Hepatitis C virus to evaluate the substance in the content of the anti-HCV effectiveness of BV. It has been discovered that melittin, apamin, and the mast cell-degranulating peptide do not directly contribute anti-HCV efficacy in BV (Sarhan et al., 2020). When the impact of BV on the bovine viral diarrhea virus was previously explored, it was shown that mellitin did not have a direct anti-viral effect, but rather enhanced the anti-viral action, when combined with apamin (Picoli et al., 2018).

Melittin is responsible for the antiviral activity of BV according to research on *herpes simplex* virus-1 and -2 (Yasin et al., 2000). As a result of these investigations, it has been stated that the molecule responsible for anti-HCV action may not be melittin, apamin or mast cell-degranulating peptide. BV may have a direct antiviral activity (Sarhan et al., 2020). Melittin acts as an antiviral agent by destroying the protective membrane envelopes that enclose viruses such as human immunodeficiency virus (HIV) (Kasozi et al., 2020).

6.3. Analgesic activity

BV acupuncture has been studied for its benefits on musculoskeletal pain such as shoulder pain and low back pain, inflammatory pain such as arthritis pain, neuropathic pain such as post-stroke pain, prostate discomfort, and chronic regional pain (Sung & Lee, 2021). Chronic low back disease or pain is a common and frequently recurring condition among people without a pathological cause, limiting movement and worsening quality of life (Andersson, 1999). In a randomized, double-blind, and placebo-controlled clinical trial, 54 volunteer patients were divided into 2 groups. While one group received just nonsteroidal anti-inflammatory

drug (NSAID), the other group received increasing quantities (0.2 mL, 0.4 mL, 0.8 mL) of BV + NSAID to the acupuncture sites once in each week. At the end of 12 week-period, when the participants' pain level was measured using the visual analogue scale, it was stated that the data of the group treated with BV acupuncture were superior. Other outcomes examined by VAS, Oswestry Disability Index (ODI), EuroQol 5 Dimension (EQ-5D), and Beck's Depression Inventory (BDI) included pain intensity, movement restriction, quality of life, and depression. It was discovered that after 3 weeks of BV acupuncture therapy, pain severity and back pain dysfunction were dramatically reduced. As a result, BV acupuncture therapy has been found to be beneficial in pain relief (Seo et al., 2017). Several investigations have shown that BV acupuncture has analgesic and antinociceptive effects that are mediated *via* α_2-adrenergic and serotonergic receptors (Kim et al., 2005; Kwon et al., 2001).

In a study, 80 Sprague–Dawley rats were subjected to collagen-induced arthritis. The tail wagging action of the experimental animals was significantly decreased after the development of rheumatoid arthritis. When compared to the non-treatment group, there was a considerable increase in tail wagging in the control group which was treated by applying simply saline to the identical acupuncture locations treated with BV acupuncture. The highest analgesic impact of BV acupuncture was observed 30 minutes after injection, and this effect was reported to persist at least 60 minutes. Because no analgesic effect was detected in the control saline-treated group, antinociceptive effect of BV acupuncture was determined to be produced by BV rather by acupuncture. In the same study, yohimbine as an indole alkaloid and α_2-adrenergic receptor antagonist along with *mu*-opioid receptor antagonist naloxone were administered to experimental animals prior to BV acupuncture administration to determine the mechanism of action of BV acupuncture treatment's analgesic effect on collagen-induced inflammatory pain. Finally, analgesic effect of BV acupuncture therapy was entirely negated by intraperitoneal administration of yohimbine. Intraperitoneal injection of the mu-opioid receptor antagonist; naloxone had no effect on analgesic efficacy on BV acupuncture. These findings imply that BVA's antinociceptive impact on inflammatory pain is specifically mediated *via* the α_2-adrenergic route rather than the opioidergic system (Baek et al., 2006). BVA owns a strong analgesic effects *via* activating spinal α_2-adrenoceptors (Kang et al., 2011). In the dorsal area of male Sprague-Dawley rats, a formalin-induced acute pain model was generated. While FOS gene expression increased in all regions of the lumbar spinal cord following formalin administration, it was inhibited early in the group treated with high-dose (0.08 mg/kg) BVA. According to this study, analgesic activity of BV may possibly be attributable to inhibition of c-FOS gene expression (Kim et al., 2003).

BVA administration (61.1 F 9.1 pg/mL) has been demonstrated in a rat model of collagen-induced rheumatoid arthritis to dramatically suppress IL-6 rise, promote apoptosis *via* a drop in BCL2 expression, and an increase in BAX and CASP3 expression in the rheumatoid synovium (Hong et al., 2005). Similarly, in a study of rheumatoid arthritis was induced in male Wistar rats with Freund's complete adjuvant (FCA) and type-2 collagen. Each experimental animal received BVA administration at a dosage of 1 mg/200 g and treatment with BV was demonstrated to reduce proinflammatory markers such as TNF-α and IL-6 (Nipate et al., 2015).

6.4. Anti-aging activity

The skin is the human body's biggest organ. Skin aging is a natural process defined by skin dehydration and wrinkles caused by genetic and cellular aging, collagen alteration as an intrinsic factor as well as extrinsic

factors such as prolonged sun exposure, UV exposure, nutrition, smoking, stress, lifestyle, using the wrong skin product, and so on. The decrease in collagen production and rise in its decomposition leads to a loss in skin flexibility and the collapse of fibroblasts, resulting in the formation of wrinkles (Han et al., 2015; Mesa-Arango et al., 2017). Facial serums containing BV at a concentration of 0.006 % were administered twice daily to the full face as 4 mL *per* application to 22 South Korean female volunteers aged 30 to 49 years with mild to moderate indications of photoaging. The overall wrinkle area on the skin, total number of wrinkles, and average wrinkle depth all decreased after 12 weeks administration of the serum. After 12 weeks, 54.6 % of the volunteers reported a significant improvement in wrinkles on their faces. A face serum containing BV has been claimed to have clinical anti-aging benefits, although the mechanism of action has not been thoroughly elucidated (Lee & Bae, 2016). On the other hand, the use of BV in the cosmetic industry has increased in recent years. Nonetheless, it is discouraged since it has a marked potential for producing allergic responses. A research group reported that the phospholipase A2 is the enzyme component of BV that produces adverse effects such as allergic reactions and erythema on the skin. Furthermore, the cytotoxicity of both BV and phospholipase A2-free BV on UV-irradiated human keratinocyte (HaCaT) and human dermal fibroblast (HDF) cell lines were investigated. While cell lines exposed to 3 µg/mL standard BV for 2 hours, it showed a severe level of cytotoxicity. Otherwise, cell lines treated with phospholipase A2-free BV showed no cytotoxicity. Depending on the concentration, both BVs boosted the formation of type-1 procollagen. Induced MMP-1 and MMP-13 secretion in cell lines treated to UV-B irradiation was reduced by concentrations of 1.5 µg/mL and 3 µg/mL of regular BV and phospholipase A2-free BV, respectively. As a result, collagen degradation was avoided. The activation of ERK1/2 and p38 signaling pathways revealed the cell signaling mechanisms behind the anti-wrinkle effects of regular and phospholipase A2-free BV. *In vitro* experiments have demonstrated that phospholipase A2-free BV is the preferable one with less cytotoxicity and better efficiency on UVB-irradiated skin cells than the original one (Lee et al., 2015).

6.5. Wound-healing activity

The wound healing process is formed into the stages as follows: hemostasis, inflammation, proliferation or re-epithelialization, neovascularization-angiogenesis, and remodeling (Guo & DiPietro, 2010; Kurek Górecka et al., 2020). *Diabetes mellitus* (DM) was induced on 45 BALB/c mice by giving streptozotocin (STZ) for 5 days. The wound-healing efficacy of BV was investigated in mice with DM-induced lesions. BV accelerated wound repair process in diabetic mice by activating collagen expression, reduced oxidative stress in diabetic wounds by activating antioxidant defense mechanisms such as GSH Px, MnSOD, catalase, and regulated angiogenesis by activating ß-defensin-2 expression. When compared to animals in the control group, it was revealed that it accelerated wound closure (Hozzein et al., 2018).

In diabetic mice, BV enhances angiogenesis of new tissues formed during the wound healing process and upregulates TGF-α and VEGF expression. In the same study, BV reduced the release of proinflammatory cytokines such as IL-1, IL-6, and TNF-α in damaged tissues and also accelerated wound healing by decreasing the expression of ATF-3 and iNOS. BV also reduced the formation of reactive oxygen species. BV-induced wound healing has been shown to reduce caspase-3, -8, and -9 activity, which triggers apoptosis during the neovascularization and angiogenesis phases of wound healing process (Badr et al., 2016; Kurek Górecka et al., 2020). According to the findings of an *in vitro* study on the human epidermal

keratinocytes (HEK) cell line, BV at low doses (1 µg/mL) stimulates the proliferation and migration of keratocyte cells, while also lowering the level of released proinflammatory cytokines such as IL-8 and TNF-α (Han et al., 2013; Kurek Górecka et al., 2020).

6.6. Anti-hyperglycemic activity

DM is a life-threatening metabolic condition defined by chronic hyperglycemia caused by abnormalities in insulin production, insulin action on its receptors or both (Kharroubi & Darwish, 2015). Hyperglycemia induces enhanced glycation between sugars and proteins' free amino groups resulting in structural and functional alterations in proteins (Sen et al., 2005). Different concentrations of BV (10, 20, and 40 µg/mL) were administered to each sample for *in vitro* investigation to determine the effect of BV on hemoglobin glycation by incubating the hemoglobin in the presence of glucose. The amount of heme was evaluated by cleaving free amino groups with florescamine. The study concluded that BV reduces glycation-induced heme breakdown in hemoglobin. Because it has a considerable antiglycation impact. It has been also proposed to be developed as a natural therapy for glycation-related problems in DM (Behroozi et al., 2014).

6.7. Effect on lupus nephritis

Lupus nephritis is a frequent and potentially fatal consequence of systemic lupus erythematosus (SLE). ZB/W F1 female mice with idiopathic glomerulonephritis, proteinuria, and renal failure were treated once a week with a subcutaneous injection of 3 mg/kg of BV diluted in saline at a concentration of 0.5 mg/mL. In the control group of 10 mice, an equal amount of saline was injected. This practice lasted 12 weeks. The kidneys and spleens of surviving mice were isolated and evaluated at the end of the research. The renal functions worsened and severe proteinuria occurred in the control group but the mean urine protein level reduced in the BV-treated group. The majority of glomeruli in the saline-treated group showed class IV and V morphology with lupus membranous nephropathy glomerulus with spherical endocapillary and mesangial hypercellularity, early crescent formation, and subepithelial spine formation with the onset of mesangial and capillary sclerosis, when the kidneys of mice were examined by light microscopy at the end. However, in the BV-treated group several glomeruli were found to have somewhat aberrant histology. Serum levels of IG2a, IgG, and IgG3 as well as levels of proinflammatory cytokines such as TNF-α and IL6 were found to be lower. When BV-injected animals were compared to saline-injected mice, the CD4+ and CD25+ regulatory T cell population was dramatically enhanced. FOXP3 expression in CD4+ T cells, which suppressed the immunological response, has also been found to be increased in mice injected with BV (H. Lee et al., 2011).

6.8. Anti-asthmatic activity

Asthma is a potentially fatal inflammatory lung disease characterized by high number of CD4+ T cells. In a study, in which an allergic asthma model was induced in Balb/c mice by ovalbumin, injection of BV (0.1 and 1 microg/mL) increased the number of regulatory T cells in mice, suppressed the production of cytokines such as IL2, IL4 and IL13, and reduced peribronchial and perivascular inflammatory cell infiltrates. It has been shown that the expression of CD4 + CD25 + and FOXP3 from natural regulatory cells rose, whereas IgE levels in experimental animals' blood decreased significantly (Choi et al., 2013; Son et al., 2007).

6.9. Effect on amyotrophic lateral sclerosis

Amyotrophil is a lateral sclerosis illness characterized by aberrant accumulation of

mutant SOD1 protein aggregates. TLR4, CD14, and TNF-α were utilized in a research, in which BV was administered into the acupuncture points of human-SOD1 G93A transgenic mice with ALS (male hSOD1G93A transgenic mice) at dose of 0.1 μg/g 3 times a week during 2 weeks. A drastical decrease was shown in neuroinflammation as well as a suppress in neuroinflammation in mouse spinal cords (Cai et al., 2015). Bees were administered at dose of 0.1 μg/g each time in a study conducted in hemizygous transgenic B6SJL mice carrying a glycine-alanine base pair mutation at codon 93 of cytosolic Cu/Zn superoxide dismutase gene (hSOD1G93A) an animal model of amyotrophic lateral sclerosis. By suppressing microglia activation and phospho-p38 MAPK production in the central nervous system, venom injection improved motor function (Yang et al., 2010).

6.10. Effect on alopecia

Alopecia is a serious condition that affects people of all ages. The impact of BV on alopecia was studied in a work by administering different concentrations (0.001 %, 0.005 %, 0.01 %) and minoxidil 2%, as a positive control, to the dorsal skin of female C57BL/6 mice for 19 days. Effect of BV on balding was studied using mouse skins and human dermal papilla cells utilizing quantitative real-time PCR and Western blot analysis. BV stimulated hair growth and slowed the transition from anagen to catagen. When compared to controls, hair growth was dose-dependently enhanced in both anagen phase mice and dexamethasone-induced catagen phase mice. BV decreased the production of SRD5A2 gene, which encodes type II 5-reductase, a key enzyme in the conversion of testosterone to dihydrotestosterone. Furthermore, BV has been demonstrated to increase human dermal papilla cells (hDPCs) and several growth factors such as insulin-like growth factor-1 receptor, vascular endothelial growth factor, fibroblast growth factor-2 and 7 in hDPCs treated with BV. Proliferation was boosted in a dose-dependent manner, when compared to control group. Finally, BV has the potential to be a powerful 5-reductase inhibitor as well as hair growth stimulator. Low doses should be used just as bee and hair stroke with its dose is on the proper track because successful venom can generate an effective side at big doses and start the entire reaction. The bee poisonings utilized in this property had no negative effects (Park et al., 2016).

6.11. Anticancer activity

Melittin, which is one of most important components of BV, boosted cell proliferation in two human leukemia cell lines, *e.g.* acute lymphoblastic leukemia (CCRF -CEM and CCL -119, ATCC TM) and chronic myelogenous leukemia K-562 (CCL -243, ATCC TM). It has been shown to have anti-leukemic properties *via* blocking and promoting cell death. Simultaneously, melittin reduced calcium pump action by blocking calmodulin. This led to a raise in Ca^{2+} concentration, which is harmful to cells and finally leads to cell death (Chu et al., 2007; Lyu et al., 2019; Nielsen, 2020).

Several researches have revealed that melittin displayed antileukemic potential by activating caspase-3 and -7 resulting in the activation of apoptosis in both cell types. It caused cell death by apoptosis. Melittin has been also shown to trigger apoptosis in the cervix HeLa cell line. It decreased the growth of HeLa cell line in a dose- and time-dependent manner according to the results of MTT experiment used to determine *in vitro* cytotoxicity. Melittin has been shown in a research to possess cytotoxic effects towards human breast cancer cell line, human hepatocellular carcinoma (Li et al., 2006), prostate cancer (Park et al., 2011), and ovarian cancer cell lines (Jo et al., 2012).

6.12. Antimicrobial activity

Melittin and PLA2, both components of BV, provide antibacterial activity by stimulating hole creation on the infecting microorganism's surface. As the holes on the microbe enlarge, the cell's integrity is compromised its genetic material is destroyed, and divisional proliferation is halted (Wehbe et al., 2019). Minimum inhibitory concentration (MIC) of BV on 16 different *Salmonella* strains isolated from poultry (*e.g. S. newport*, *S. isangi*, *S. enterica* subsp. *salama*, *S. bardo*, *S. montevideo*, *S. infantis, S. stanleyville*, *S. ndolo*, *S. dabou*, *S. typhimurium*, and *S. enteritidis*) was determined using broth microdilution method. Each cell line was treated with BV diluted with water at a concentration of 4.096 µg/mL. Although the MIC value was generally 512 µg/mL, where *S. enterica* subsp*. salama* had the highest MIC value of 1024 µg/mL and one of the *S. typhimurium* lines had the lowest MIC value as 256 µg/mL. Simultaneously, apitoxin inhibited biofilm development in *Salmonella* and substantially eliminated preexisting biofilms. Biofilm production was decreased by BV from 68.22% to 27.66%. As a result, researchers have underlined that combining apitoxin with other antimicrobial medicines can lead to the creation of novel and effective treatments (Arteaga et al., 2019).

In a relevant study, disc diffusion assay technique was used to test antibacterial activity of the venom obtained from *A. mellifera*, which was dried through lyophilization and subsequently purified, at 25, 50, 75, and 100 µg/mL concentrations. In this experiment, gentamicin (10 µg/disc) was utilized as a positive control. Antimicrobial activity of BV against *Escherichia coli* and *Staphylococcus aureus* strains was dose-dependent, but not observed against *Pseudomonas aeruginosa* and *Bacillus subtilis.* At a concentration of 100 µg/mL, BV had even a greater antibacterial activity than that of gentamicin, which was employed as a positive control against *E. coli* (29.06 ± 1.31 mm) and *S. aureus* (17.51 ± 1.07 mm). In the same study, the LD_{50} value of BV was estimated to be 177.8 µg/mouse as a result of the investigation. As a result, at non-toxic and safe quantities, BV has a very good antimicrobial impact (Babaie et al., 2020).

7. BV dosage forms and components

BV has recently been employed in cosmetic products such as face serum, moisturizer, and face mask, owing to its anti-aging and anti-acne properties. Lyophilized BV powders are stored in sterilized glass vials and dissolved with physiological saline at health centers that provide BV treatment. Melittin possesses anticancer action, as previously stated. It is extremely toxic and hemolyzes red blood cells when administered into the human body at anticancer doses. Melittin was loaded into a dual secured nano-sting (DSNS) produced by the combination of a zwitterionic glycol chitosan and disulfide bonds in a research. In a research of multiple cancer cell lines, melittin-loaded DSNS at 5 mM concentration may kill nearly 100 percent of cancer cells while having no hemolytic impact (Cheng et al., 2015). The toxicity, non-specificity, and fast degradation of cytotoxic peptides such melittin make *in vivo* administration challenging. Perfluorocarbon nanoparticles loaded with melittin demonstrated an anticancer impact in a research by directly targeting cancer cells and inducing death in these cells (Soman et al., 2009).

8. Pharmacokinetic properties of BV and its components

Tc-labeled melittin was administered intravenously to C57BL/6 experimental mice both freely and in a nanoparticle carrier in a study to determine the pharmacokinetic properties of melittin, the most important component of BV. Blood samples were collected from experimental animals at various time intervals. The plasma half-lives of free melittin and nanoparticled melittin were determined to be 0.79 ± 0.05 minutes

and 4.22 ± 1.48 minutes, respectively. Their distribution volumes were determined to be 9.32 ± 1.54 ml and 2.94 ± 0.31 ml, respectively. By examining the tissue samples, it was discovered that free melittin immediately settled on the cell membrane and induced hemolysis, however, melittin loaded on nanoparticle did not. Melittin-loaded nanoparticles suppressed tumor development more efficiently than free melittin. Half-life and effect duration were prolonged by adding Melittin into the nanoparticle system. Because it may be particularly targeted to tumor cells, its action on tumor cells is enhanced and its toxicity is lowered indirectly (Soman et al., 2009). Another research evaluated the pharmacokinetic differences between intravenous and oral administration of BV in powder form. According to the statistics, impact of BV administered orally is quite modest since it passes *via* the gastrointestinal tract. The loss of impact might be related to enzymatic reactions and degradation in the digestive system's acidic environment (Xing et al., 2003).

9. Stability of BV

A study found that diluting BV 3000 and 4000 times can keep it stable for 12 months at ambient temperature and refrigerator temperature. However, in the same investigation, diluted BV maintained at ambient temperature for 12 months did not demonstrate antibacterial action against *Staphylococcus aureus*, although diluted BV stored in a refrigerator did. Impact of the component responsible for the antibacterial activity of BV reduces at room temperature, according to this study (Kang et al., 2003). Another study, conducted in 2021, assessed stability of melittin, a component of BV known to be responsible for several biological effects such as anticancer, antiviral, and anti-inflammatory. Melittin concentration of identical BV held at ambient temperature and in the refrigerator for 6 months was quantified using a reversed phase high performance liquid chromatography (HPLC) technique with a photo-diode array (PDA) detector in this study, and no significant difference was found. Melittin's remarkable stability, even at room temperature, lowers the storage costs for BV makers (Flanjak et al., 2021).

10. Economic importance of BV

A gram of BV costs between $30 and $300 depending on its purity, according to 2009 research. In recent years, BV has grown in appeal in both the pharmaceutical and cosmetic industries. In Türkiye, there is currently no regulation governing the manufacture of BV. As a result, the number of BV producers is extremely low. Although the use of bee products in cosmetics and health care has only just begun in Turkish, European, and Well Eastern nations are far advanced in this area. Because BV is not widely used or used in Türkiye, the number of BV manufacturers is limited. This market has not emerged for the manufacturers that are already manufacturing. According to a research conducted in Portugal, the profitability rate of facilities that continue production using traditional methods utilized in the manufacturing of BV is quite low, and it is extremely expensive for those who want to enter this field. As a result, the profit generated from BV production may be raised by adhering to existing BV production methods, improving the yield of the product acquired, and lowering the cost (Serrinha et al., 2019).

Conclusion

The use of bee products, particularly BV, in curing diseases and protecting people's well-being has been used since ancient Egypt and Rome. The growing awareness of nature in the world has made bee products, especially BV, increasingly important for curing diseases and protecting the well-being of people. BV therapy is very popular,

particularly in Far East countries such as South Korea and China; it is also practiced in several European countries nowadays. In Türkiye, BV therapy has been becoming more popular. As a result, the number of apitherapy clinics in Türkiye has increased in recent years. It is important to conduct a BV allergy test before beginning any type of treatment involving BV and to ensure that BV is standardized. The majority of BV is composed of melittin, a polypeptide with a polypeptide structure, followed by the PLA2. The anti-inflammatory action of BV has attracted the most attention from researchers, and several scientific studies have been conducted on the issue. Melittin was considered to be responsible for these biological activities in research on the biological activities of BV, and many investigations on melittin have been conducted. According to the findings, melittin exhibits anticancer, antiviral, anti-inflammatory, neuroprotective, and antibacterial properties. BV is now being used not just for medicinal purposes, but also for cosmetic purposes. BV is being employed in cosmetic preparations to benefit from its anti-aging, skin-regenerating, and moisture-retaining properties. More research is needed to completely understand the biochemical pathways of BV.

Abbreviations

ACE: Angiotensin-converting enzyme, **AKT:** Alpha-serine/Threonine Kinase, **ALS**: Amyotrophic lateral sclerosis**, AP-1:** Activator protein 1, **ATF3:** Activating Transcription Factor 3, **BAX**: BCL2-Associated X Protein, **BCL2:** B-cell lymphoma 2, **BDI**: Beck's Depression Inventory, **BV:** Bee venom, **BVA:** Bee venom acupuncture, **BV-PLA2:** Bee venom phospholipase A2, **CASP3:** Caspase-3**, CD-4**: Cluster of differentiation 4, **CFU:** Colony-forming units, **COVID19**: Coronavirus disease 2019 **Da**: Dalton, **DR 4/5**: Death receptors 4/5 **ERK1/2**: Extracellular signal-regulated kinase ½, **EQ-5D**: EuroQol 5 Dimension, **FCA**: Freund's complete adjuvant, **FOS:** *Fos* Proto-Oncogene **GSH Px:** Glutathione peroxidase, ***HaCaT:*** Cultured Human Keratinocyte cells, **HCV**: Hepatitis C virus, **HDF**: Human dermal fibroblast cell line, **HEK:** Human epidermal keratinocytes cell line, **iNOS:** Inducible nitric oxide synthase, **ICR:** Institute of Cancer Research, **IC_{50}:** The half maximal inhibitory concentration, **IgE:** Immunoglobulin E, ***IFN-γ***: Interferon gamma, **IL-1ß:** Interleukin-1 Beta, **IL-2**: Interleukin -2, **IL-4:** Interleukin -4, **IL-6**: Interleukin -6, **IL-8**: Interleukin -8, **IL-13**: Interleukin -13, **LD_{50}:** Lethal Dose, 50%, **MAPK**: Mitogen-activated protein kinase, **MCD peptide:** Mast cell degranulating peptide, **MIC**: Minimum inhibitory concentration, **MMP-1**: Matrix metalloproteinase-1, **MMP-13**: Matrix metalloproteinase-13, **MnSOD:** Manganese-dependent superoxide dismutase, **MTT**: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, **NEMO**: Nuclear factor kappa-B essential modulator **NF-κB :** Nuclear factor kappa B, **NSAID:** Nonsteroidal anti-inflammatory medicine, **ODI:** Oswestry Disability Index, **PBS:** Phosphate buffer solution, **PBV™ :** Purified bee venom**, PCR:** Polymerase chain reaction, **PDGFRβ**: Platelet-derived growth factor receptor beta, **PLA2:** Phospholipase A2, **PLCγ1**: Phospholipase Cγ1, **ROS**: Reactive oxygen species**, SARS-CoV-2:** Severe acute respiratory syndrome coronavirus 2, **SLE**: Systemic lupus erythematosus, **SRD5A2**: Steroid 5 alpha reductase alpha polypeptide 2, **STZ**: Streptozotocin, **TGF-α:** Transforming growth factor alpha, **TLR-2**: Toll-like receptor 2 **TLR-4:** Toll-like receptor 4, **TNF-α**: Tumor necrosis factor-α, **VAS**: Visual analogue scale, **VEGF**: Vascular endothelial growth factor

References

1. An, H. J., Lee, W. R., Kim, K. H., Kim, J. Y., Lee, S. J., Han, S. M., Lee, K. G., Lee, C. K., & Park, K. K. (2014). Inhibitory effects of bee venom on *Propionibacterium acnes*-induced inflammatory skin disease in an animal model. *International Journal of Molecular Medicine*, *34*(5), 1341-1348. https://doi.org/10.3892/ijmm.2014.1933
2. Andersson, G. B. (1999). Epidemiological features of chronic low-back pain. *The Lancet*, *354*(9178), 581-585. https://doi.org/10.1016/S0140-6736(99)01312-4
3. Arteaga, V., Lamas, A., Regal, P., Vázquez, B., Miranda, J. M., Cepeda, A., & Franco, C. M. (2019). Antimicrobial activity of apitoxin from *Apis mellifera* in *Salmonella enterica* strains isolated from poultry and its effects on motility, biofilm formation and gene expression. *Microbial Pathogenesis*, *137*, 103771. https://doi.org/10.1016/j.micpath.2019.103771
4. Azam, M. N. K., Ahmed, M. N., Biswas, S., Ara, N., Rahman, M. M., Hirashima, A., & Hasan, M. N. (2018). A review on bioactivities of honey bee venom. *Annual Research & Review in Biology*, 1-13. https://doi.org/10.9734/ARRB/2018/45028
5. Babaie, M., Mehrabi, Z., & Mollaei, A. (2020). Partial purification and characterization of

antimicrobial effects from snake (*Echis carinatus*), scorpion (*Mesosobuthus epues*) and bee (*Apis mellifera*) venoms. *Iranian Journal of Medical Microbiology*, *14*(5), 460-477. https://doi.org/10.30699/ijmm.14.5.460

6. Badr, G., Hozzein, W. N., Badr, B. M., Al Ghamdi, A., Saad Eldien, H. M., & Garraud, O. (2016). Bee venom accelerates wound healing in diabetic mice by suppressing activating transcription factor-3 (ATF-3) and inducible nitric oxide synthase (iNOS)-mediated oxidative stress and recruiting bone marrow-derived endothelial progenitor cells. *Journal of Cellular Physiology*, *231*(10), 2159-2171. https://doi.org/10.1002/jcp.25328
7. Baek, Y. H., Huh, J. E., Lee, J. D., & Park, D. S. (2006). Antinociceptive effect and the mechanism of bee venom acupuncture (Apipuncture) on inflammatory pain in the rat model of collagen-induced arthritis: Mediation by α2-Adrenoceptors. *Brain Research*, *1073*, 305-310. https://doi.org/10.1016/j.brainres.2005.12.086
8. Behroozi, J., Divsalar, A., & Saboury, A. A. (2014). Honey bee venom decreases the complications of diabetes by preventing hemoglobin glycation. *Journal of Molecular Liquids*, *199*, 371-375. https://doi.org/10.1016/j.molliq.2014.09.034
9. Bellik, Y. (2015). Bee venom: its potential use in alternative medicine. *Anti-infective Agents*, *13*(1), 3-16. https://doi.org/10.2174/2211352513666150318234624
10. Cai, M., Choi, S. M., & Yang, E. J. (2015). The effects of bee venom acupuncture on the central nervous system and muscle in an animal hSOD1G93A mutant. *Toxins*, *7*(3), 846-858. https://doi.org/10.3390/toxins7030846
11. Chan, W., He, B., Wang, X., & He, M. L. (2020). Pandemic COVID-19: Current status and challenges of antiviral therapies. *Genes & Diseases*, *7*(4), 502-519. https://doi.org/https://doi.org/10.1016/j.gendis.2020.07.001
12. Chen, M., Aoki Utsubo, C., Kameoka, M., Deng, L., Terada, Y., Kamitani, W., Sato, K., Koyanagi, Y., Hijikata, M., & Shindo, K. (2017). Broad-spectrum antiviral agents: secreted phospholipase A2 targets viral envelope lipid bilayers derived from the endoplasmic reticulum membrane. *Scientific Reports*, *7*(1), 1-8. https://doi.org/10.1038/s41598-017-16130-w
13. Cheng, B., Thapa, B., Remant, K., & Xu, P. (2015). Dual secured nano-melittin for the safe and effective eradication of cancer cells. *Journal of Materials Chemistry B*, *3*(1), 25-29. https://doi.org/ 10.1039/c4tb01401d
14. Cheng, Y., & Ren, X. (2004). Arrhythmia by bee sting acupuncture. *Journal of Clinical Acupuncture and Moxibustion*, *20*, 54.
15. Cherbuliez, T. (2013). Apitherapy–the use of honeybee products. In *Biotherapy-History, Principles and Practice* (pp. 113-146). Springer.
16. Cherniack, E. P., & Govorushko, S. (2018). To bee or not to bee: the potential efficacy and safety of bee venom acupuncture in humans. *Toxicon*, *154*, 74-78. https://doi.org/10.1016/j.toxicon.2018.09.013
17. Choi, M. S., Park, S., Choi, T., Lee, G., Haam, K. K., Hong, M. C., Min, B. I., & Bae, H. (2013). Bee venom ameliorates ovalbumin induced allergic asthma *via* modulating CD4+ CD25+ regulatory T cells in mice. *Cytokine*, *61*(1), 256-265. https://doi.org/10.1016/j.cyto.2012.10.005
18. Chu, S.-T., Cheng, H.-H., Huang, C.-J., Chang, H.-C., Chi, C.-C., Su, H.-H., Hsu, S.-S., Wang, J.-L., Chen, I.-S., & Liu, S.-I. (2007). Phospholipase A2-independent Ca^{2+} entry and subsequent apoptosis induced by melittin in human MG63 osteosarcoma cells. *Life Sciences*, *80*(4), 364-369. https://doi.org/10.1016/j.lfs.2006.09.024
19. De Graaf, D. C., Brochetto Braga, M. R., de Abreu, R. M. M., Blank, S., Bridts, C. H., De Clerck, L. S., Devreese, B., Ebo, D. G., Ferris, T. J., & Hagendorens, M. M. (2021). Standard methods for *Apis mellifera* venom research. *Journal of Apicultural Research*, 1-31. https://doi.org/10.1080/00218839.2020.1801073
20. Dudler, T., Chen, W. Q., Wang, S., Schneider, T., Annand, R. R., Dempcy, R. O., Crameri, R., Gmachl, M., Suter, M., & Gelb, M. H. (1992). High-level expression in *Escherichia coli* and rapid purification of enzymatically active honey bee venom phospholipase A2. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, *1165*(2), 201-210. https://doi.org/10.1016/0005-2760(92)90188-2
21. Fenard, D., Lambeau, G., Valentin, E., Lefebvre, J. C., Lazdunski, M., & Doglio, A. (1999). Secreted phospholipases A 2, a new class of HIV inhibitors that block virus entry into host cells. *The Journal of Clinical Investigation*, *104*(5), 611-618. https://doi.org/10.1172/JCI6915
22. Flanjak, I., Primorac, L., Stokanović, M. C., Puškadija, Z., Rajs, B. B., & Kovačić, M. (2021). Melittin stability in honey bee venom under different storage conditions measured with RP-HPLC-PDA method. *Fisheries, Game Management and Beekeeping*.
23. Guo, S. a., & DiPietro, L. A. (2010). Factors affecting wound healing. *Journal of Dental Research*, *89*(3), 219-229. https://doi.org/10.1177/0022034509359125

24. Han, H. J., Lee, J. H., Park, S. H., Choi, H. J., Yang, I. S., Mar, W. C., Kang, S. K., & Lee, H. J. (2000). Effect of bee venom and its melittin on apical transporters of renal proximal tubule cells. *Kidney and Blood Pressure Research*, *23*(6), 393-399. https://doi.org/10.1159/000025988
25. Han, S. M., Hong, I. P., Woo, S. O., Chun, S. N., Park, K. K., Nicholls, Y. M., & Pak, S. C. (2015). The beneficial effects of honeybee-venom serum on facial wrinkles in humans. *Clinical Interventions in Aging*, *10*, 1587. https://doi.org/10.2147/CIA.S84940
26. Han, S. M., Pak, S. C., Nicholls, Y. M., & Macfarlane, N. (2016). Evaluation of anti-acne property of purified bee venom serum in humans. *Journal of Cosmetic Dermatology*, *15*(4), 324-329. https://doi.org/10.1111/jocd.12227
27. Han, S. M., Park, K. K., Nicholls, Y. M., Macfarlane, N., & Duncan, G. (2013). Effects of honeybee (*Apis mellifera*) venom on keratinocyte migration *in vitro*. *Pharmacognosy Magazine*, *9*(35), 220. https://doi.org/10.4103/0973-1296.113271
28. Hong, S. J., Rim, G. S., Yang, H. I., Yin, C. S., Koh, H. G., Jang, M. H., Kim, C. J., Choe, B. K., & Chung, J. H. (2005). Bee venom induces apoptosis through caspase-3 activation in synovial fibroblasts of patients with rheumatoid arthritis. *Toxicon*, *46*(1), 39-45. https://doi.org/10.1016/j.toxicon.2005.03.015
29. Hossen, M., Shapla, U. M., Gan, S. H., & Khalil, M. (2017). Impact of bee venom enzymes on diseases and immune responses. *Molecules*, *22*(1), 25. https://doi.org/10.3390/molecules22010025
30. Hozzein, W. N., Badr, G., Badr, B. M., Allam, A., Al Ghamdi, A., Al-Wadaan, M. A., & Al-Waili, N. S. (2018). Bee venom improves diabetic wound healing by protecting functional macrophages from apoptosis and enhancing Nrf2, Ang-1 and Tie-2 signaling. *Molecular Immunology*, *103*, 322-335. https://doi.org/10.1016/j.molimm.2018.10.016
31. Jo, M., Park, M. H., Kollipara, P. S., An, B. J., Song, H. S., Han, S. B., Kim, J. H., Song, M. J., & Hong, J. T. (2012). Anti-cancer effect of bee venom toxin and melittin in ovarian cancer cells through induction of death receptors and inhibition of JAK2/STAT3 pathway. *Toxicology and Applied Pharmacology*, *258*(1), 72-81. https://doi.org/10.1016/j.taap.2011.10.009
32. Kang, M.-S., Byun, I.-j., Lee, S.-N., & Kim, K.-H. (2003). A study on the stability of diluted bee venom solution. *Journal of Pharmacopuncture*, *6*(2), 105-111. https://doi.org/10.3831/KPI.2003.6.2.105
33. Kang, S.-Y., Kim, C.-Y., Roh, D.-H., Yoon, S.-Y., Park, J.-H., Lee, H.-J., Beitz, A. J., & Lee, J.-H. (2011). Chemical stimulation of the ST36 acupoint reduces both formalin-induced nociceptive behaviors and spinal astrocyte activation via spinal alpha-2 adrenoceptors. *Brain Research Bulletin*, *86*(5-6), 412-421. https://doi.org/10.1016/j.brainresbull.2011.08.012
34. Kasozi, K. I., Niedbała, G., Alqarni, M., Zirintunda, G., Ssempijja, F., Musinguzi, S. P., Usman, I. M., Matama, K., Hetta, H. F., Mbiydzenyuy, N. E., Batiha, G. E. S., Beshbishy, A. M., & Welburn, S. C. (2020). Bee venom—a potential complementary medicine candidate for SARS-CoV-2 infections. *Frontiers in Public Health*, *8*. https://doi.org/10.3389/fpubh.2020.594458
35. Kemeny, D. M., Dalton, N., Lawrence, A. J., Pearce, F. L., & Vernon, C. A. (1984). The purification and characterisation of hyaluronidase from the venom of the honey bee, *Apis mellifera*. *European Journal of Biochemistry*, *139*(2), 217-223. https://doi.org/10.1111/j.1432-1033.1984.tb07997.x.
36. Kharroubi, A. T., & Darwish, H. M. (2015). Diabetes mellitus: The epidemic of the century. *World Journal of Diabetes*, *6*(6), 850. https://doi.org/10.4239/wjd.v6.i6.850
37. Kim, H.-W., Kwon, Y.-B., Ham, T.-W., Roh, D.-H., Yoon, S.-Y., Lee, H.-J., Han, H.-J., Yang, I.-S., Beitz, A. J., & Lee, J.-H. (2003). Acupoint stimulation using bee venom attenuates formalin-induced pain behavior and spinal cord fos expression in rats. *Journal of Veterinary Medical Science*, *65*(3), 349-355. https://doi.org/10.1292/jvms.65.349
38. Kim, H. W., Kwon, Y. B., Han, H. J., Yang, I. S., Beitz, A. J., & Lee, J. H. (2005). Antinociceptive mechanisms associated with diluted bee venom acupuncture (apipuncture) in the rat formalin test: involvement of descending adrenergic and serotonergic pathways. *Pharmacological Research*, *51*(2), 183-188. https://doi.org/10.1016/j.phrs.2004.07.011
39. Kim, H. Y., Jo, M. J., Nam, S. Y., Kim, K. M., Choi, M. B., & Lee, Y. H. (2020). Evaluating the effects of honey bee (*Apis mellifera* L.) venom on the expression of insulin sensitivity and inflammation-related genes in co-culture of adipocytes and macrophages. *Entomological Research*, *50*(5), 236-244. https://doi.org/10.1111/1748-5967.12431
40. Kim, J., Ochoa, M. T., Krutzik, S. R., Takeuchi, O., Uematsu, S., Legaspi, A. J., Brightbill, H. D., Holland, D., Cunliffe, W. J., & Akira, S. (2002). Activation of toll-like receptor 2 in acne triggers inflammatory cytokine responses. *The Journal of Immunology*, *169*(3), 1535-1541. https://doi.org/10.4049/jimmunol.169.3.1535
41. Kim, J. Y., Leem, J., & Park, K. K. (2020). Antioxidative, antiapoptotic, and anti-inflammatory effects of apamin in a murine

model of lipopolysaccharide-induced acute kidney injury. *Molecules*, *25*(23), 5717. https://doi.org/10.3390/molecules25235717

42. Ko, S. H., Oh, H. M., Kwon, D. Y., Yang, J. E., Kim, B. J., Ha, H. J., Lim, E. J., Oh, M. S., Son, C. G., & Lee, E. J. (2022). Incidence rate of bee venom acupuncture related anaphylaxis: a systematic review. *Toxins*, *14*(4), 238. https://doi.org/10.3390/toxins14040238
43. Kurek Górecka, A., Komosinska Vassev, K., Rzepecka Stojko, A., & Olczyk, P. (2020). Bee venom in wound healing. *Molecules*, *26*(1), 148. https://doi.org/10.3390/molecules26010148
44. Kwon, Y.-B., Kang, M.-S., Han, H.-J., Beitz, A. J., & Lee, J.-H. (2001). Visceral antinociception produced by bee venom stimulation of the Zhongwan acupuncture point in mice: role of α2 adrenoceptors. *Neuroscience Letters*, *308*(2), 133-137. https://doi.org/10.1016/s0304-3940(01)01989-9
45. Lee, E. J., Ahn, Y. C., Kim, Y. I., Oh, M. S., Park, Y. C., & Son, C. G. (2020). Incidence rate of hypersensitivity reactions to bee-venom acupuncture. *Frontiers in Pharmacology*, 1575. https://doi.org/10.3389/fphar.2020.545555
46. Lee, G., & Bae, H. (2016). Anti-inflammatory applications of melittin, a major component of bee venom: detailed mechanism of action and adverse effects. *Molecules*, *21*(5), 616. https://doi.org/10.3390/molecules21050616
47. Lee, H., Lee, E. j., Kim, H., Lee, G., Um, E. J., Kim, Y., Lee, B. Y., & Bae, H. (2011). Bee venom-associated Th1/Th2 immunoglobulin class switching results in immune tolerance of NZB/W F1 murine lupus nephritis. *American Journal of Nephrology*, *34*(2), 163-172. https://doi.org/10.1159/000329731
48. Lee, W. R., Kim, K. H., An, H. J., Kim, J. y., Chang, Y. C., Chung, H., Park, Y. Y., Lee, M. L., & Park, K. k. (2014). The protective effects of melittin on *Propionibacterium acnes*–induced inflammatory responses *in vitro* and *in vivo*. *Journal of Investigative Dermatology*, *134*(7), 1922-1930. https://doi.org/10.1038/jid.2014.75
49. Lee, W. R., Park, J. H., Kim, K. H., Park, Y. Y., Han, S. M., & Park, K. k. (2011). Protective effects of melittin on transforming growth factor-β1 injury to hepatocytes *via* anti-apoptotic mechanism. *Toxicology and Applied Pharmacology*, *256*(2), 209-215. https://doi.org/10.1016/j.taap.2011.08.012
50. Li, B., Gu, W., Zhang, C., Huang, X. Q., Han, K. Q., & Ling, C. Q. (2006). Growth arrest and apoptosis of the human hepatocellular carcinoma cell line BEL-7402 induced by melittin. *Oncology Research and Treatment*, *29*(8-9), 367-371. https://doi.org/10.1159/000094711
51. Lyu, C., Fang, F., & Li, B. (2019). Anti-tumor effects of melittin and its potential applications in clinic. *Current Protein and Peptide Science*, *20*(3), 240-250. https://doi.org/10.2174/1389203719666180612084615
52. Männle, H., Hübner, J., & Münstedt, K. (2020). Beekeepers who tolerate bee stings are not protected against SARS-CoV-2 infections. *Toxicon*, *187*, 279-284. https://doi.org/10.1016/j.toxicon.2020.10.004
53. Mesa-Arango, A. C., Flórez-Muñoz, S. V., & Sanclemente, G. (2017). Mechanisms of skin aging. *Latreia*, *30*(2), 160-170.
54. Moreno, M., & Giralt, E. (2015). Three valuable peptides from bee and wasp venoms for therapeutic and biotechnological use: melittin, apamin and mastoparan. *Toxins*, *7*(4), 1126-1150. https://doi.org/10.3390/toxins7041126
55. Musarra Pizzo, M., Pennisi, R., Ben Amor, I., Mandalari, G., & Sciortino, M. T. (2021). Antiviral activity exerted by natural products against human viruses. *Viruses*, *13*(5), 828. https://doi.org/https://doi.org/10.3390/v13050828
56. Nielsen, V. G. (2020). The anticoagulant effect of *Apis mellifera* phospholipase A2 is inhibited by CORM-2 *via* a carbon monoxide-independent mechanism. *Journal of Thrombosis and Thrombolysis*, *49*(1), 100-107. https://doi.org/10.1007/s11239-019-01980-0
57. Nipate, S., Hurali, P. B., & Ghaisas, M. (2015). Evaluation of anti-inflammatory, anti-nociceptive, and anti-arthritic activities of Indian *Apis dorsata* bee venom in experimental animals: biochemical, histological, and radiological assessment. *Immunopharmacology and Immunotoxicology*, *37*(2), 171-184. https://doi.org/10.3109/08923973.2015.1009996
58. Park, M. H., Choi, M. S., Kwak, D. H., Oh, K. W., Yoon, D. Y., Han, S. B., Song, H. S., Song, M. J., & Hong, J. T. (2011). Anti-cancer effect of bee venom in prostate cancer cells through activation of caspase pathway *via* inactivation of NF-κB. *The Prostate*, *71*(8), 801-812. https://doi.org/10.1002/pros.21296
59. Park, S., Erdogan, S., Hwang, D., Hwang, S., Han, E. H., & Lim, Y.-H. (2016). Bee venom promotes hair growth in association with inhibiting 5α-reductase expression. *Biological and Pharmaceutical Bulletin*, b16-00158. https://doi.org/10.1248/bpb.b16-00158
60. Pattabhiramaiah, M., Ramesh, K., KV, V., & Muniswamy Reddy, S. (2020). Computational analysis of phospholipaseA2 in the honey bee venom. *Journal of Apicultural Research*, *59*(4), 706-721. https://doi.org/10.1080/00218839.2020.1754589

61. Patterson, R. A., & Stankewicz, H. A. (2021). Penicillin allergy. In *StatPearls [Internet]*. StatPearls Publishing.
62. Pawlak, M., Klupczynska, A., Kokot, Z. J., & Matysiak, J. (2020). Extending metabolomic studies of *Apis mellifera* venom: LC-MS-based targeted analysis of organic acids. *Toxins*, *12*(1), 14. https://doi.org/10.3390/toxins12010014
63. Picoli, T., Peter, C. M., Vargas, G. D., Hübner, S. O., Lima, M. d., & Fischer, G. (2018). Antiviral and virucidal potential of melittin and apamin against bovine herpesvirus type 1 and bovine viral diarrhea virus. *Pesquisa Veterinária Brasileira*, *38*, 595-604. https://doi.org/10.1590/1678-5150-PVB-4758
64. Rady, I., Siddiqui, I. A., Rady, M., & Mukhtar, H. (2017). Melittin, a major peptide component of bee venom, and its conjugates in cancer therapy. *Cancer Letters*, *402*, 16-31. https://doi.org/10.1016/j.canlet.2017.05.010
65. Rakha, M., Tawfiq, R. A., Sadek, M., Anwer, M., Salama, S., Mohamed, A., El-Hendy, M., El-Said, S. E., Ahmed, N., & Mekawi, K. (2018). Neurotherapeutic effects of bee venom in a rotenone-induced mouse model of Parkinson's disease. *Neurophysiology*, *50*(6), 445-455. https://doi.org/10.1007/s11062-019-09777-w
66. Sarhan, M., El-Bitar, A. M., & Hotta, H. (2020). Potent virucidal activity of honeybee "*Apis mellifera*" venom against Hepatitis C Virus. *Toxicon*, *188*, 55-64. https://doi.org/10.1016/j.toxicon.2020.10.014
67. Sen, S., Kar, M., Roy, A., & Chakraborti, A. S. (2005). Effect of nonenzymatic glycation on functional and structural properties of hemoglobin. *Biophysical Chemistry*, *113*(3), 289-298. https://doi.org/10.1016/j.bpc.2004.05.005
68. Seo, B. K., Han, K., Kwon, O., Jo, D. J., & Lee, J. H. (2017). Efficacy of bee venom acupuncture for chronic low back pain: a randomized, double-blinded, sham-controlled trial. *Toxins*, *9*(11), 361. https://doi.org/10.3390/toxins9110361
69. Serrinha, V., Correia, S. D., & Marques, G. (2019). Productivity and economic analysis of a new intensive collector in the Portuguese market with implication of open innovation perspective. *Journal of Open Innovation: Technology, Market, and Complexity*, *5*(3), 71. https://doi.org/10.3390/joitmc5030071
70. Shimpi, R., Chaudhari, P., Deshmukh, R., Devare, S., Bagad, Y., & Bhurat, M. (2016). A review: pharmacotherapeutics of bee venom. *The World Journal of Pharmaceutical Sciences*, *5*(7), 656-567.
71. Sığ, A. K., Güney, M., Özlem, Ö., & Hüseyin, Ş. (2019). Bee venom: a medical perspective. *Turkish Journal of Clinics and Laboratory*, *10*(3), 414-421. https://doi.org/10.18663/tjcl.451586
72. Soman, N. R., Baldwin, S. L., Hu, G., Marsh, J. N., Lanza, G. M., Heuser, J. E., Arbeit, J. M., Wickline, S. A., & Schlesinger, P. H. (2009). Molecularly targeted nanocarriers deliver the cytolytic peptide melittin specifically to tumor cells in mice, reducing tumor growth. *The Journal of Clinical Investigation*, *119*(9), 2830-2842. https://doi.org/10.1172/JCI38842
73. Son, D. J., Lee, J. W., Lee, Y. H., Song, H. S., Lee, C. K., & Hong, J. T. (2007). Therapeutic application of anti-arthritis, pain-releasing, and anti-cancer effects of bee venom and its constituent compounds. *Pharmacology & Therapeutics*, *115*(2), 246-270. https://doi.org/10.1016/j.pharmthera.2007.04.004
74. Sung, S. H., Kim, J. W., Han, J. E., Shin, B. C., Park, J. K., & Lee, G. (2021). Animal venom for medical usage in pharmacopuncture in Korean medicine: current status and clinical implication. *Toxins*, *13*(2), 105. https://doi.org/10.3390/toxins13020105
75. Sung, S. H., & Lee, G. (2021). Bee venom acupuncture effects on pain and its mechanisms: an updated review. *Toxins*, *13*(9), 608. https://doi.org/10.3390/toxins13090608
76. Wehbe, R., Frangieh, J., Rima, M., El Obeid, D., Sabatier, J. M., & Fajloun, Z. (2019). Bee venom: overview of main compounds and bioactivities for therapeutic interests. *Molecules*, *24*(16), 2997. https://doi.org/10.3390/molecules24162997
77. Xing, L., Liping, X., Rongqing, Z., Dawei, C., & Lan, S. (2003). A sensitive enzyme-linked immunosorbent assay for evaluating the concentration of bee venom in rat plasma. *Journal of Pharmacy and Pharmacology*, *55*(10), 1359-1363. https://doi.org/10.1211/0022357022025
78. Yang, E. J., Jiang, J. H., Lee, S. M., Yang, S. C., Hwang, H. S., Lee, M. S., & Choi, S.-M. (2010). Bee venom attenuates neuroinflammatory events and extends survival in amyotrophic lateral sclerosis models. *Journal of Neuroinflammation*, *7*(1), 1-12. https://doi.org/10.1186/1742-2094-7-69
79. Yang, W., Hu, F. l., & Xu, X. f. (2020). Bee venom and SARS-CoV-2. *Toxicon*, *181*, 69. https://doi.org/10.1016/j.toxicon.2020.04.105
80. Yasin, B., Pang, M., Turner, J., Cho, Y., Dinh, N., Waring, A., Lehrer, R., & Wagar, E. (2000). Evaluation of the inactivation of infectious *Herpes simplex* virus by host-defense peptides. *European Journal of Clinical Microbiology and Infectious Diseases*, *19*(3), 187-194. https://doi.org/10.1007/s100960050457
81. Ye, M., Chung, H.-S., Lee, C., Yoon, M. S., Yu, A. R., Kim, J. S., Hwang, D.-S., Shim, I., & Bae, H. (2016). Neuroprotective effects of bee venom phospholipase A2 in the 3xTg AD mouse model of Alzheimer's disease. *Journal of*

Neuroinflammation, *13*(1), 1-12. https://doi.org/10.1186/s12974-016-0476-z

82. Yong, W. H., Wyman, S., & Levy, J. A. (1990). Optimal conditions for synthesizing complementary DNA in the HIV-1 endogenous reverse transcriptase reaction. *AIDS 4*(3), 199-206. https://doi.org/10.1097/00002030-199003000-00004
83. Zhang, J., Shi, D., Wang, L., Liu, R., & Zhang, L. (1995). Investigation of anaphylaxis by bee sting acupuncture in 9 case. *Shanghai Journal of Acupuncture and Moxibustion*, *3*, 126.