e-ISSN: 2621 - 4814

Borneo Journal of PHARMACY

Volume 5 Issue 3 August 2022

Accredited at SINTA 2 until February 2025
by Ministry of Research and Technology / National Research and Innovation Agency, Indonesia
No: 148/M/KPT/2020.

Institute for Research and Community Services
Universitas Muhammadiyah Palangkaraya

BORNEO JOURNAL OF PHARMACY

Borneo J Pharm
e-ISSN: 2621-4814

Volume 5 Issue 3 August 2022

Department of Pharmacy
Faculty of Health Sciences
Universitas Muhammadiyah Palangkaraya

PUBLISHED BY

Institute for Research and Community Services
Universitas Muhammadiyah Palangkaraya
RTA Milono St. Km. 1,5 Palangka Raya 73111
bjop@umpr.ac.id
http://journal.umpr.ac.id/index.php/bjop

EDITORIAL WORDS

apt. Mohammad Rizki Fadhil Pratama, S.Farm., M.Si.

Editor in Chief
Borneo J Pharm

Assalamu'alaikum Wr. Wb.

Alhamdulillahirabbil 'alamin. The next edition of **Borneo Journal of Pharmacy** (*Borneo J Pharm*), has been published at August 2022. This edition contains ten articles: Pharmacognosy-Phytochemistry, Pharmaceutical, Microbiology Pharmacy, Natural Product Development, Clinical-Community Pharmacy, and Management Pharmacy. This edition includes writings from four countries: Costa Rica, Indonesia, Netherlands, and Nigeria. The authors come from several institutions, including Universitas Ahmad Dahlan, K24 Indonesia, Institut Sains dan Teknologi Nasional, Universidad de Costa Rica, Kebbi State University of Science and Technology, Aliero, Federal University, Birnin Kebbi, Sekolah Tinggi Teknologi Industri dan Farmasi Bogor, IPB University, National Research and Innovation Agency Republic of Indonesia, Poltekkes Kemenkes Bandung, Sekolah Tinggi Ilmu Farmasi Riau, Universitas Islam Negeri Maulana Malik Ibrahim, Universitas Airlangga, Universitas Hang Tuah, Universitas Katolik Widya Mandira, University of Groningen, Universitas Gadjah Mada, Universitas Hasanuddin, Universitas Syiah Kuala, Poltekkes Kemenkes Maluku, and Universitas Islam Sultan Agung.

Editorial boards are fully aware that there are still room for improvement in this edition, hence with all humility willing to accept constructive suggestions and feedback for improvements to the publication for the next editions. The editorial board would like to thank all editors and reviewers, and contributors of the scientific articles who have provided the repetoire in this issue. We hope that all parties, especially the contributors, could re-participate for the publication in the next edition on November 2022.

Wassalamu'alaikum Wr. Wb.

Palangka Raya, August 2022

Editor-in-Chief

Table of Contents
BORNEO JOURNAL OF PHARMACY
Borneo J Pharm – e-ISSN: 2621-4814

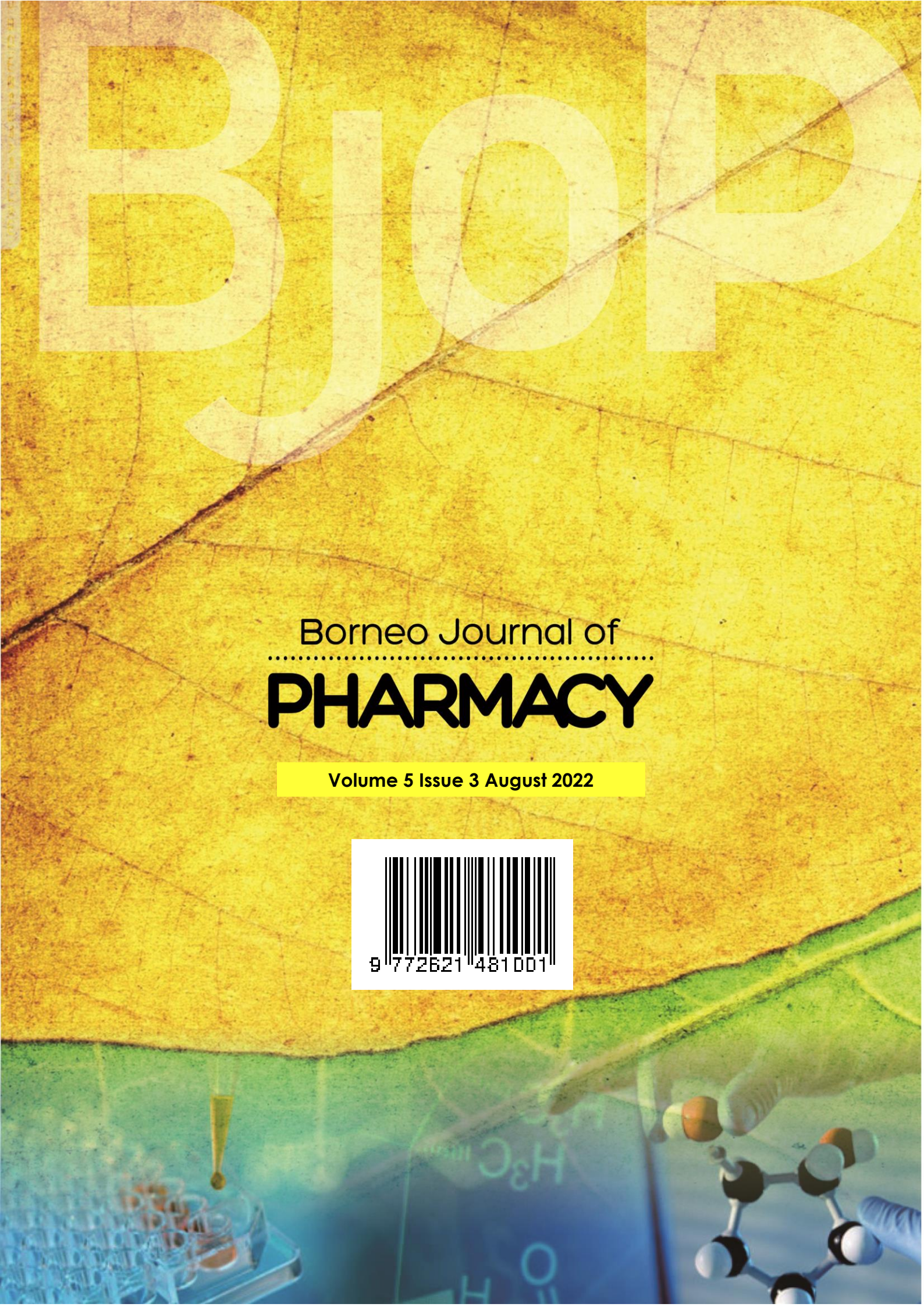
Volume 5 Issue 3 August 2022

Author Guidelines

BORNEO JOURNAL OF PHARMACY

Borneo J Pharm – e-ISSN: 2621-4814

1. Writing is the result of research or review of critical analysis study in the field but not limited to Pharmacology-Toxicology, Pharmacognosy-Phytochemistry, Pharmaceutical, Analytical Pharmacy-Medicinal Chemistry, Natural Product Development, Clinical-Community Pharmacy, Management Pharmacy, and other Pharmacy aspects which have never been published in other scientific publication media.
2. Manuscripts that have been published in the form of a preprint on several platforms such as arXiv, INA-RXiv, SSRN, and ResearchGate are welcome to publish.
3. The manuscript is written in English with Book Antiqua font 10 pt, two-column, density 1.15 spaces, on A4 paper with a top-down margin of 2.5 cm and right-left margins 1.5 cm.
4. The manuscript is typed with MS-Word program using the provided template and saved in .doc or .docx format.
5. The manuscript is typed strictly with the following rules:
   a. The title, short and clear, written in English. The title length does not exceed 20 words.
   b. Authors, listed without mention of title or degree, under the name including the affiliation and complete address, and email of the corresponding author. The author's name, which consists of one word, must be repeated twice for first and last names. To author with names of more than two words, the author's last name consists of only one word, the rest is written as the first name.
   c. Abstract, written in English which is a summary of the article. The abstract is created in one paragraph and a maximum of 250 words with Book Antiqua font 9 pt, single column, and space 1. Points that must be listed in the abstract include the research objectives, methods, results, conclusions, and keywords. The number of keywords is between three and five keywords.
   d. Introduction, contain background, reasons for the importance of underlying research or hypotheses, general approaches and objectives of the research and relevant literature review. The number of paragraphs not restricted but the contents must include background, objectives, positions on previous research, as well as the novelty of the research. The little theoretical basis which indirectly related to the contents of the article could be included without sub-chapters required. Citation is written in occurrence number on the manuscript (Vancouver style).
   e. Methodology, Research method consists of 2 sub-chapters including materials and methods. If data analysis is specific, or the way of analysis is novel, then data analysis subchapter could be added. The sub-chapters are written without numbering or bullet.
   f. Results and Discussion, at least contain three subjects (1) description of results, (2) interpretation or explanation of results, and (3) comparative of results with previous studies results. If the subchapters very length could be made sub-sub-chapters with Arabic numbering. Description of the results can be in the form of tables and figures with a serial number (Table uses a sequence of Roman numerals and placed at the top, while figures using Arabic numeric sequence and placed in the below).
   g. Conclusion, made in a single paragraph without the citation contains the final conclusion and suggestions for advanced research.
   h. References, a list of recently selected topics is published last five years (the minimum percentage and a minimum number of references are different for each type of manuscript). The bibliography is written in chronological order with the serial number (Vancouver style) and suggested using a reference management application such as EndNote, Mendeley, Zotero, and other applications.
6. Authors whose manuscripts are published are free of charge for both Publication cost and article processing charge.
7. Authors will get full access to the article published on e-journal of Universitas Muhammadiyah Palangkaraya at the website http://journal.umpr.ac.id/index.php/bjop.


BJOP
Borneo Journal of
PHARMACY
Volume 5 Issue 3 August 2022
9 772621 481001

Borneo Journal of Pharmacy
Vol 5 Issue 3 August 2022
Page 194 – 201

http://journal.umpalangkaraya.ac.id/index.php/bjop/article/view/3221
DOI: https://doi.org/10.33084/bjop.v5i3.3221
e-ISSN: 2621-4814

Research Article

Determination of the Active Chemical Compounds and the Antibacterial Activity of Various Fractions of *Lawsonia inermis* L.

Sri Mulyaningsih [1*]

Febriyati Adji Rachmadani [2]

[1] Department of Pharmacy, Universitas Ahmad Dahlan, Yogyakarta, Special Region of Yogyakarta, Indonesia

[2] K24 Indonesia, Yogyakarta, Special Region of Yogyakarta, Indonesia

*email: sri.mulyaningsih@pharm.uad.ac.id

Keywords:
Ethyl acetate
Lawsonia inermis L
Naphthoquinones
Staphylococcus aureus
TLC-bioautography

Abstract

Lawsonia inermis L., or henna leaves, are usually used to treat wounds on the skin. *Lawsonia inermis* contain naphthoquinones, flavonoids, tannins, and saponins. The antibacterial activity of *L. inermis* leaf extract in various solvents has been extensively studied. However, which component is responsible for the antibacterial activity is still unknown. This study was intended to investigate the antibacterial effect of *L. inermis* fractions against *Staphylococcus aureus* and to discover the antibacterial chemical class in the most active fraction. The methanol extract was fractionated with *n*-hexane and ethyl acetate subsequently. The antibacterial activity of various fractions was tested using the well diffusion method. TLC-bioautography was used to identify the class of active chemicals as antibacterial agents. Antibacterial activity against *S. aureus* was highest in the ethyl acetate fraction. TLC-bioautography of the ethyl acetate fraction showed inhibition areas at Rf values of 0.25 and 0.53, respectively, indicating the naphthoquinones and phenolic compounds groups. In conclusion, naphthoquinones and phenolic compounds are suggested to contribute to the antibacterial effect of the ethyl acetate fraction of *L. inermis* leaves.

Received: January 31th, 2022
1st Revised: May 7th, 2022
2nd Revised: July 19th, 2022
Accepted: July 20th, 2022
Published: August 31th, 2022

INTRODUCTION

Lawsonia inermis, or known as *henna* (English name), has local names such as *hennastrauch* (Germany), *hena/mendhi* (Pakistan, India), and *inai/pacar kuku* (Indonesia, Malaysia). It is one of the familiar plants widely found in Asia, including in Indonesia[1]. Generally, the leaves are used by the community as a natural reddish brown dye for coloring nails, hair, and skin. The community often uses *L. inermis* leaves to treat wounds and skin inflammation[2,3].

Lawsonia inermis leaves contain large amounts of chemical compounds such as lawsone, flavonoids, tannins, coumarins, sterols, and terpenoids[4]. According to the phytochemical analysis[5], all of the extracts contained naphthoquinones, saponins, flavonoids, and steroids. Lawsone (2-hydroxy-1,4-naphthoquinone), a kind of naphthoquinone, has been identified as the major component in *L. inermis*[6,7].

Many studies have investigated the antimicrobial activity of *L. inermis* leaves extract in various solvents. Usman and Rabiu[5] reported that the aqueous extract of *L. inermis* leaves inhibited *Staphylococcus aureus* and *Epidermophyton floccosum*. The *L. inermis* extract inhibited some microbial isolates at 1000 µg/mL concentrations. The most significant antimicrobial activity of methanol, ethanol, and aqueous *L. inermis* extract against some human pathogenic bacteria, and some fungi were possessed by methanol and ethanolic extracts[8,9]. However, many investigations reported that methanol extract exhibited promising antibacterial activity against some pathogenic bacteria from clinical isolated[10-12]. Leaves extract of *L. inermis* has also been reported to possess good biofilm inhibition and antibacterial activity, which can be explored to develop new drugs for MDR pathogens[13].

How to cite: Mulyaningsih S, Rachmadani FA. Determination of the Active Chemical Compounds and the Antibacterial Activity of Various Fractions of Lawsonia inermis L. Borneo J Pharm. 2022;5(3):194-201. doi:10.33084/bjop.v5i3.3221

Although *L. inermis* was reported to have acted as an antibacterial agent, the information on which compound is responsible for the antibacterial activity is still unclear. In this study, the methanol extract of *L. inermis* leaves was fractionated with *n*-hexane and ethyl acetate to obtain *n*-hexane, ethyl acetate, and methanol fractions. The purpose of the fractionation is to separate the compounds in the extract into the solvent according to their polarity. Non-polar compounds were screened out with *n*-hexane, semi-polar compounds were sorted out with ethyl acetate, and polar compounds were taken with methanol[14]. The three fractions were then tested against *S. aureus* and continued with TLC-bioautography. This study's objective was to comprehend and determine the antibacterial activity of the active fraction of *L. inermis* leaves and the class of active compounds as an antibacterial agent from the most active fraction against *S. aureus*.

MATERIALS AND METHODS

Materials

Plant materials were obtained from Merapi Farma Herbal Yogyakarta, and the identification of the plant was carried out by the Biology Laboratory, Faculty of Science and Applied Technology, Universitas Ahmad Dahlan, Yogyakarta, with the voucher specimen number 087/Lab.bio/B/VI/2019. *Staphylococcus aureus* ATCC 25923, Mueller Hinton Agar (Oxoid), Brain Heart Infusion medium (Oxoid), 1% $BaCl_2$ (Merck), 1% H_2SO_4 (Merck), NaCl 0.9% (Merck), 1% Dimethylsulfoxide (Merck), Vancomycin 1% (Vancep), and silica plate GF_{254} (Merck). The instruments used were digital balance (Ohaus), oven (Binder), micropipettes (Soccorex), biosafety cabinet (Monmouth Scientific), incubator (Binder), autoclave (Shenan), TLC chamber (Camag), UV lamps, and glassware (Pyrex).

Methods

Preparation of methanol extract

A total of 2 kg of *L. inermis* leaves were washed and dried in the oven. The dried *L. inermis* leaves were then ground with a blender and sieved with a 50-mesh sieve. An amount of 250 g of *L. inermis* leaves powder was macerated with 1000 mL of methanol. The maceration process was carried out at room temperature for the first six hours while shaking, then allowed to stand for 18 hours. Remaceration was done in the same manner. Afterward, the macerate was filtrated using a Buchner funnel. The filtrate was then evaporated with a rotary evaporator until a thick extract was obtained[15].

Fractionation of methanol extract of L. inermis

A total of 15 g of *L. inermis* extract was subsequently dissolved in methanol and fractionated with *n*-hexane and ethyl acetate. Each *n*-hexane fraction and ethyl acetate fraction were evaporated with a rotary evaporator to get the *n*-hexane, ethyl acetate, and methanol fraction. Every fraction was weighed to give the yield of fractionation.

Antibacterial activity test against S. aureus

The well diffusion method was used to conduct the antibacterial activity test. A sterile cotton swab was used to apply the *S. aureus* bacterium suspension with a 1 x 10^8 CFU/mL density to the Mueller Hinton agar surface. Then the surface of the agar is perforated and dripped with test and control samples. Next, the plate was incubated at 37°C for 24 hours. After incubation, the diameter of the inhibition zones was measured.

Phytochemical compound testing of the ethyl acetate fraction by reaction test[16]

The test solution was made in a concentration of 1% w/v by dissolving 250 mg of the ethyl acetate fraction of L. inermis in 25 mL of distilled water. The test was carried out to determine the presence of naphthoquinone, flavonoid, tannin, and saponin compounds.

Naphthoquinone test

As much as 1 mL of the test solution was added to a few drops of 1 N NaOH, a positive solution containing naphthoquinone will show a red color.

Flavonoid test

The test solution was dropped on filter paper, then treated with ammonia vapor. If it causes a yellow color, it indicates the presence of flavonoids.

Tannin test

A positive solution containing naphthoquinone will show a red color when one mL of the test solution is added to a few drops of 1 N NaOH.

Saponin test

A total of 1 mL of the test solution was shaken vigorously for 10 seconds. If the foam is formed for not less than 10 minutes as high as 3-10 cm and by the addition of 2 N HCl the foam does not disappear, it is positive for saponins.

TLC-Bioautography

As the mobile phase, the ethyl acetate fraction of *L. inermis* leaves was separated using TLC with chloroform : acetone : formic acid (6 : 1.5 : 0.5). The surface medium of MH agar was sprayed with a bacterial suspension evenly. After that, the silica gel plate was placed on the surface of the MHA agar medium in an inverted position and left for 30 minutes to allow diffusion based on the reference with minor modifications[17]. Then the plate was removed, and the petri dish was incubated at 37°C for 24 hours. After incubation, the inhibition zone was observed. The inhibition zone that appears was measured by the Rf value and compared with the chromatogram detected with spraying reagents to determine the group of active compounds.

RESULTS AND DISCUSSION

Yield of extraction and fractionation of L. inermis leaves

The maceration of *L. inermis* leaves obtained 47.96 g of methanol extract with a yield of 19.18%. In the study by Sharma and Goel[18], the yield of methanol extract of *L. inermis* nails was 17%. The extraction yield we obtained is greater than that of the previous study due to several factors, such as geographical conditions, sampling time, or other factors.

The fractionation using *n*-hexane obtained a *n*-hexane fraction of 1.4610 g with a yield of 9.74%. The *n*-hexane fraction had a greenish color due to the presence of chlorophyll. With a yield of 25.55%, the ethyl acetate fraction displayed a reddish-brown color of 3.8333 g. The residue in the form of methanol fraction is 8.0585 g with a yield of 53.72%.

Antibacterial activity of the fractions of L. inermis against S. aureus

The antibacterial activity test was performed on MHA media seeded with *S. aureus* using the well diffusion method. **Table I** reveals that the inhibitory zone diameter of the methanol extract from *L. inermis* leaves is 11.33 mm. The *n*-hexane, ethyl acetate, and methanol fractions result in 8.33 mm, 9.50 mm, and 0 mm, respectively. Remarkably, the methanol extract had the most significant inhibitory zone, though the methanol fraction had none. According to Nwodo *et al*.[19], fractionation occasionally led to increased activity but occasionally led to decreased activity. This represents a situation where fractionation leads to loss of activity, suggesting that components of the extract may have acted synergistically or additively to produce the activity observed in the extract. Another study found that some fractions of *Tamarindus indica* showed no activity against *type P. aeruginosa* and *E. coli* strains, unlike the extract.

The antibacterial activity of the methanol extract was greater compared to each fraction. The highest antibacterial activity was confirmed in crude methanol extract, possibly due to all the antibacterial compounds in its fractions[20]. These results indicate that the active chemical compounds as antibacterial agents were spread into these fractions and were not collected in one certain fraction. Previous studies showed that the chemical compounds contained in plants provide a synergistic or additive effect in causing pharmacological effects[21]. If these compounds are separated, it will cause a decrease in their pharmacological activity. However, the ethyl acetate fraction had the greatest antibacterial activity compared to the other fractions. The positive control in the antibacterial activity test was vancomycin because it is sensitive to *S. aureus*. As a

negative control, 1% DMSO was utilized to dissolve practically all polar and non-polar substances. There was no bactericidal action in 1% DMSO. **Table I** shows the diameter of the inhibitory zones of the methanol extract and the fractions.

Table I. Diameter of inhibition zones of methanol extract and various fractions against *S. aureus* using well diffusion method

Sample tested (w/v)	Diameter of inhibition zone (mean± SD in mm)
Methanol extract 10%	11.33 ± 0.29
n-hexane 10%	8.33 ± 0.52
Ethyl acetate 10%	9.50 ± 0.87
Methanol fraction 10%	0.00 ± 0.00
Vancomycin 1%	21.00 ± 0.00
DMSO 1%	0.00 ± 0.00

Furthermore, the antibacterial activity of the ethyl acetate fraction of *L. inermis* leaves was tested at different concentrations to determine the concentration that could inhibit the growth of bacteria. The concentrations of ethyl acetate fraction tested were 5, 15, and 20 %w/v. **Table II** shows that the diameter of the inhibition zone increased as the concentration of the ethyl acetate fraction was raised. The 20% ethyl acetate fraction produced the largest inhibition diameter of 10.67 mm. Statistical analysis with the Kruskal-Wallis test showed that there were differences in the antibacterial activity of each concentration tested.

Table II. Diameter of inhibition zones of the ethyl acetate fraction against *S. aureus* using well diffusion method

Concentration of ethyl acetate fraction (w/v)	Diameter of inhibition zone (mean ± SD in mm)
Vancomycin 1%	21.00 ± 0.00
DMSO 1%	0.00 ± 0.00
5%	8.25 ± 0.43
15%	9.67 ± 0.72
20%	10.67 ± 0.80

The phytochemical content of ethyl acetate fraction of L. inermis leaves

The results of the phytochemical screening test (**Table III**) show that the ethyl acetate fraction of *L. inermis* leaves contains naphthoquinones, flavonoids, and tannins. In the naphthoquinone test, when the ethyl acetate fraction of *L. inermis* leaves was dripped with 1 N NaOH solution, the color changed to brownish red due to the presence of a chromophore group in the ethyl acetate fraction of *L. inermis* leaves so that the addition of a hydroxyl group from NaOH will give a red color[6,15]. When testing for flavonoids, a more intense yellow color appears on filter paper that has been treated with ammonia vapor, indicating the presence of flavonoid[22].

The test on tannin compounds, when added to the gelatin solution in the ethyl acetate fraction of *L. inermis* leaves, forms a precipitate due to the nature of the tannins, which can precipitate protein so that the tannin test with the addition of gelatin solution, which is a protein will be precipitated by the tannins[23]. In the saponin test, our finding showed that within 10 minutes, the foam slowly disappeared when HCl was added, indicating that the ethyl acetate fraction of *L. inermis* leaves did not contain saponin[16].

Table III. The phytochemical screening of ethyl acetate fraction of *L. inermis*

Reaction test	Result	Presence
Naphthoquinone	Brownish red	Present
Flavonoid	Yellow	Present
Tannin	Brown precipitate	Present
Saponin	No foam	Absent

TLC-Bioautograaby of ethyl acetate fraction of L. inermis

The results of the TLC-bioautography of the ethyl acetate fraction of *L. inermis* leaves can be seen in **Figures 1** and **2**. **Figure 1** shows two inhibition zones formed on MHA inoculated *S. aureus* with Rf values of 0.25 and 0.53. After the TLC plate was sprayed with 10% KOH, a spot with the Rf value of 0.25 appeared to be a positive red-brown color, indicating the presence of naphthoquinone compounds (**Figure 2** and **Table IV**). The appearance of a reddish-brown color is due to the addition of

a hydroxyl group from KOH[15]. After the plate was sprayed with $FeCl_3$, a spot with the Rf value of 0.53 (**Figure 2** and **Table IV**) showed a blue-black color, indicating the presence of phenolic compounds[16]. The reaction forms a blue-black color due to the formation of complex compounds between metal atoms of iron (Fe) and non-metal atoms. The presence of phenolic compounds is in line with previous research, which stated that *L. inermis* contains phenolics[11,24]. Husni *et al*.[25] reported that the ethanol extract of *L. inermis* leaves has a total phenolic content of 16.02 g/100 g.

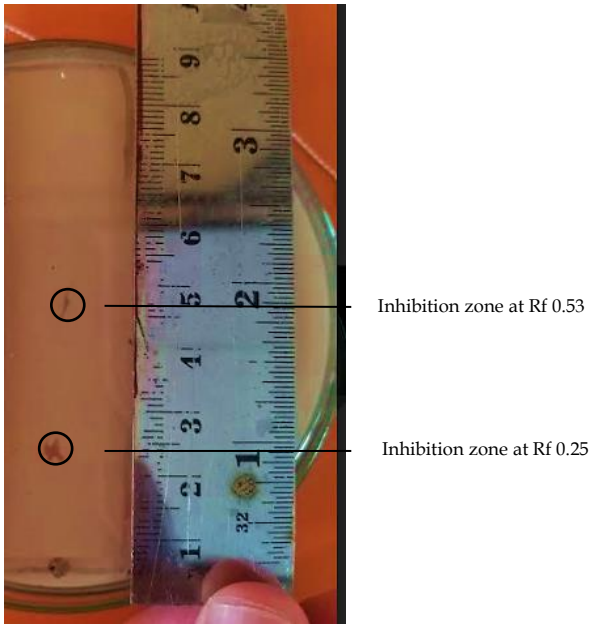


Figure 1. The bioautography result of the ethyl acetate fraction of *L. inermis* leaves on Mueller Hinton Agar inoculated by *S. aureus*. The inhibition zones are depicted with black circles.

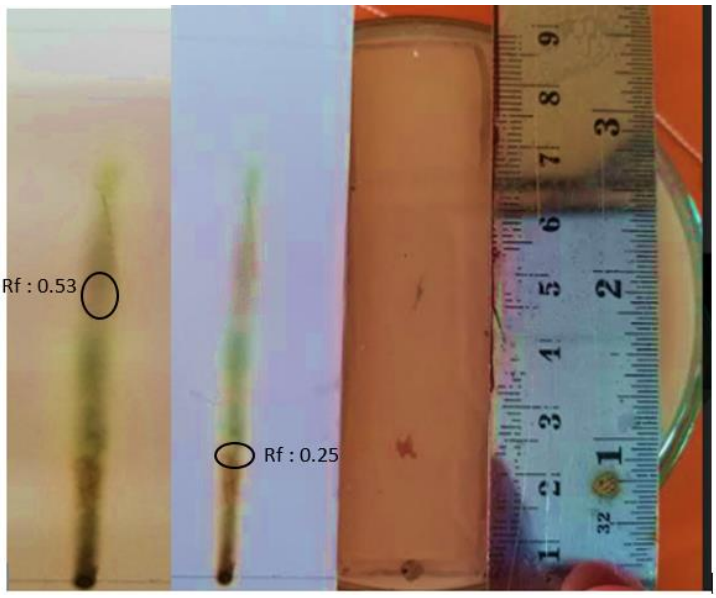


Figure 2. The chomatogram of ethyl acetate fraction of *L. inermis* leaves after sprayed with $FeCl_3$ (**a**), and with KOH 10% (**b**).

Table IV. Result of TLC-bioautography of the ethyl acetate fraction of *L. inermis* leaves

Rf	Detection	Color	Chemical group
0.25	KOH 10%	Brownish red	Naphthoquinone
0.53	$FeCl_3$	Blue black	Phenolic compounds

The mechanism of phenolic compounds in inhibiting bacterial growth is to irreversibly bind to nucleophilic amino acids from proteins, causing protein inactivation and becoming non-functional while also inactivating adhesins and enzymes on microbial membranes. The presence of phenolic groups with a high protein binding affinity can inhibit microbial enzymes while also boosting membrane affinity, resulting in increased antibacterial action[26]. Luis *et al*.[27] investigated the mechanism of action of a phenolic compound and hypothesized that it was linked to polyphenol-membrane contact. The presence of a phenolic compound was connected to increased permeability and depolarization of the cell layer, as well as a decrease in respiratory action in the *S. aureus* ATCC 25923 strain. The component of activity of a phenolic compound is connected to cell layer damage and changes in the vigorous metabolism of *S. aureus* cells[8,27]. A phenolic compound suppresses a hemolysin secretion in *S. aureus*; a membrane-dependent activity further supports the initial findings[28]. Simple phenols' activities are thought to be mediated through contact with sulfhydryl groups in microbial enzymes, inhibiting those enzymes or nonspecific protein interactions[29].

Naphthoquinones are found naturally in various plants and are considered promising antibacterial agents. Increased ROS production, followed by apoptotic cell death, is the mechanism of action for this antibacterial agent. Various naphthoquinone compounds have pharmacological effects, including antibacterial, anticancer, antitubercular, antimalarial, and trypanocidal properties. Naphthoquinone analogs are highly lethal to infected cells due to their capacity to create reactive oxygen species (ROS) and can inhibit cellular enzymes involved in apoptosis and cell proliferation. Consequently, these compounds serve as models for developing clinical antibacterial drugs[30]. Another study confirmed 2-hydroxy-1,4-naphthoquinone found in *L. inermis* as the main compound that may be an antibacterial agent[31]. However, its specific mechanism of action needs further research. Although the class of compounds with antibacterial action was identified in this investigation, the actual name of the active chemical cannot be determined. Therefore, additional investigation is required to identify and isolate the active substance.

CONCLUSION

These results indicate that the ethyl acetate fraction of *L. inermis* leaves contains naphthoquinones, flavonoids, and phenolic compounds that can inhibit bacterial growth. The results also suggest that other phytochemical compounds may contribute to the antibacterial activity of *L. inermis* leaves, and further study needs to be done to explore them.

ACKNOWLEDGMENT

We want to thank Nurma Harfita Sari for assisting with this research. The authors received no financial support for the study or publication of this article.

AUTHORS' CONTRIBUTION

Sri Mulyaningsih: designed, directed, and managed the study; drafted manuscript preparation; edited and reviewed article. **Febriyati Adji Rachmadani**: collected data.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Charoensup R, Duangyod T, Palanuvej C, Ruangrungsi N. Pharmacognostic Specifications and Lawsone Content of Lawsonia inermis Leaves. Pharmacognosy Res. 2017;9(1):60-4. doi:10.4103/0974-8490.199775

2. Sharma RK, Goel A, Bhatia AK. Lawsonia Inermis a Plant with Cosmetic and Medical Benefits. Int J Appl Sci Biotechnol. 2016;4:15-20. doi:10.3126/ijasbt.v4i1.14728

3. Leela K, Singh ARJ. Bioactive Compound Studies of Lawsonia inermis L. (Henna) –Its Ethnomedicinal and Pharmacological Applications: A Review. Int J Mod Trends Sci Technol. 2020;6(9):187-200. doi:10.46501/IJMTST060929

4. Semwal RB, Semwal DK, Combrinck S, Cartwright-Jones C, Viljoen A. Lawsonia inermis L. (henna): ethnobotanical, phytochemical and pharmacological aspects. J Ethnopharmacol. 2014;155(1):80-103. doi:10.1016/j.jep.2014.05.042

5. Usman R, Rabiu U. Antimicrobial activity of Lawsonia inermis (henna) extracts. Bayero J Pure Appl Sci. 2019;11(1):167-71. doi:10.4314/bajopas.v11i1.27S

6. Garg R, Tripathi R, Batav N, Singh R. Phytochemical High Performance Thin Layer Chromatography based Estimation of Lawsone in Lawsonia inermis (Henna) obtained from Two Natural Habitats and Dye Products Collected from Local Market. Med Aromat Plants. 2017;6:3. doi:10.4172/2167-0412.1000290

7. Xavier MR, Santos MMS, Queiroz MG, Silva MSdL, Goes AJS, De Morais Jr MA. Lawsone, a 2-hydroxy-1,4-naphthoquinone from Lawsonia inermis (henna), produces mitochondrial dysfunctions and triggers mitophagy in Saccharomyces cerevisiae. Mol Biol Rep. 2020;47(2):1173-85. doi:10.1007/s11033-019-05218-3

8. Miklasińska-Majdanik M, Kępa M, Wojtyczka RD, Idzik D, Wąsik TJ. Phenolic Compounds Diminish Antibiotic Resistance of Staphylococcus Aureus Clinical Strains. Int J Environ Res Public Health. 2018;15(10):2321. doi:10.3390/ijerph15102321

9. Al-Snafi AE. A Review on Lawsonia Inermis: A Potential Medicinal Plant. Int J Curr Pharm Res. 2019;11(5):1-13. doi:10.22159/ijcpr.2019v11i5.35695

10. Shahabinejad S, Kariminik A. Antibacterial activity of methanol extract of Lawsonia inermis against uropathogenic bacteria. MicroMedicine. 2019;7(2):31-6. doi:10.5281/zenodo.3473381

11. Akhtar J, Bashir F, Bi S. Scientific Basis for the Innovative Uses of Henna (Lawsonia inermis L.) mentioned by Unani Scholars in different ailments. J Complement Altern Med Res. 2021;14(1):1-21. doi:10.9734/jocamr/2021/v14i130234

12. Nigussie D, Davey G, Legesse BA, Fekadu A, Makonnen E. Antibacterial activity of methanol extracts of the leaves of three medicinal plants against selected bacteria isolated from wounds of lymphoedema patients. BMC Complement Med Ther. 2021;21(1):2. doi:10.1186/s12906-020-03183-0

13. Ag T, Kumar MS, Shivannavar CT Gaddad SM. Antibacterial and anti-biofilm activities of crude extracts of Lawsonia inermis against Methicillin Resistant Staphylococcus aureus. Asian J Pharm Clin Res. 2016;9(6):263-5. doi:10.22159/ajpcr.2016.v9i6.14362

14. Abubakar AR, Haque M. Preparation of Medicinal Plants: Basic Extraction and Fractionation Procedures for Experimental Purposes. J Pharm Bioallied Sci. 2020;12(1):1-10. doi:10.4103/jpbs.jpbs_175_19

15. Zainab Z, Muthoharoh A. Penapisan Fitokimia, Penetapan Kadar Naftokuinon total, dan Aktivitas Antifungi Fraksi Tidak Larut Etil Asetat Ekstrak Etanol Daun Pacar Kuku (Lawsonia inermis L.) TERHADAP Candida albicans ATCC. Pharmaciana. 2015;5(2):199-208. doi:10.12928/pharmaciana.v5i2.2371

16. Shaikh JR, Patil M. Qualitative tests for preliminary phytochemical screening: An overview. Int J Chem Stud. 2020;8(2):603-8. doi:10.22271/chemi.2020.v8.i2i.8834

17. Kusumaningtyas E, Astuti E, Darmono D. Sensitivitas Metode Bioautografi Kontak dan Agar Overlay dalam Penentuan Senyawa Antikapang. J Ilmu Kefarmasian Indones. 2008;6(2):75-80.

18. Sharma R, Goel A. Identification of Phytoconstituents in Lawsonia inermis Linn. Leaves Extract by GC-MS and their Antibacterial Potential. Pharmacogn J. 2018;10(6):1101-8. doi:10.5530/pj.2018.6.187

19. Nwodo UU, Ngene AA, Iroegbu CU, Obiiyeke G. Effects of fractionation on antibacterial activity of crude extracts of Tamarindus indica. African J Biotechnol. 2010;9(42):7108-13. doi:10.5897/AJB09.1662

20. Voukeng IK, Nganou BK, Sandjo LP, Celik I, Beng VP, Tane P, et al. Antibacterial activities of the methanol extract, fractions and compounds from Elaeophorbia drupifera (Thonn.) Stapf. (Euphorbiaceae). BMC Complement Altern Med. 2017;17(1):28. doi:10.1186/s12906-016-1509-y

21. Mundy L, Pendry B, Rahman M. Antimicrobial resistance and synergy in herbal medicine. J Herb Med. 2016;6(2):53-8. doi:10.1016/j.hermed.2016.03.001

22. Sembiring EN, Elya B, Sauriasari R. Phytochemical screening, total flavonoid and total phenolic content and antioxidant activity of different parts of Caesalpinia bonduc (L.) Roxb. Pharmacogn J. 2018;10(1):123-7. doi:10.5530/pj.2018.1.22

23. Dhanani T, Shah S, Gajbhiye NA, Kumar S. Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of Withania somnifera. Arab J Chem. 2017;10(S1):1193-9. doi:10.1016/j.arabjc.2013.02.015

24. Hadef KZ, Boufeldja W. Antimicrobial activity of lawsonia inermis leaf extract collected from south of algeria touat (Adrar) and tidikelt (in salah). Asian J Plant Sci. 2020;15(1):9-16. doi:10.3923/jps.2020.9.16

25. Husni E, Suharti N, Atma APT. Karakterisasi Simplisia dan Ekstrak Daun Pacar Kuku (Lawsonia inermis Linn) serta Penentuan Kadar Fenolat Total dan Uji Aktivitas Antioksidan. J Sains Farm Klin. 2018;5(1):12-6. doi:10.25077/jsfk.5.1.12-16.2018

26. Bouarab-Chibane L, Forquet V, Lantéri P, Clément Y, Léonard-Akkari L, Oulahal N, et al. Antibacterial Properties of Polyphenols: Characterization and QSAR (Quantitative Structure-Activity Relationship) Models. Front Microbiol. 2019;10:829. doi:10.3389/fmicb.2019.00829

27. Luís Â, Silva F, Sousa S, Duarte AP, Domingues F. Antistaphylococcal and biofilm inhibitory activities of gallic, caffeic, and chlorogenic acids. Biofouling. 2014;30(1):69–79. doi:10.1080/08927014.2013.845878

28. Cowan M. Plant products as antimicrobial agents. Clin Microbiol Rev. 1999;12(4):564-82. doi:10.1128/cmr.12.4.564

29. Aldulaimi OA. General Overview of Phenolics from Plant to Laboratory, Good Antibacterials or Not. Pharmacogn Rev. 2017;11(22):123-7. doi:10.4103/phrev.phrev_43_16

30. Ravichandiran P, Sheet S, Premnath D, Kim AR, Yoo DJ. 1,4-Naphthoquinone Analogues: Potent Antibacterial Agents and Mode of Action Evaluation. Molecules. 2019;24(7):1437. doi:10.3390/molecules24071437

31. Pour AP, Farahbakhs H. Lawsonia inermis L. leaves aqueous extract as a natural antioxidant and antibacterial product. Nat Prod Res. 2020;34(23):3399-403. doi:10.1080/14786419.2019.1569006

Borneo Journal of Pharmacy
Vol 5 Issue 3 August 2022
Page 202 – 208

http://journal.umpalangkaraya.ac.id/index.php/bjop/article/view/3694
DOI: https://doi.org/10.33084/bjop.v5i3.3694
e-ISSN: 2621-4814

Research Article

Tyrosinase Inhibition Activity and Phytochemical Screening of *Melaleuca leucadendron* L. Leaves

Munawarohthus Sholikha*

Ainun Wulandari

Department of Pharmacy, Institut Sains Teknologi Nasional, South Jakarta, Jakarta Capital Special Region, Indonesia

*email: mona.farmasi@istn.ac.id

Keywords:
Melaleuca leucadendron L
Phytochemical screening
Tyrosinase

Abstract

Melaleuca leucadendron L. is a plant whose almost all parts (bark, leaves, twigs, and fruit) can be used as medicine, such as antioxidants, antifungals, sedative effects, and anti-hyaluronidase. This research was conducted to determine tyrosinase inhibition activity and compound content of *M. leucadendron* leaves. Maceration of *M. leucadendron* leaves was done in methanol, then carried out liquid-liquid fractionation with n-butanol, chloroform, and water. Methanol extract, butanol fraction, chloroform fraction, and water fraction were tested for phytochemical screening and tyrosinase inhibition using L-DOPA substrate with an ELISA plate well reader. The results of the tyrosinase inhibition activity test at concentrations of 100, 1000 and 10000 µg/mL respectively showed that methanol extract 29.532%, 55.227%, 89.583%; butanol fraction 29.313%, 59.174%, 94.737%, chloroform fraction 21.820%, 24.671%; 53.765%; water fraction 24,086%, 47.661%, 91.118%. Inhibition of the tyrosinase enzyme is shown through the IC_{50} value from methanol extract, butanol fraction and water fraction, and kojic acid as a positive control, respectively 645.438 µg/mL, 517.935 µg/mL, 669.403 µg/mL, 50.064 µg/mL. Phytochemical screening showed that the extract and fraction contained tannins, flavonoids, saponins, terpenes, and steroids. These results indicate that the butanol fraction is more potent as an anti-tyrosinase agent than the others.

Received: June 29th, 2022
1st Revised: August 4th, 2022
Accepted: August 14th, 2022
Published: August 31th, 2022

INTRODUCTION

The skin is the outermost part of the human body that plays a vital role in body protection. One of the functions of the skin is to protect the body from UV rays[1]. Excessive ultraviolet (UV) light exposure increases the contribution of free radicals known as reactive oxygen species (ROS)[2]. Reactive oxygen species increase pigmentation and cause oxidative stress-induced damage to the melanocytes[3]. Hyperpigmentation disorders are diseases in which patches of skin are darker than the normal surrounding skin, resulting from the upregulated activity of melanin synthesis, increased numbers of melanocytes, and decreased decomposition of melanosomes[4]. Hyperpigmentation conditions can also be caused by certain drugs, hormonal changes, or autoimmune conditions[5].

The enzyme that plays an essential role in the melanin synthesis pathway is tyrosinase. Tyrosinase has the activity of tyrosine hydroxylation, oxidation of L-DOPA (3,4-dihydroxyphenylalanine), and oxidation of hydroxyindole[6]. In the process of melanogenesis, tyrosinase acts as a catalyst in two different reactions, the hydroxylation of tyrosine to L-DOPA and the oxidation of L-DOPA to dopaquinone[7]. Dopaquinone is a highly reactive compound that can spontaneously polarize to form melanin[8].

Bleach acts as an inhibitor of melanin production and is a competitive tyrosinase inhibitor. Various tyrosinase inhibitors are found in cosmetic ingredients to prevent hyperpigmentation, including hyaluronic acid, arbutin, kojic acid, mercury, and

How to cite: Sholikha M, Wulandari A. Tyrosinase Inhibition Activity and Phytochemical Screening of Melaleuca leucadendron L. Leaves. Borneo J Pharm. 2022;5(3):202-8. doi:10.33084/bjop.v5i3.3694

hydroquinone[9]. This compound has immense whitening power despite having severe side effects such as carcinogenesis, hepatotoxicity, and dermatitis[10]. Hydroquinone not only inhibits tyrosinase activity and destroys melanosomes but also causes necrosis of melanocytes by modifying the membrane structure[11]. This is the potential mechanism of action of hydroquinone as a skin-lightening agent and its toxicity mechanism. The use of this ingredient in cosmetics has been banned since 2001 because of the high risk of carcinogenesis in case of prolonged exposure to hydroquinone[12].

Several researchers prefer to identify inhibitors from natural sources due to their less toxicity and better bioavailability, especially for food, cosmetic and medicinal applications[13]. The class of flavonoid compounds that have tyrosinase inhibitory activity is quercetin from the flavonol group. Flavonoid compounds have tyrosinase inhibitor and chelating activity Cu, where the hydroxyl groups on the A and rings B inhibit the action of tyrosinase[14]. Besides flavonoids, other polyphenols, also known as tyrosinase inhibitors, include coumarin, stilbenes derivatives[15], terpenoid derivatives[16], and lignans[17].

In previous studies, the antioxidant activity test of *Melaleuca leucadendron* L. leaves was extracted with methanol and then fractionated with chloroform and butanol. The total phenolic that has been carried out in previous studies was 289.23 ± 5.21 µg GAE/g in methanol extract, 107.36 ± 1.88 µg GAE/g in chloroform extract, and 508.43 ± 2.33 µg GAE/g in butanol extract. While the IC_{50}- obtained in the *M. leucadendron* extract as an antioxidant was 14.5 µg/mL in methanol extract, 50.3 µg/mL in chloroform extract, and 10.1 µg/mL in butanol extract[18]. Based on previous studies, the tyrosinase inhibition test on *M. leucadendron* leaves has never been carried out. This test is necessary because a high total phenolic and a low IC_{50} value of antioxidants can act as anti-tyrosinase. We hope this research can contribute to developing new safe, efficient anti-tyrosinase agents to prevent hyperpigmentation disorders.

MATERIALS AND METHODS

Materials

Melaleuca leucadendron dry leaves were collected from Balai Penelitian Tanaman Rempah dan Obat (Balitro), which has been identified at the Botanical Garden Plant Conservation Center, Lembaga Ilmu Pengetahuan Indonesia (LIPI), number of the certificate B-1222/IPH3/KS/X/2020 (**Figure 1**). Chemical reagents such as methanol 75%, L-DOPA (Sigma), tyrosinase (Sigma), kojic acid (Sigma), chloroform, butanol, distilled water, HCl 2 N, Dragendorff reagent, Mayer reagent, Wagner reagent, Bouchardat reagent, HCl, $NaNO_2$ 5%, $AlCl_3$ 10%, NaOH 1 N, $FeCl_3$ 1%, NaOH 2 N, ether, H_2SO_4, potassium dihydrogen phosphate, dimethyl sulfoxide (DMSO) (Merck), and phosphate buffer (pH 6.5). At the same time, the equipment used includes a digital analytical scale, rotary vacuum evaporator, multi-well plate reader (ELISA), multilevel fractionation device, pH meter, and incubator.

a **b**

Figure 1. *Melaleuca leucadendron* leaves (**a**) simplicia powder of *M. leucadendron* leaves (**b**).

Methods

Extraction and fractination

Melaleuca leucadendron dry leaves were ground to obtain 500 g of sample powder for extraction. Methanol 75% (5 L) was used as the solvent in the maceration extraction of the samples for 3 x 24 hours. The crude methanol extracts were then dried using a rotary evaporator. Liquid-liquid fractionation was conducted using distilled water, butanol, and chloroform to the methanol extract to obtain fractions with different polarities, then dried using a rotary evaporator.

Phytochemical screening

Extracts and three fractions were carried out in a phytochemical screening test to identify alkaloids, flavonoids, tannins, saponins, steroids/triterpenoids using the classical method[19].

Tyrosinase inhibitory assay

Tyrosinase inhibitory activity was evaluated based on inhibition of the sample (diluted in DMSO) to diphenolase activity. The assay was carried out using an ELISA plate well reader with tyrosinase enzyme, L-DOPA as the substrates, phosphate buffer pH 6.5, with three repetitions[20]. Kojic acid was used as a positive control. The following **Equation 1** can calculate the percentage of tyrosinase inhibitory activity:

$$\text{Inhibition (\%)} = [1 - \frac{(A-C)}{(B-D)}] \text{ x } 100\% \quad \ldots [1]$$

In which,

A: Absorbance of the sample

B: Absorbance of blank

C: Absorbance of sampel control

D: Absorbance of blank control

The IC_{50} value can be calculated using a linear regression equation, sample concentration (x-axis), and %-inhibition (y-axis). From the equation $y = a \ln (x) + b$, the IC_{50} value can be calculated using the **Equation 2**.

$$\ln IC_{50} = \frac{50-b}{a} \quad \ldots [2]$$

RESULTS AND DISCUSSION

The yields of the extracts and their respective fractions are presented in **Table I**. The extraction method is maceration because the equipment used is simple and easy. Methanol 75% was used as a solvent because it can attract the highest phenolic compounds and has a low antioxidant IC_{50} value[18]. During maceration, stirring is carried out so that the pollen liquid penetrates the cell wall and enters the cell cavity containing the active substance. The difference in concentration between the solution inside and outside the cell causes a more concentrated solution to be pushed out so that the metabolite compound can be extracted entirely[21].

Based on the results of phytochemical screening obtained on *M. leucadendron* leaves extract containing flavonoids, saponins, tannins, and steroids/triterpenoids and negative results in the alkaloid test (**Table II**). Previous research[22] showed that *M. leucadendron's* methanol fraction contains alkaloid compounds, flavonoids, saponins, tannins, steroids, and triterpenoids. The difference in results obtained is due to the use of hexane solvent when maceration. The water fraction shows negative results in the steroid/triterpenoid test; this is because terpenoids can be extracted using non-polar solvents (ether, hexane, chloroform), while in the form of glycosides (generally from triterpenes) the solubility is more remarkable in polar solvents (ethanol, methanol).

Table I. The yield of the extract and fraction of *M. leucadendron* leaves

Extracts/Fraction	Powder weight (g)	Extract weight (g)	Yield (%)
Methanol	500	82.02	16.04
Chloroform	40	12.6	31.5
Water	40	21	52.5
Butanol	40	6.5	16.25

Table II. Phytochemical screening test results of *M. leucadendron* leaves

Test	Sample			
	ME	BF	CF	WF
Saponin	+	+	+	+
Alkaloid				
Mayer	-	-	-	-
Wagner	-	-	-	-
Dragendorff	-	-	-	-
Tannins	+	+	+	+
Flavonoids	+	+	+	+
Steroids-terpenoids	+	+	+	-

ME: methanol extract; BF: butanol fraction; CF: chloroform fraction; WF: water fraction

The activity of tyrosinase inhibitors is one of the parameters of skin lightening agents. The mechanism of tyrosinase inhibitors is to decrease skin pigmentation by inhibiting the catalytic of the enzyme to the pigmentation associated with melanin production in the melanogenesis pathway[23]. Kojic acid is used as a positive control because it is one of the tyrosinase inhibitors used as a cosmetic ingredient. It is a skin protector from the ultraviolet sun and can whiten the skin. Kojic acid prevents the formation of melanin in human melanocytes due to the reversible inhibition of tyrosinase, but it has some side effects, such as skin irritability and instability[24].

The butanol fraction had the highest tyrosinase inhibition value (94.737±0.767%) at 1000 µg/mL concentration. However, kojic acid as a positive control show better inhibition at a concentration of 500 µg/mL with tyrosinase inhibition value of 91.155±0.228%. Measurement of the IC_{50} value was carried out on methanol extract, butanol fraction, water fraction, and kojic acid, while the chloroform fraction was not carried out because of the low percentage value of inhibition at a concentration of 1000 µg/mL (**Table III**).

Table III. Tyrosinase inhibitory of *M. leucadendron* leaves

Sample	Concentrations (µg/mL)	Tyrosinase Inhibition (%)
Methanol extract	100	29.532 ± 0.713
	1000	55.227 ± 1.081
	10000	89.583 ± 0.110
Butanol fraction	100	29.313 ± 0.920
	1000	59.174 ± 1.299
	10000	94.737 ± 0.767
Chloroform fraction	100	21.820 ± 1.245
	1000	24.671 ± 1.245
	10000	53.765 ± 0.444
Water fraction	100	24.086 ± 1.271
	1000	47.661 ± 0.228
	10000	91.118 ± 0
Kojic acid	500	91.155 ± 0.228

Values are expressed as mean ± SD of triplicate measurements

The IC_{50} values of tyrosinase inhibition of all samples are presented in **Table IV**. Butanol fraction had the lowest IC_{50} value with 517.94 µg/mL. Nevertheless, kojic acid as a positive control was more potent with IC_{50} 50.06 µg/mL. The butanol fraction is more active than other extracts and fractions, while in previous research[18], the total phenolic content was reported to be more significant, 508.43±2.33 µg GAE/g extract, and the antioxidant IC_{50} value of 4.8 µg/mL. From the research results, it can be seen that there is a correlation between total phenolic and antioxidant activity with tyrosinase activity.

Table IV. IC_{50} of tyrosinase inhibitory of *M. leucadendron* leaves

Sample	Concentrations (µg/mL)	Tyrosinase inhibition (%)	IC_{50} (µg/mL)
Methanol extract	500	47.11±0.39	645.44
	1000	54.18±0.51	
	1500	58.38±0.53	
	2000	58.18±0.84	
	2500	59.71±0.75	
Butanol fraction	500	48.85±0.57	517.94
	1000	60.83±0.84	
	1500	69.65±0.71	
	2000	73.18±0.29	
	2500	74.37±1.03	
Water fraction	500	48.23±0.25	669.40
	1000	51.66±0.53	
	1500	59.91±0.58	
	2000	66.63±0.85	
	2500	67.99±0.54	
Kojic acid	31.25	33.23±0.41	50.06
	62.5	51.74±0.84	
	125	79.20±0.08	
	250	89.65±0.14	
	500	94.84±0.05	

Values are expressed as mean ± SD of triplicate measurements

CONCLUSION

Inhibition of the tyrosinase enzyme is shown through the IC_{50} value from methanol extract, butanol fraction, and water fraction was 645.44 µg/mL, 517.94 µg/mL, 669.40 µg/mL, respectively. As a positive control, the IC_{50} value of kojic acid was 50.06 µg/mL. Phytochemical screening showed that the extract and fraction of *M. leucadendron* leaves contained tannins, flavonoids, saponins, terpenes, and steroids. These results indicate that the butanol fraction of *M. leucadendron* leaves is the most potent anti-tyrosinase agent compared to the others.

ACKNOWLEDGMENT

The authors are thankful to acknowledge the Ministry of Research, Technology, and Higher Education, Republic of Indonesia, for support via Research Grant (26/E1/KPT/2020).

AUTHORS' CONTRIBUTION

Munawarohthus Sholikha: conceptualization, methodology, get a grant for funding, supervised the experimental works, writing and review. **Ainun Wulandari**: contributed in the experimental works and assisted for manuscript writing.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

REFERENCES

1. D'Orazio J, Jarrett S, Amaro-Ortiz A, Scott T. UV radiation and the skin. Int J Mol Sci. 2013;14(6):12222-48. doi:10.3390/ijms140612222

2. Pizzino G, Irrera N, Cucinotta M, Pallio G, Mannino F, Arcoraci V, et al. Oxidative Stress: Harms and Benefits for Human Health. Oxid Med Cell Longev. 2017;2017:8416763. doi:10.1155/2017/8416763

3. Upadhyay PR, Starner RJ, Swope VB, Wakamatsu K, Ito S, Abdel-Malek ZA. Differential Induction of Reactive Oxygen Species and Expression of Antioxidant Enzymes in Human Melanocytes Correlate with Melanin Content: Implications on the Response to Solar UV and Melanoma Susceptibility. Antioxidants. 2022;11(6):1204. doi:10.3390/antiox11061204

4. Xing X, Dan Y, Xu Z, Xiang L. Implications of Oxidative Stress in the Pathogenesis and Treatment of Hyperpigmentation Disorders. Oxd Med Cell Longev. 2022;2022:7881717. doi:10.1155/2022/7881717

5. Desai SR. Hyperpigmentation therapy: a review. J Clin Aesthet Dermatol. 2014;7(8):13-7.

6. da Silva AP, Silva NdF, Andrade EHA, Gratieri T, Setzer WN, Maia JGS, et al. Tyrosinase inhibitory activity, molecular docking studies and antioxidant potential of chemotypes of Lippia origanoides (Verbenaceae) essential oils. PLoS One. 2017;12(5):e0175598. doi:10.1371/journal.pone.0175598

7. Pillaiyar T, Manickam M, Namasivayam V. Skin whitening agents: medicinal chemistry perspective of tyrosinase inhibitors. J Enzyme Inhib Med Chem. 2017;32(1):403-25. doi:10.1080/14756366.2016.1256882

8. Ito S, Wakamatsu K. Chemistry of mixed melanogenesis--pivotal roles of dopaquinone. Photochem Photobiol. 2008;84(3):582-92. doi:10.1111/j.1751-1097.2007.00238.x

9. Zolghadri S, Bahrami A, Khan MTH, Munoz-Munoz J, Garcia-Molina F, Garcia-Canovas F, et al. A comprehensive review on tyrosinase inhibitors. J Enzyme Inhib Med Chem. 2019;34(1):279-309. doi:10.1080/14756366.2018.1545767

10. David S, Hamilton JP. Drug-induced Liver Injury. US Gastroenterol Hepatol Rev. 2010;6:73-80.

11. Boo YC. Arbutin as a Skin Depigmenting Agent with Antimelanogenic and Antioxidant Properties. Antioxidants. 2021;10(7):1129. doi:10.3390/antiox10071129

12. Owolabi JO, Fabiyi OS, Adelakin LA, Ekwerike MC. Effects of Skin Lightening Cream Agents - Hydroquinone and Kojic Acid, on the Skin of Adult Female Experimental Rats. Clin Cosmet Investig Dermatol. 2020;13:283-9. doi:10.2147/ccid.s233185

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

14. El-Nashar HAS, El-Din MIG, Hritcu L, Eldahshan OA. Insights on the Inhibitory Power of Flavonoids on Tyrosinase Activity: A Survey from 2016 to 2021. Molecules. 2021;26(24):7546. doi:10.3390/molecules26247546

15. Obaid RJ, Mughal U, Naeem N, Sadiq A, Alsantali RI, Jassas RS, et al. Natural and synthetic flavonoid derivatives as new potential tyrosinase inhibitors : a systematic review. RSC Advances. 2021;11:22159–98. doi:10.1039/D1RA03196A

16. Ashraf Z, Rafiq M, Nadeem H, Hassan M, Afzal S, Waseem M, et al. Carvacrol derivatives as mushroom tyrosinase inhibitors; synthesis, kinetics mechanism and molecular docking studies. PLoS One. 2017;12(5):e0178069. doi:10.1371/journal.pone.0178069

17. Wu B, Zhang X, Wu X. New lignan glucosides with tyrosinase inhibitory activities from exocarp of Castanea henryi. Carbohydr Res. 2012;355:45–9. doi:10.1016/j.carres.2012.04.009

18. Surh J, Yun JM. Antioxidant and anti-inflammatory activities of butanol extract of Melaleuca leucadendron L. Prev Nutr Food Sci. 2012;17(1):22–8. doi:10.3746/pnf.2012.17.1.022

19. Sembiring EN, Elya B, Sauriasari R. Phytochemical screening, total flavonoid and total phenolic content and antioxidant activity of different parts of Caesalpinia bonduc (L.) Roxb. Pharmacogn J. 2018;10(1):123–7. doi:10.5530/pj.2018.1.22

20. Arifianti AE, Anwar E, Nurjanah. Tyrosinase Inhibitor and Antioxidant Activity of Seaweed Powder from Fresh and Dried Sargassum plagyophyllum. J Pengolahan Hasil Perikanan Indones. 2017;20(3):488-93. doi:10.17844/jphpi.v20i3.19769

21. Uddin MS, Ferdosh S, Akanda MJH, Ghafoor K, Rukshana AH, Ali ME, et al. Techniques for the extraction of phytosterols and their benefits in human health: a review. Sep Sci Technol. 2018;53(14):2206-23. doi:10.1080/01496395.2018.1454472

22. Khongsai S, Vittaya L. Solvent Effect on Phytochemical Screening of Melaleuca leucadendra Linn. and Syzygium cinerea. Rajamangala Univ Technol Srivijaya Res J. 2019;12(1):112–9.

23. Song Y, Chen S, Li L, Zeng Y, Hu X. The Hypopigmentation Mechanism of Tyrosinase Inhibitory Peptides Derived from Food Proteins: An Overview. Molecules. 2022;27(9):2710. doi:10.3390/molecules27092710

24. Phasha V, Senabe J, Ndzotoyi P, Okole B, Fouche G, Chuturgoon A. Review on the Use of Kojic Acid—A Skin-Lightening Ingredient. Cosmetics. 2022;9(3):64. doi:10.3390/cosmetics9030064

Borneo Journal of Pharmacy
Vol 5 Issue 3 August 2022
Page 209 – 228

http://journal.umpalangkaraya.ac.id/index.php/bjop/article/view/3262
DOI: https://doi.org/10.33084/bjop.v5i3.3262
e-ISSN: 2621-4814

Research Article

Metabolite Profiling of the Environmental-Controlled Growth of *Marsilea crenata* Presl. and Its *In Vitro* and *In Silico* Antineuroinflammatory Properties

Burhan Ma'arif [1*]
Faisal Akhmal Muslikh [2]
Dilla Amalia [1]
Anisah Mahardiani [1]
Luthfi Achmad Muchlasi [1]
Pramudita Riwanti [3]
Maximus Markus Taek [4]
Hening Laswati [5]
Mangestuti Agil [6]

[1] Department of Pharmacy, Universitas Islam Negeri Maulana Malik Ibrahim, Malang, East Java, Indonesia

[2] Master Program of Pharmaceutical Science, Universitas Airlangga, Surabaya, East Java, Indonesia

[3] Department of Pharmacy, Universitas Hang Tuah, Surabaya, East Java, Indonesia

[4] Department of Chemistry, Universitas Katolik Widya Mandira, Kupang, East Nusa Tenggara, Indonesia

[5] Department of Physical Medicine and Rehabilitation, Universitas Airlangga, Surabaya, East Java, Indonesia

[6] Department of Pharmaceutical Science, Universitas Airlangga, Surabaya, East Java, Indonesia

*email: burhan.maarif@farmasi.uin-malang.ac.id

Keywords:
Environmental-controlled growth
HMC3 microglia cells
Marsilea crenata Presl.
Neuroinflammatory
Phytoestrogens

Abstract

This study was aimed to evaluate the metabolite contents and antineuroinflammatory potential of *Marsilea crenata* Presl. grown under a controlled environmental condition. The antineuroinflammatory test has been carried out *in vitro* using ethanolic extract of *M. crenata* leaves on HMC3 microglia cells. An *in silico* approach was applied to predict the active compounds of the extract. The HMC3 microglia cells were induced with IFNγ to create prolonged inflammatory conditions and then treated with 96% ethanolic extract of the *M. crenata* leaves of 62.5, 125, and 250 µg/mL. The expression of MHC II was analyzed using the ICC method with the CLSM instrument. Metabolites of the extract were profiled using UPLC-QToF-MS/MS instrument and MassLynx 4.1 software. *In silico* evaluation was conducted with molecular docking on 3OLS protein using PyRx 0.8 software, and physicochemical properties of the compounds were analyzed using SwissADME webtool. The ethanolic extract of *M. crenata* leaves could reduce the MHC II expression in HMC3 microglia cells in all concentrations with the values 97.458, 139.574, and 82.128 AU. The result of metabolite profiling found 79 compounds in the extract. *In silico* evaluation showed that 19 compounds gave agonist interaction toward 3OLS, and three met all parameters of physicochemical analysis. The ethanolic extract of the environmental-controlled growth of *M. crenata* leaves antineuroinflammatory activity on HMC3 microglia cells. The extract was predicted to contain some phytoestrogen compounds which act as 3OLS agonists.

Received: February 8th, 2022
1st Revised: May 13th, 2022
2nd Revised: July 3rd, 2022
3rd Revised: July 27th, 2022
Accepted: July 31th, 2022
Published: August 31th, 2022

How to cite: Ma'arif B, Muslikh FA, Amalia D, Mahardiani A, Muchlasi LA, Riwanti P, et al. Metabolite Profiling of the Environmental-Controlled Growth of Marsilea crenata Presl. and Its In Vitro and In Silico Antineuroinflammatory Properties. Borneo J Pharm. 2022;5(3):209-28. doi:10.33084/bjop.v5i3.3262

INTRODUCTION

The prevalence of postmenopausal women worldwide is gotten higher over several decades[1]. This increase is closely related to a direct decline in quality of life[2,3]. Women in the postmenopause phase will experience various disease complaints caused by estrogen deficiency conditions, one of which is a neurodegenerative disorder[4].

Neuroinflammation is one of the leading causes of neurodegenerative disorders arising from estrogen deficiency[5,6]. Neuroinflammation occurs due to an increase in activated microglia in the M1 polarization situation so that it can increase the expression of proinflammatory signaling factors such as major histocompatibility complex II (MHC II) and other inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and nitric oxide (NO) in the brain[7,8]. Increased inflammatory cytokines will reduce synapsis and plasticity functions of neuron cells and induce cell death[9].

Celecoxib, ibuprofen, minocycline, and aspirin are some medicines used to treat neuroinflammation[10,11]. However, those medicines appear with potential side effects in the form of gastrointestinal tract (GIT) disorders such as nausea, gastritis, abdominal pain, and digestive tract bleeding, as well as other side effects such as dizziness, hypertension, headache, and vertigo[12-14]. These side effects encourage the need for various studies on potential new drug sources with minimal side effects, such as phytoestrogens[15-18]. Phytoestrogens are a group of natural plant products with a structure and function similar to 17β-estradiol[19-21].

Marsilea crenata Presl. is one of the plants used as the typical food by the local community in East Java, Indonesia[22]. Ethanol 96% extract of *M. crenata* leaves been tested by radioimmunoassay (RIA) and shows a high estrogen-like substance[23]. Other previous studies also have shown that this plant contains phytoestrogen group compounds, such as flavonoids, that can act as an anti-inflammatory and has other estrogenic activity for maintaining human body homeostasis[24-28].

In this research, we cultivated *M. crenata* under external controlled factors to gain a standardized raw material of this plant. Cultivation was carried out in Kediri, East Java, Indonesia. This area is a lowland of 0-200 m above sea level (masl), with humidity of 60-90% and an average temperature of 23.8-30.7°C[29]. The plant was cultivated in a greenhouse under controlled nutrition, soil, irrigation, and some environmental factors, to produce better quality and quantity of the raw material of this plant[30-33].

This study aimed to prove the antineuroinflammatory activity of the 96% ethanolic extract of the leaves of *M. crenata* grown under controlled environmental conditions in inhibiting HMC3 microglia cells. This inhibition was observed in the situation of M1 polarization of these cells, with a decrease of MHC II expression as an indicator. This study also aimed to predict phytoestrogen compounds that play a role in the antineuroinflammatory activity through metabolite profiling using ultra-performance liquid chromatography – quadrupole time of flight – mass spectrometry (UPLC-QToF-MS/MS) and *in silico* studies of those compounds on estrogen receptors β (ERβ), which, in this case using protein 3OLS from protein data bank (PDB).

MATERIALS AND METHODS

Materials

Plant materials

The leaves of *M. crenata* were harvested from a controlled farming (greenhouse) in Kediri, East Java, Indonesia. The characteristics of the plants were two weeks old, the stem height was approximately 17 cm, the leaf width was approximately 2 cm, and the color of the leaves was dark green. The leaves were then identified at Unit Pelayanan Teknis (UPT) Materia Medika, Batu, East Java, Indonesia, with the determination key of 1a-17b-18a-1 and identification letter 074/368/102.7/2017. The specification of the cultivation area and the external factors are listed in **Table I**.

Table I. Specification of location and external factors in the cultivation of *M. crenata*

Parameter	Value
Region	Pagu, Kediri, East Java, Indonesia
Height	Lowland, 0-200 masl
Rainfall	130-150 mm per year, with the rainy day number average for 6-15 days
Climate	Tropical with 2 seasons
Temperature	Average maximum temperature of 30.7°C Average minimum temperature of 23.8°C Annual average temperature of 27.2°C
Humidity	60-90%
Irrigation	Ground water
Growing Media	Not submerged with water
Fertilizer	Organic fertilizer with specification: C Organic ≥ 15% C/N Ratio : 15-25 pH : 4-9 Water Content : 8-20%
Plantation Location	Green house

Chemicals

Fetal bovine serum (FBS), penicillin, streptomycin, Eagle's minimum essential medium (EMEM), dimethyl sulfoxide (DMSO), tween 80, phosphate-buffered saline (PBS), paraformaldehyde (PFA), bovine serum albumin (BSA), fluorescein isothiocyanate (FITC) anti-rabbit secondary antibody were purchased from Sigma-Aldrich (St. Louis, USA). MHC II anti-rabbit secondary antibody was purchased from Abcam (Cambridge, UK). Ethanol 96%, dichloromethane, methanol, acetonitrile, and formic acid were purchased from Merck (Darmstadt, Germany).

Hardware and software

The hardware used was an A416MA-EB422TS personal computer with Intel Celeron. The software used is the operating system Windows® 10. ChemDraw Ultra 12.0 was used in drawing 2D structures. Avogadro 1.0.1 was used in structural optimization. The docking process was done with AutoDock Vina using PyRx 0.8. Visualization of docking results was performed using Biovia Discovery Studio 2016.

Methods

Extraction

About 500 g of powdered leaves of *M. crenata* were extracted with 96% ethanol using the ultrasonic method with Soltec Sonica 5300EP S3 for 3 x 10 minutes, filtered, and evaporated at 50°C using a Heidolph G3 rotary evaporator. A 25 g of the 96% ethanolic extract was obtained and subjected to further test and analysis.

Cell culture

HMC3 microglia cells (ATCC® CRL-3304™) were cultured using complete media in 25 cm^2-sized flasks which contained 10% of FBS and a mixture of 1% of penicillin-streptomycin in a ±5 mL media EMEM. Cells in the flask were then incubated in a 5% CO_2 ThermoScientific Hera Cell 150i incubator at 37°C for a week. The cells were then placed into a 24-well microplate after the confluence was approximately 80%.

Measurement of MHC II expression

As much as 40 mg of the 96% ethanolic extract was suspended in the mixture of 0.5% DMSO and 0.5% Tween 80 to produce the mother liquor of 4,000 µg/mL; it was then diluted in the concentration of 62.5; 125; 250 µg/mL. About 40 µL of genistein was added with the culture media reaching 0.8 mL to produce genistein with the concentration of 50 µM, which was used as the positive control. Induction of IFN-γ was performed after cells were cultured on a 24-well microplate and reached 80% confluence. After induction of 10 ng IFN-γ for 24 hours, the cells were rinsed with PBS and then treated with 50 µM genistein for 48 hours. The cells were then fixated with 4% PFA, Triton X-100, and blocking buffer, and also primary and secondary antibodies were added. They were, afterward, rinsed using PBS and visualized MHC II with CLSM (Olympus Fluoview Ver. 4.2a.) 488 nm[34].

Metabolite profiling

The process of metabolite profiling was conducted at Pusat Laboratorium Forensik Badan Reserse Kriminal Kepolisian Negara Republik Indonesia (Puslabfor Bareskrim Polri) by using UPLC-QToF-MS/MS instrument. 96% ethanolic extract was prepared using dichloromethane and methanol solvents through the solid phase extraction (SPE) method. After that, 5 µL was equally injected into ACQUITY UPLC® H-Class System (Waters, USA) with the detector of MS Xevo G2-S QToF (Waters, USA). Samples were separated in the column of ACQUITY BEH C18 (1.7 µm × 2.1 mm × 50 mm) with acetonitrile of + 0.05% of formic acid and water + 0.05% of formic acid as the mobile phase, with the flow rate of 0.2 mL/minute. The analysis result of UPLC-QToF-MS/MS was processed using MassLynx 4.1 software to get chromatogram data and m/z spectrum from each detected peak. The detected compounds were then confirmed using the online database of ChemSpider (http://www.chemspider.com), PubChem (https://pubchem.ncbi.nlm.nih.gov), and MassBank (https://massbank.eu/MassBank).

In silico study

X-ray protein from ERβ was attained from the protein data bank (http://www.rcsb.org) with the PDB ID 3OLS. The antineuroinflammatory impact to be evaluated in this study is the antineuroinflammatory effect that emerges from phytoestrogens in plants, replacing the role of estrogen in the ER-dependent pathway and not through other mechanisms. ERβ also has a higher amount than other receptors, is more sensitive to estrogen binding, and plays the most role in regulating nerve cell homeostasis[35,36].

The initial preparation was performed to separate native ligand (17β-estradiol) from 3OLS protein using Biovia Discovery Studio Visualizer 2016 and saved in Sybyl mol2 format. Metabolite profiling compounds drawn 2D using ChemDraw Ultra 12.0 and saved in mol format. Internal validation was performed by adding 3OLS and 17-estradiol ligands and then docking them with PyRx 0.8 software. Native ligand and the result compounds of metabolite profiling were then optimized with Avogrado 1.0.1 using the MMFF94 method. Then, molecular docking was conducted using PyRx 0.8 with the AutoDock Vina method to simulate the docking process. All compound files were imported into the PyRx 0.8 software and converted to pdbqt format automatically. The determination of the grid box includes setting location according with grid center x = 11.1148, y = -35.813, and z = 12.1403 with dimension of 25 x 25 x 25 Å. Setting the exhaustiveness to number 8 and the work grid through Autogrid to 17-estradiol ligand completed the operation, yielding a binding affinity value and a molecular docking compound in the form of pdbqt. The result of complex visualization between receptor and ligand was observed using Biovia Discovery Studio Visualizer 2016 to see the interaction that occurred. To see the compound's potential as oral medicine, then the compounds which had the agonist interaction were processed with physicochemical analysis using the SwissADME web tool to find out the penetration capability.

RESULTS AND DISCUSSION

Measurement of MHC II expression

Figure 1 was the visualization result using the CLSM instrument, which displayed MHC II fluorescence intensity in the figure. IFNγ induction for 24 hours can activate nuclear factor kappa B (NF-κB) through toll-like receptor 4 (TLR4), which can affect cell protein synthesis, so that it can activate the microglia in M1 polarization and change its morphology into amoeboid, which cause the appearance of the inflammatory mediator, one of them is MHC II[16,17,37,38]. IFNγ plays the role of activating the microglia and can increase MHC II molecule expression as the transcription activator. MHC II plays a role in producing the exogen antigen, activating the T helper cell through the receptor, and secreting several cytokines to manage the immune response[39,40]. The strongest intensity was seen on the negative control, and the weakest intensity was seen on the positive control. In the negative control, treatment was not given, so it caused the HMC3 microglia cells to stay active on M1 polarization and produced high MHC II fluorescence intensity. All treatment groups showed lower MHC II fluorescence intensity compared to the negative control.

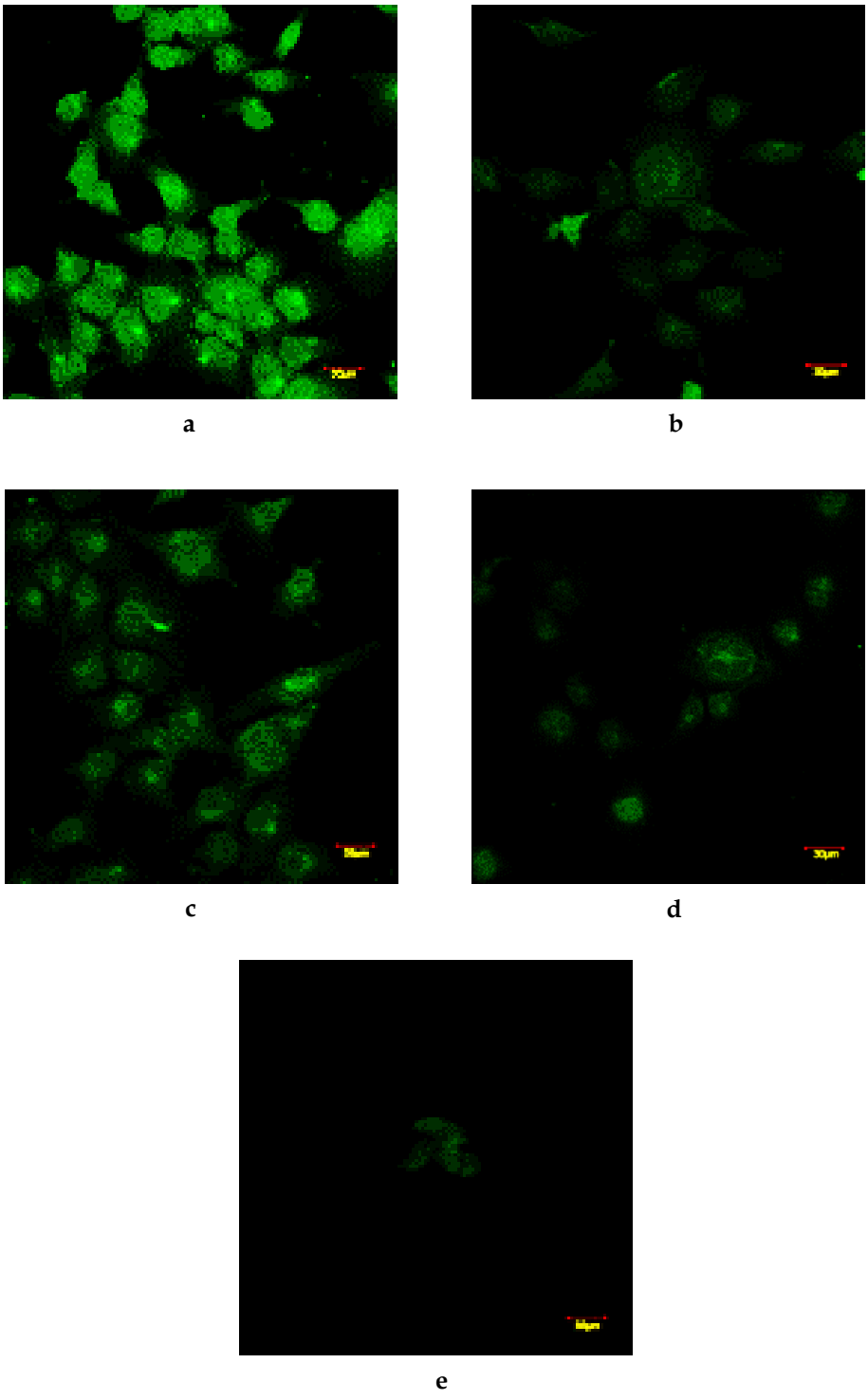


Figure 1. MHC II fluorescence intensity on HMC3 microglia cells (**a**) negative control; (**b**) 62.5 µg/mL; (**c**) 125 µg/mL; (**d**) 250 µg/mL; and (**e**) genistein.

Furthermore, **Figure 2** shows MHC II expressions comparison in the concentration of 62.5; 125; 250 µg/mL with negative control and positive control. In all concentrations, MHC II expression was lower compared to the negative control. The result of ANOVA test showed significant difference in MHC II expression reduction among all concentrations on negative control ($p=0.000$; $p=0.000$; and $p=0.000$) and on positive control ($p=0.000$; $p=0.000$; and $p=0.000$).

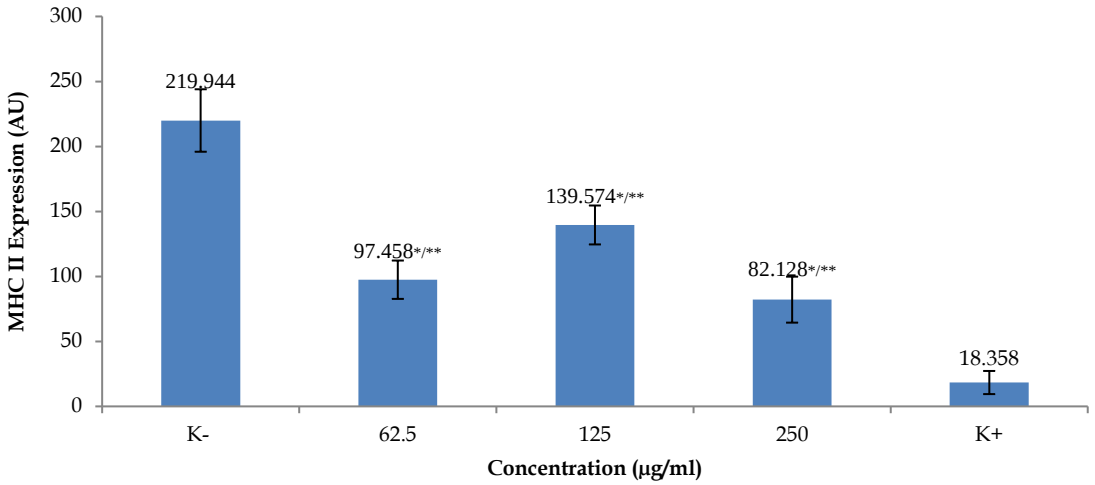


Figure 2. Expression of MHC II in 96% ethanol extract of *M. crenata* leaves. The * sign indicates a significant difference from the negative control (K-), while the ** sign indicates a significant difference from the positive control (K+).

In **Figure 2**, concentration increase was not accompanied by MHC II expression decrease. This probably happened because of the Non-Monotonic Dose Response (NMDR) phenomenon in HMC3 microglia cells, which was indicated by various slope values in the given concentration ranges[41]. NMDR often occurred in research by using hormone treatment or sample used as the hormone substitute, in this case, was phytoestrogen compound in 96% ethanol extract of the environmental controlled growth *M. crenata* leaves.

The affinity level difference between the hormone or sample that substituted the hormone and the receptor could cause difficulty in predicting the response following the increased concentration. Another factor causing NMDR was receptor downregulation and receptor desensitization; this may happen because the increasing concentration on the sample could cause the compound to bond with other receptors except for ER or make ER insensitive in bonding with the compound. However, the moment the concentration was continuously increased, it could increase the number of ER degraded and unequal to the produced ER, which caused the cell to produce ER massively and could increase the bond of the compound with ER and the activity response[41,42].

A 62.5 µg/mL concentration was the optimum concentration for reducing MHC II fluorescence intensity. This was because the concentration treatment showed a significant difference from the negative control and a big fluorescence intensity difference between the negative control value and the MHC II expression value of 97.458 Arbitrary Unit (AU). Concentrations 62.5 and 250 µg/mL were the concentrations that also could reduce MHC II expression well but did not differ from each other significantly in statistics, so 62.5 µg/mL was chosen as the most optimum concentration because the small concentration could produce a similar effect with the concentration of 250 µg/mL. The result showed that giving 96% ethanol extract to the controlled environmental growth of M. *crenata* leaves treatment reduced the number of MHC II, which was observed from the expression reduction.

This *in vitro* activity test was carried out to know the activity potential of a plant that was given to a cell[43]. The result of the in vitro test showed that the 96% ethanol extract of *M. crenata* leaves had antineuroinflammatory activity, which was indicated by a significant MHC II expression decrease. Then, to predict the compound contained in 96% ethanol extract of *M. crenata* leaves, metabolite profiling was conducted[44], and to predict the compound which had antineuroinflammatory activity, *in silico* analysis was carried out[45].

Metabolite profiling

The metabolite profiling result of 96% ethanol extract of the controlled environmental growth of M. crenata leaves by using UPLC-QToF-MS/MS instrument on dichloromethane and methanol solvents in the form of total ion chromatogram (TIC)

can be seen in **Figure 3**. In contrast, the value of retention time (RT), % area, m/z, molecule formula, and the compound's name can be overviewed in **Tables II** and **III**.

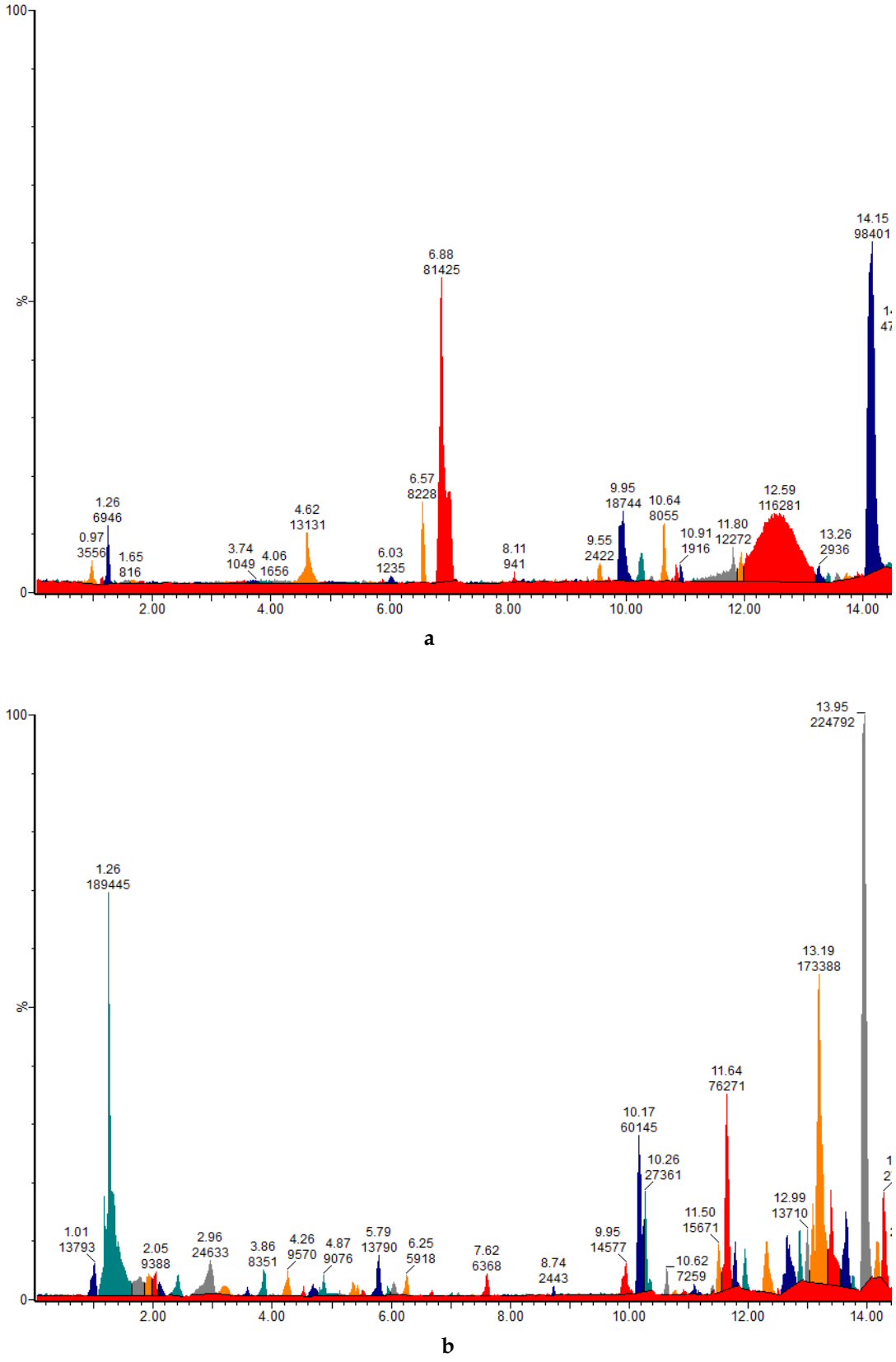
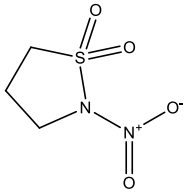
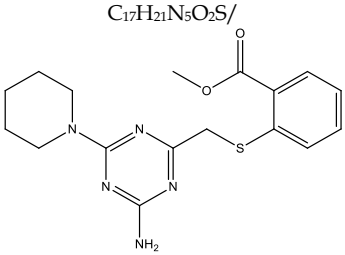
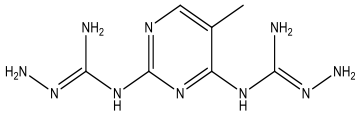
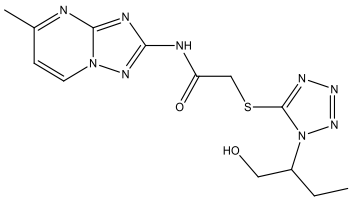
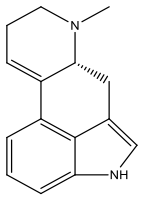
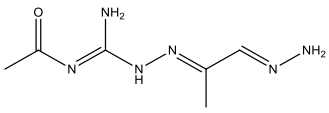
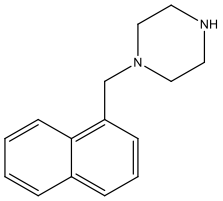
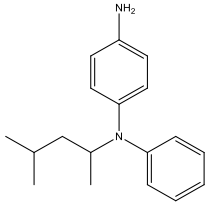


Figure 3. TIC of 96% ethanol extract of *M. crenata* leaves in solvent (**a**) dichloromethane and (**b**) methanol.

Table II. Prediction of compounds in 96% ethanol extract of *M. crenata* leaves in dichloromethane solvent

No.	RT (min)	% Area	m/z	Molecular Formula and Structure	Compound Name
1	0.971	0.8040	166.0053	$C_3H_6N_2O_4S$ 	2-Nitro-1,2-thiazolidine 1,1-dioxide
2	1.255	1.5705	359.1417	$C_{17}H_{21}N_5O_2S$/ 	Methyl 2-({[4-amino-6-(1-piperidinyl)-1,3,5-triazin-2-yl]methyl}sulfanyl)benzoate
3	4.058	0.3745	238.1403	$C_7H_{14}N_{10}$ 	N'',N'''''-(5-Methyl-2,4-pyrimidinediyl)dicarbonohydrazonic diamide
4	5.554	0.0696	363.1224	$C_{13}H_{17}N_9O_2S$ 	2-{[1-(1-Hydroxy-2-butanyl)-1H-tetrazol-5-yl]sulfanyl}-N-(5-methyl[1,2,4]triazolo[1,5-a]pyrimidin-2-yl)acetamide
5	6.028	0.3691	224.1309	$C_{15}H_{16}N_2$ 	6-Methyl-9,10-didehydroergoline
6	6.407	0.0311	184.1070	$C_6H_{12}N_6O$ 	N-{(E)-Amino[2-(1-hydrazono-2-propanylidene)hydrazino] methylene}acetamide
7	6.882	20.2706	226.1475	$C_{15}H_{18}N_2$ 	1-(1-Naphthylmethyl) piperazine
8	8.115	0.2128	268.1938	$C_{18}H_{24}N_2$ 	N-(4-Methyl-2-pentanyl)-N-phenyl-1,4-benzenediamine
9	9.548	0.5447	284.0996	$C_9H_8N_{12}$	5-Methyl-N-[1-(1H-1,2,4-triazol-5-yl)-1H-tetrazol-5-yl][1,2,4]triazolo[1,5-a]pyrimidin-2-amine

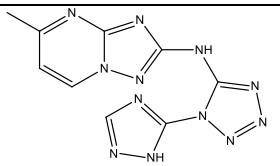
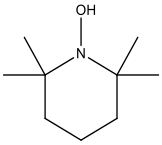
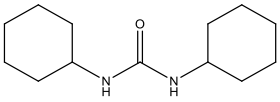
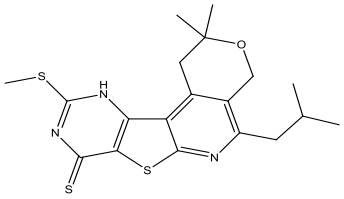
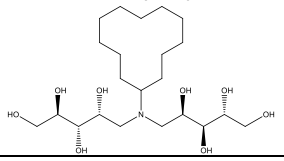
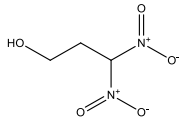
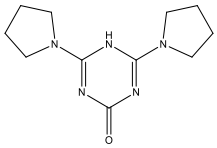
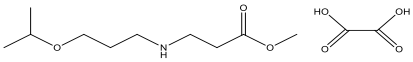
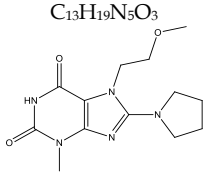
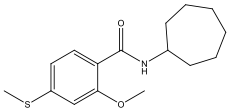
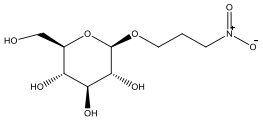
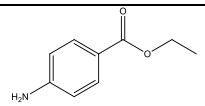
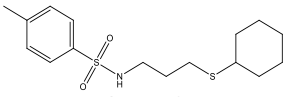
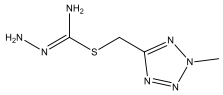
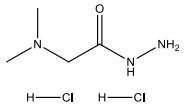
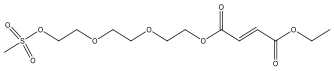
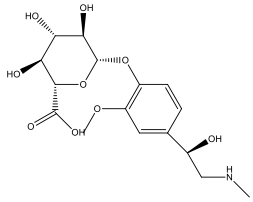
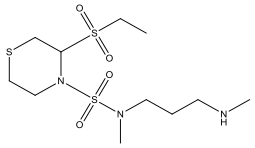
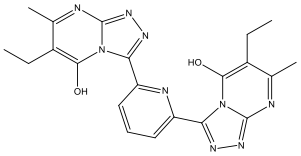
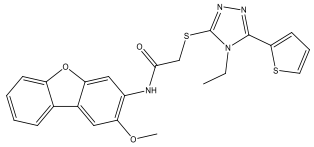
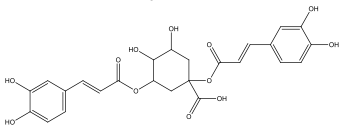
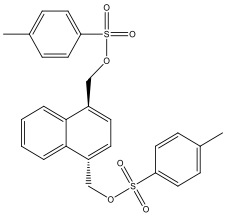
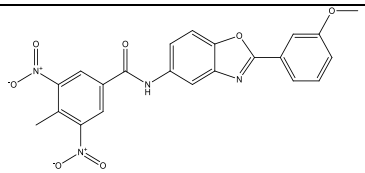
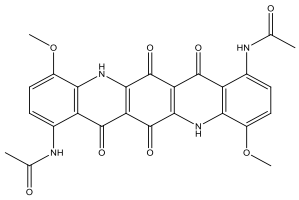
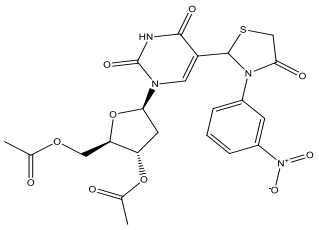
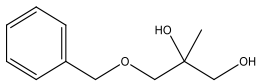
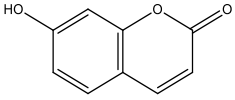
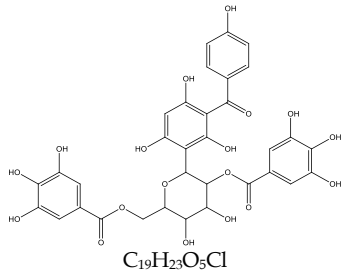
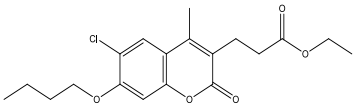
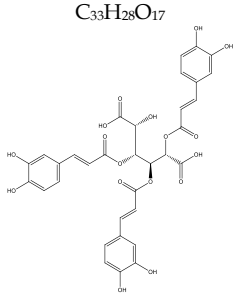
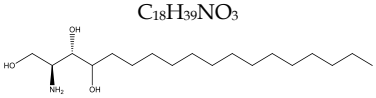
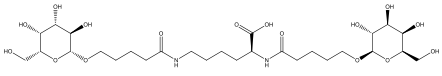
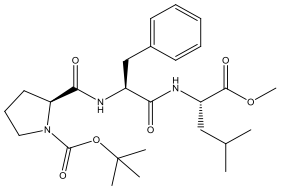
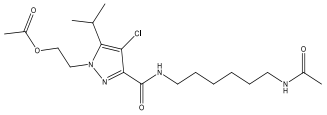
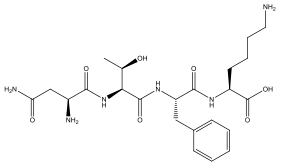
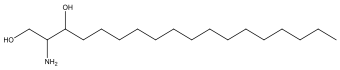
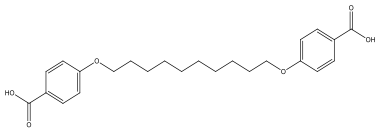
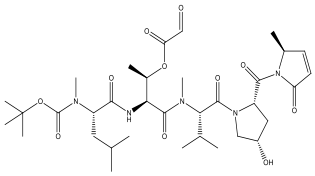
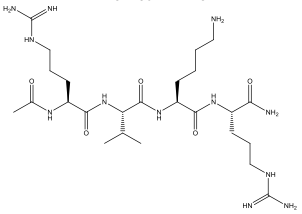
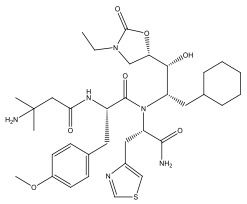
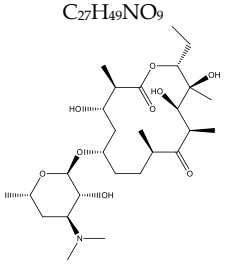
					
10	10.243	1.2945	157.1468	$C_9H_{19}NO$	2,2,6,6-Tetramethyl-4-piperidinol
					
11	10.422	0.1290	224.1886	$C_{13}H_{24}N_2O$	1,3-Dicyclohexylurea
					
12	13.256	0.6638	405.1003	$C_{19}H_{23}N_3OS_3$	5-Isobutyl-2,2-dimethyl-10-(methylsulfanyl)-1,4-dihydro-2H-pyrano[4'',3'':4',5']pyrido [3',2':4,5]thieno[3,2-d]pyrimidine-8(11H)-thione
					
13	13.551	0.2674	451.3150	$C_{22}H_{45}NO_8$	(2R,3S,4R,2'R,3'S,4'R)-5,5'-(Cyclododecylimino)di(1,2,3,4-pentanetetrol)
					

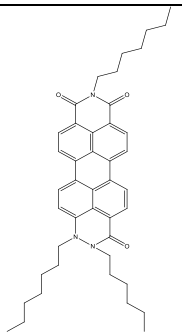
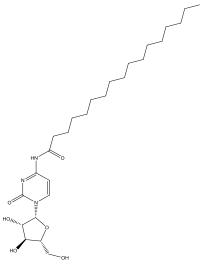
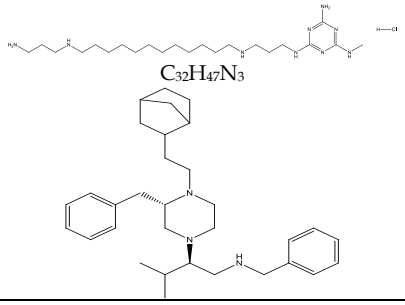
Table III. Prediction of compounds in 96% ethanol extract of *M. crenata* leaves in methanol solvent

No.	RT (min)	%Area	m/z	Molecular Formula and Structure	Compound Name
1	1.013	1.1178	150.0282	$C_3H_6N_2O_5$	3,3-Dinitro-1-propanol
					
2	1.255	15.3535	235.1431	$C_{11}H_{17}N_5O$	4,6-Di(1-pyrrolidinyl)-1,3,5-triazin-2(5H)-one
					
3	1.930	0.8740	293.1473	$C_{12}H_{23}NO_7$	Methyl N-(3-isopropoxypropyl)-β-alaninate ethanedioate (1:1)
					
4	2.046	0.7608	293.1491	$C_{13}H_{19}N_5O_3$	7-(2-Methoxyethyl)-3-methyl-8-(1-pyrrolidinyl)-3,7-dihydro-1H-purine-2,6-dione
					
5	2.109	0.3141	293.1454	$C_{16}H_{23}NO_2S$	N-Cycloheptyl-2-methoxy-4-(methylsulfanyl)benzamide
					
6	2.425	0.7979	267.0956	$C_9H_{17}NO_8$	Miserotoxin
					
7	2.962	1.9964	165.0790	$C_9H_{11}NO_2$	Benzocaine

					
8	3.183	0.4959	327.1324	$C_{16}H_{25}NO_2S_2$	N-[3-(Cyclohexylsulfanyl) propyl]-4-methylbenzene sulfonamide
					
9	3.858	0.6768	187.0639	$C_4H_9N_7S$	(2-Methyl-2H-tetrazol-5-yl)methyl carbamo hydrazonothioate
					
10	4.016	0.0231	189.0435	$C_4H_{13}N_3OCl_2$	Girard Reagent D dihydrochloride
					
11	4.258	0.7756	354.0980	$C_{13}H_{22}O_9S$	2,2-Dioxido-3,6,9-trioxa-2λ6-thiaundecan-11-yl ethyl (2E)-2-butenedioate
					
12	4.532	0.1558	373.1070	$C_{16}H_{23}NO_9$	(-)-Metanephrine glucuronide
					
13	4.870	0.7356	359.1002	$C_{11}H_{25}N_3O_4S_3$	3-(Ethylsulfonyl)-N-methyl-N-[3-(methylamino)propyl]-4-thiomorpholinesulfonamide
					
14	5.133	0.0955	431.1820	$C_{21}H_{21}N_9O_2$	3,3'-(2,6-Pyridinediyl)bis(6-ethyl-7-methyl[1,2,4]triazolo [4,3-a]pyrimidin-5-ol)
					
15	5.354	0.4942	464.0975	$C_{23}H_{20}N_4O_3S_2$	2-{[4-Ethyl-5-(2-thienyl)-4H-1,2,4-triazol-3-yl]sulfanyl}-N-(2-methoxydibenzo[b,d]furan-3-yl)acetamide
					
16	5.533	0.1073	516.1268	$C_{25}H_{24}O_{12}$	1,3-Bis{[(2E)-3-(3,4-dihydroxy phenyl)-2-propenoyl]oxy}-4,5-dihydroxycyclohexane carboxylic acid
					
17	5.786	1.1176	498.1174	$C_{26}H_{26}O_6S_2$	(1S,4S)-1,4-Dihydronaphthalene-1,4-diylbis(methylene) bis(4-methylbenzenesulfonate)
					
18	5.944	0.1634	448.1023	$C_{22}H_{16}N_4O_7$	N-[2-(3-Methoxyphenyl)-1,3-benzoxazol-5-yl]-4-methyl-3,5-dinitrobenzamide

					
19	6.049	0.3460	516.1278	$C_{26}H_{20}N_4O_8$	N,N'-(4,11-Dimethoxy-6,7,13,14-tetraoxo-5,6,7,12,13,14-hexahydroquinolino[2,3-b]acridine-1,8-diyl)diacetamide
					
20	6.249	0.4796	534.1054	$C_{22}H_{22}N_4O_{10}S$	3',5'-Di-O-acetyl-2'-deoxy-5-[3-(3-nitrophenyl)-4-oxo-1,3-thiazolidin-2-yl]uridine
					
21	6.682	0.1119	196.1099	$C_{11}H_{16}O_3$	3-(Benzyloxy)-2-methyl-1,2-propanediol
					
22	7.019	0.0473	162.0317	$C_9H_6O_3$	Umbelliferone
					
23	7.124	0.0409	712.1271	$C_{33}H_{28}O_{18}$	1,5-Anhydro-2,6-bis-O-(3,4,5-trihydroxybenzoyl)-1-[2,4,6-trihydroxy-3-(4-hydroxybenzoyl)phenyl]hexitol
					
24	7.345	0.0231	366.1232	$C_{19}H_{23}O_5Cl$	Ethyl 3-(7-butoxy-6-chloro-4-methyl-2-oxo-2H-chromen-3-yl)propanoate
					
25	7.619	0.5161	696.1328	$C_{33}H_{28}O_{17}$	3,4,5-Tris-O-[(2E)-3-(3,4-dihydroxyphenyl)-2-propenoyl]-D-glucaric acid
					
26	10.264	2.2174	317.2935	$C_{18}H_{39}NO_3$	(2S,3S)-2-Amino-1,3,4-octadecanetriol
					
27	10.622	0.5883	670.3158	$C_{28}H_{50}N_2O_{16}$	(2S)-2,6-Bis[(5-{[(2R,3R,4S,5R,6R)-3,4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2H-pyran-2-yl]oxy}pentanoyl)amino]hexanoic acid
					

28	10.781	0.0919	489.2842	$C_{26}H_{39}N_3O_6$ 	Methyl 1-{[(2-methyl-2-propanyl)oxy]carbonyl}prolylphenylalanyl leucinate
29	10.906	0.1012	241.2775	$C_{16}H_{35}N$ 	Cetylamine
30	11.085	0.2922	414.2036	$C_{19}H_{31}N_4O_4Cl$ 	2-{3-[(6-Acetamidohexyl) carbamoyl]-4-chloro-5-isopropyl-1H-pyrazol-1-yl}ethyl acetate
31	11.402	0.0841	508.2642	$C_{23}H_{36}N_6O_7$ 	Asparaginylthreonylphenylalanyllysine
32	11.497	1.2700	301.2985	$C_{18}H_{39}NO_2$ 	Safingol
33	11.644	6.1813	414.2043	$C_{24}H_{30}O_6$ 	4,4'-[1,10-Decanediylbis(oxy)] dibenzoic acid
34	11.781	0.9642	693.3952	$C_{34}H_{55}N_5O_{10}$ 	(2R,3S)-4-[{(2S)-1-[(2S,4S)-4-Hydroxy-2-{[(2S)-2-methyl-5-oxo-2,5-dihydro-1H-pyrrol-1-yl]carbonyl}-1-pyrrolidinyl]-3-methyl-1-oxo-2-butanyl} (methyl)amino]-3-[(N-methyl-N-{[(2-methyl-2-propanyl)oxy] car bonyl}-L-leucyl)amino]-4-oxo-2-butanyl oxoacetate
35	12.297	2.1360	598.4022	$C_{25}H_{50}N_{12}O_5$ 	N2-Acetyl-L-arginyl-L-valyl-L-lysyl-L-argininamide
36	12.497	0.0803	700.3621	$C_{35}H_{52}N_6O_7S$ 	N-(3-Amino-3-methylbutanoyl)-O-methyl-L-tyrosyl-N-{(1R,2S)-3-cyclohexyl-1-[(5S)-3-ethyl-2-oxo-1,3-oxazolidin-5-yl]-1-hydroxy-2-propanyl}-3-(1,3-thiazol-4-yl)-L-alaninamide
37	12.877	1.1958	531.3407	$C_{27}H_{49}NO_9$ 	(3R,4S,6S,9R,11R,12R,13S,14R)-6-{[(2S,3R,4S,6S)-4-(Dimethylamino)-3-hydroxy-6-methyltetrahydro-2H-pyran-2-yl]oxy}-14-ethyl-4,12,13-trihydroxy-3,9,11,13-tetramethyloxacyclotetradecane-2,10-dione (non-p referred name)
38	12.993	1.1111	671.4090	$C_{44}H_{53}N_3O_3$	1,2,9-Triheptyl-1,2-dihydroisoquinolino[4',5',6':6,5,10]anthra[2,1,9-def]cinnoline-3,8,10(9H)-trione

39	13.193	14.0522	495.3313		$C_{26}H_{45}N_3O_6$	1-(β-D-Arabinofuranosyl)-4-(heptadecanoylamino)-2(1H)-pyrimidinone
40	13.646	2.6446	473.3724		$C_{22}H_{48}N_9Cl$	N2-[3-({12-[(3-Aminopropyl)amino]dodecyl}amino)propyl]-N4-methyl-1,3,5-triazine-2,4,6-triamine hydrochloride (1:1)
41	13.951	18.2182	473.3770		$C_{32}H_{47}N_3$	(2R)-N-Benzyl-2-{(3S)-3-benzyl-4-[2-(bicyclo[2.2.1]hept-2-yl)ethyl]-1-piperazinyl}-3-methyl-1-butanamine

The result of metabolite profiling showed a total of 79 compounds in dichloromethane and methanol solvents which consisted of 54 known and 25 unknown compounds. The use of the two solvents aimed at eluting the extract optimally in the column of UPLC-QToF-MS/MS[27]. The compound peak analyzed from the result of metabolite profiling was the RT peak between 0 and 14 minutes. The RT peak above 14 minutes could not be considered because the peak was generally impure, like the peak produced from solvent or degradant. From the total of detected 79 compounds, not all peaks in TIC could be identified in the process of metabolite profiling. This was shown by the appearance of 25 unknown compounds. Unknown compounds could not be identified in the database; these compounds could be in the form of impure compounds or degradant which were still detected by the instrument or new compounds which were still not listed in the database, especially unknown compounds which had high content[27,46].

In silico study

The 79 compounds from metabolite profiling of 96% ethanol extract of the controlled environmental growth *M. crenata* leaves were then analyzed through molecular docking using PyRx 0.8 software and AutoDock Vina as the docking simulator. Based on the native ligand test (17β-estradiol) using 3OLS protein, the value of RMSD 1.238 Å was retrieved, which showed that RMSD <2 Å means the docking protocol could be used in the docking process of resulting compounds from metabolite profiling using 3OLS protein[47,48]. After that, the bond between native ligand and compound towards 3OLS protein was visualized using Biovia Discovery Visualizer 2016 software. Based on the analysis of molecular docking 17β-estradiol result on 3OLS protein, it was found that the compound was categorized as an ERβ agonist compound if it met several parameters similar to 17β-estradiol. These parameters consisted of a pharmacophore cluster that bonded His 475 amino acid to Glu 305 amino acid or Arg 346 amino acid, which can be viewed in **Figure 4**. The type of bond to the amino acid also influences the bonds' stability. This interaction describes the binding strength of the ligand to the receptor. The

hydrogen bonds can stabilize the interaction between ligands and receptors, in addition to van der walls and electrostatic bonds[49]. Besides, they also had a pharmacophore distance similar to 17β-estradiol, approximately 10.862 Å. The similarity in pharmacophore distance from pharmacophore cluster on 17β-estradiol could be used as a guideline to predict other compounds with the same pharmacological effects[50-52]. The analysis using Discovery Studio Visualizer 2016 of 79 compounds resulted from metabolite profiling of 96% ethanol extract of the controlled environmental growth of *M. crenata* leaves can be seen in **Table IV**.

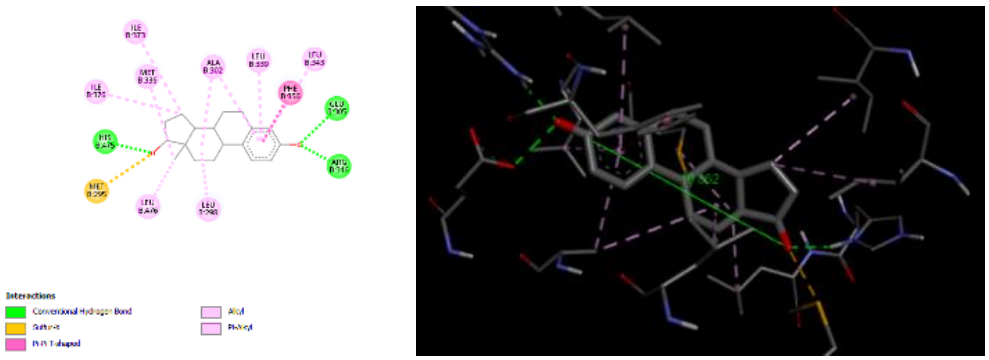


Figure 4. Interaction of 17β-estradiol with 3OLS protein.

Table IV. 17β-estradiol and compounds in 96% ethanol extract of *M. crenata* leaves which are agonists against ERβ

No.	Compound Name	% Area	*Binding Affinity* (kkal/ mol)	Amino Acid (Type of Bond)	Pharmacophore Distance (Å)
-	17β-estradiol	-	-10.5	His 475 (Hydrogen) Glu 305 (Hydrogen) Arg 346 (Hydrogen)	10.862
1	Methyl 2-({[4-amino-6-(1-piperidinyl)-1,3,5-triazin-2-yl]methyl} sulfanyl)benzoate	1.5705	-3.8	His 475 (Pi-Alkyl) Glu 305 (Carbon)	8.596
2	N'',N''''-(5-Methyl-2,4-pyrimidinediyl) dicarbonohydrazonic diamide	0.3745	-7.1	His 475 (Unfavorable) Arg 346 (Unfavorable) Glu 305 (Salt Bridge)	9.521
3	N-{(E)-Amino[2-(1-hydrazono-2-propanylidene) hydrazino]methylene}acetamide	0.0311	-3.6	His 475 (Unfavorable) Glu 305 (Attractive Charge)	6.649
4	N-(4-Methyl-2-pentanyl)-N-phenyl-1,4-benzenediamine	0.2128	-7.9	His 475 (Alkyl) Glu 305 (Hydrogen)	10.05
5	7-(2-Methoxyethyl)-3-methyl-8-(1-pyrrolidinyl)-3,7-dihydro-1H-purine-2,6-dione	0.7608	-4.5	His 475 (Pi-Alkyl) Glu 305 (Hydrogen)	7.898
6	N-Cycloheptyl-2-methoxy-4-(methylsulfanyl) benzamide	0.3141	-2.2	His 475 (Unfavorable) Arg 346 (Sulfur)	11.430
7	Miserotoxin	0.7979	-6.1	His 475 (Unfavorable) Glu305 (Carbon) Arg 346 (Hydrogen)	10.377
8	(2-Methyl-2H-tetrazol-5-yl)methyl carbamo hydrazonothioate	0.6768	-5.3	His 475 (Unfavorable) Glu 305 (Carbom)	8.124

9	2,2-Dioxido-3,6,9-trioxa-2λ6-thiaundecan-11-yl ethyl (2E)-2-butenedioate	0.7756	-3.5	His 475 (Hydrogen) Arg 346 (Hydrogen)	10.887
10	(-)-Metanephrine glucuronide	0.1558	-2.8	His 475 (Hydrogen) Glu 305 (Hydrogen)	10.377
11	3-(Ethylsulfonyl)-N-methyl-N-[3-(methylamino)propyl]-4-thiomorpholinesulfonamide	0.7356	-5.6	His 475 (Hydrogen) Glu 305 (Hydrogen)	8.436
12	2-{[4-Ethyl-5-(2-thienyl)-4H-1,2,4-triazol-3-yl]sulfanyl}-N-(2-methoxydibenzo[b,d]furan-3-yl)acetamide	0.4942	1.9	His 475 (Unfavorable) Glu 305 (Unfavorable)	10.936
13	(1S,4S)-1,4-Dihydronaphthalene-1,4-diylbis(methylene) bis(4-methylbenzenesulfonate)	1.1176	4	His 475 (Hydrogen) Glu305 (Unfavorable) Arg 346 (Hydrogen)	11.179
14	3',5'-Di-O-acetyl-2'-deoxy-5-[3-(3-nitrophenyl)-4-oxo-1,3-thiazolidin-2-yl]uridine	0.4796	20.1	His 475 (Hydrogen) Glu 305 (Unfavorable)	10.025
15	1,5-Anhydro-2,6-bis-O-(3,4,5-trihydroxybenzoyl) -1-[2,4,6-trihydroxy-3-(4-hydroxybenzoyl)phenyl]hexitol	0.0409	85.6	His 475 (Pi-Sigma) Glu 305 (Attractive charge)	10.019
16	Ethyl 3-(7-butoxy-6-chloro-4-methyl-2-oxo-2H-chromen-3-yl)propanoate	0.0231	-4.5	His 475 (Hydrogen) Glu 305 (Carbon)	10.063
17	3,4,5-Tris-O-[(2E)-3-(3,4-dihydroxyphenyl)-2-propenoyl]-D-glucaric acid	0.5161	20.4	His 475 (Hydrogen) Glu 305 (Hydrogen) Arg 346 (Hydrogen)	11.026
18	N2-Acetyl-L-arginyl-L-valyl-L-lysyl-L-argininamide	2.1360	11.1	His 475 (Unfavorable) Glu 305 (Hydrogen)	10.268
19	N-(3-Amino-3-methylbutanoyl)-O-methyl-L-tyrosyl-N-{(1R,2S)-3-cyclohexyl-1-[(5S)-3-ethyl-2-oxo-1,3-oxazolidin-5-yl]-1-hydroxy-2-propanyl}-3-(1,3-thiazol-4-yl)-L-alaninamide	0.0803	26.3	His 475 (Unfavorable) Glu 305 (Hydrogen)	12.6

The result of *in silico* analysis showed that 19 compounds had the agonist characteristics towards 3OLS protein, which meant that those compounds belonged to phytoestrogen. To predict the compound potential as oral medicine, ERβ agonist compounds were then selected using the SwissADME web tool to identify the physicochemical properties of the compounds (The result of the SwissADME test can be seen on https://doi.org/10.5281/zenodo.6904891). The parameters used in the physicochemical analysis were molecule weight < 500 g/mol, HBD (hydrogen binding donors) < 5, HBA (hydrogen binding acceptors) < 10, TPSA ≤ 140 Å[53], met Lipinski rule of five[54], and BBB permeant "yes"[55]. The TPSA value showed the compounds' capability to penetrate the cell membrane, and the BBB permeant showed the compound's capability to penetrate the blood-brain barrier[53,55]. Lipinski's rule of five was used to predict the similarity of compounds and medicine, which had a specific biological activity designed for oral treatment[53].

The analysis result of the TPSA parameter found 11 compounds that met those criteria. Based on the analysis of the Lipinski rule of five, it was found that 14 compounds met those criteria. At the same time, the analysis result of the BBB permeant parameter found that three compounds met those criteria. This indicates that these three compounds may influence the

CNS. Thus, the result of the physicochemical analysis showed that three ERβ agonist compounds met all parameters: TPSA, Lipinski rule of five, and BBB permeant, which can be seen in **Table V**.

Table V. Agonist compound that met all parameters of physicochemical analysis

No.	Compound name	Molecule weight (g/mol)	HBD	HBA	BBB Permeant	TPSA ≤ 140	Lipinski Rule of 5
1.	N-(4-Methyl-2-pentanyl)-N-phenyl-1,4-benzenediamine	268.40	1	0	Yes	29.26	Yes
2.	N-Cycloheptyl-2-methoxy-4-(methylsulfanyl)benzamide	293.42	1	2	Yes	63.63	Yes
3.	Ethyl 3-(7-butoxy-6-chloro-4-methyl-2-oxo-2H-chromen-3-yl)propanoate	366.84	0	5	Yes	65.74	Yes

The result of the physicochemical analysis above implied that those compounds were categorized as phytoestrogen, which was indicated by the agonist interaction with 3OLS protein and had the potential to be developed as antineuroinflammatory medicine given orally, which was shown by meeting physicochemical analysis parameters[56,57]. The correlation of these research findings was to prove that 96% ethanol extract of the controlled environmental growth of *M. crenata* leaves had *in vitro* antineuroinflammatory activity, which was shown by a significant decrease in MHC II expression, and supported by the prediction of 19 secondary metabolite compounds as the result of metabolite profiling on 96% ethanol extract of the controlled environmental growth of *M. crenata* which had *in silico* antineuroinflammatory activity and three of those compounds had the potential to be developed as oral medicine. The correlation result showed that the use of cultivated *M. crenata* had the advantage of decreasing MHC II expression significantly and contained more active compounds because of external factors control which could affect the compound content of the plant[58].

CONCLUSION

The 96% ethanol extract of the environmental-controlled growth of *M. crenata* has an antineuroinflammatory activity through MHC II expression inhibition on HMC3 microglia cells, with an optimum concentration of 62.5 µg/mL and a value of 97.458 AU. This extract was predicted to contain 19 phytoestrogen compounds with agonist characteristics on ERβ, and three met all parameters of physicochemical analysis, including BBB permeant.

ACKNOWLEDGMENT

None.

AUTHORS' CONTRIBUTION

All authors have an equal contribution in carrying out this study.

DATA AVAILABILITY

The result of the SwissADME test can be seen on https://doi.org/10.5281/zenodo.6904891.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest in this research.

REFERENCES

1. Kalhan M, Singhania K, Choudhary P, Verma S, Kaushal P, Singh T. Prevalence of Menopausal Symptoms and its Effect on Quality of Life among Rural Middle Aged Women (40-60 Years) of Haryana, India. Int J Appl Basic Med Res. 2020;10(3):183-8. doi:10.4103/ijabmr.ijabmr_428_19

2. Webster AD, Finstad DA, Kurzer MS, Torkelson CJ. Quality of life among postmenopausal women enrolled in the Minnesota Green Tea Trial. Maturitas. 2018;108:1-6. doi:10.1016/j.maturitas.2017.10.013

3. Silva TR, Oppermann K, Reis FM, Spritzer PM. Nutrition in Menopausal Women: A Narrative Review. Nutrients. 2021;13(7):2149. doi:10.3390/nu13072149

4. Dalal PK, Agarwal M. Postmenopausal syndrome. Indian J Psychiatry. 2015;57(Suppl 2):S222-32. doi:10.4103/0019-5545.161483

5. Kwon HS, Koh SH. Neuroinflammation in neurodegenerative disorders: the roles of microglia and astrocytes. Transl Neurodegener. 2020;9(1):42. doi:10.1186/s40035-020-00221-2

6. Au A, Feher A, McPhee L, Jessa A, Oh S, Einstein G. Estrogens, inflammation and cognition. Front Neuroendocrinol. 2016;40:87–100. doi:10.1016/j.yfrne.2016.01.002

7. Matt SM, Johnson RW. Neuro-immune dysfunction during brain aging: new insights in microglial cell regulation. Curr Opin Pharmacol. 2016;26:96–101. doi:10.1016/j.coph.2015.10.009

8. Arcuri C, Mecca C, Bianchi R, Giambanco I, Donato R. The pathophysiological role of microglia in dynamic surveillance, phagocytosis and structural remodeling of the developing CNS. Front Mol Neurosci. 2017;10:191. doi:10.3389/fnmol.2017.00191

9. Prieto GA, Cotman CW. Cytokines and cytokine networks target neurons to modulate long-term potentiation. Cytokine Growth Factor Rev. 2017;34:27-33. doi:10.1016/j.cytogfr.2017.03.005

10. Radtke FA, Chapman G, Hall J, Syed YA. Modulating neuroinflammation to treat neuropsychiatric disorders. Biomed Res Int. 2017;2017:5071786. doi:10.1155/2017/5071786

11. Dong Y, Li X, Cheng J, Hou L. Drug development for alzheimer's disease: Microglia induced neuroinflammation as a target? Int J Mol Sci. 2019;20(3):558. doi:10.3390/ijms20030558

12. Wixey JA, Reinebrant HE, Buller KM. Post-insult ibuprofen treatment attenuates damage to the serotonergic system after hypoxia-ischemia in the immature rat brain. J Neuropathol Exp Neurol. 2012;71(12):1137–48. doi:10.1097/nen.0b013e318277d4c7

13. Garrido-Mesa N, Zarzuelo A, Gálvez J. Minocycline: Far beyond an antibiotic. Br J Pharmacol. 2013;169(2):337–52. doi:10.1111/bph.12139

14. Zheng X, Yue P, Liu L, Tang C, Ma F, Zhang Y, et al. Efficacy between low and high dose aspirin for the initial treatment of Kawasaki disease: Current evidence based on a meta-analysis. PLoS One. 2019;14(5):e0217274. doi:10.1371/journal.pone.0217274

15. Rietjens IMCM, Louisse J, Beekmann K. The potential health effects of dietary phytoestrogens. Br J Pharmacol. 2017;174(11):1263-80. doi:10.1111/bph.13622

16. Jantaratnotai N, Utaisincharoen P, Sanvarinda P, Thampithak A, Sanvarinda Y. Phytoestrogens mediated anti-inflammatory effect through suppression of IRF-1 and pSTAT1 expressions in lipopolysaccharide-activated microglia. Int Immunopharmacol. 2013;17(2):483–8. doi:10.1016/j.intimp.2013.07.013

17. Villa A, Vegeto E, Poletti A, Maggi A. Estrogens, neuroinflammation, and neurodegeneration. Endocr Rev. 2016;37(4):372–402. doi:10.1210/er.2016-1007

18. Ma'arif B, Fitri H, Saidah NL, Najib LA, Yuwafi AH, Atmaja RRD, et al. Prediction of compounds with antiosteoporosis activity in Chrysophyllum cainito L. leaves through in silico approach. J Basic Clin Physiol Pharmacol. 2021;32(4):803–8. doi:10.1515/jbcpp-2020-0393

19. Desmawati D, Sulastri D. Phytoestrogens and Their Health Effect. Open AccessMaced J Med Sci. 2019;7(3):495-9. doi:10.3889/oamjms.2019.044

20. Liu T, Li N, Yan YQ, Liu Y, Xiong K, Liu Y, et al. Recent advances in the anti-aging effects of phytoestrogens on collagen, water content, and oxidative stress. Phytother Res. 2020;34(3):435-47. doi:10.1002/ptr.6538

21. Ma'arif B, Aditama AP. Activity of 96% Ethanol Extract of Chrysophyllum Cainito L. in Increasing Vertebrae Trabecular Osteoblast Cell Number in Male Mice. Asian J Pharm Clin Res. 2019;12(1):286-8. doi:10.22159/ajpcr.2019.v12i1.28994

22. Agil M, Laswati H, Kuncoro H, Ma'arif B. In silico Analysis of Phytochemical Compounds in Ethyl Acetate Fraction of Semanggi (Marsilea crenata Presl.) Leaves As Neuroprotective Agent. Res J Pharm Technol. 2020;13(8):3745–52. doi:10.5958/0974-360x.2020.00663.0

23. Putra HL. Green clover Potentiates Delaying the Increment of Imbalance Bone Remodeling Process in Postmenopausal Women. Folia Medica Indonesiana. 2011;47(2):112–7.

24. Nurjanah, Azka A, Abdullah A. Aktivitas Antioksidan Dan Komponen Bioaktif Semanggi Air (Marsilea Crenata). Asian J Innov Entrep. 2012;1(3):152–8. doi:10.20885/ajie.vol1.iss3.art2

25. Ma 'arif B, Agil M, Laswati H. Phytochemical Assessment on N-Hexane Extract and Fractions of Marsilea crenata Presl. Leaves through GC-MS. Trad Med J. 2016;21(2):77–85. doi:10.22146/tradmedj.12821

26. Ma'arif B, Agil M, Laswati H. Alkaline phosphatase activity of Marsilea crenata Presl. extract and fractions as marker of MC3T3-E1 osteoblast cell differentiation. J Appl Pharm Sci. 2018;8(3):55–9. doi:10.7324/JAPS.2018.8308

27. Ma'arif B, Mirza DM, Suryadinata A, Muchlisin MA, Laswati H, Agil M. Metabolite Profiling of 96% Ethanol Extract from Marsilea crenata Presl. Leaves Using UPLC-QToF-MS/MS and Anti-Neuroinflammatory Predicition Activity with Molecular Docking. J Trop Pharm Chem. 2019;4(6):261–70. doi:10.25026/jtpc.v4i6.213

28. Ma'arif B, Agil M, Laswati H. The enhancement of Arg1 and activated ERβ expression in microglia HMC3 by induction of 96% ethanol extract of Marsilea crenata Presl. leaves. J Basic Clin Physiol Pharmacol. 2019;30(6):20190284. doi:10.1515/jbcpp-2019-0284

29. Agil M, Kusumawati I, Purwitasari N. Phenotypic Variation Profile of Marsilea crenata Presl. Cultivated in Water and in the Soil. J Botany. 2017;2017:7232171. doi:10.1155/2017/7232171

30. Opačić N, Radman S, Uher SF, Benko B, Voća S, Žlabur JŠ. Nettle Cultivation Practices-From Open Field to Modern Hydroponics: A Case Study of Specialized Metabolites. Plants. 2022;11(4):483. doi:10.3390/plants11040483

31. Fussy A, Papenbrock J. An Overview of Soil and Soilless Cultivation Techniques-Chances, Challenges and the Neglected Question of Sustainability. Plants. 2022;11(9):1153. doi:10.3390/plants11091153

32. Chen SL, Yu H, Luo HM, Wu Q, Li CF, Steinmetz A. Conservation and sustainable use of medicinal plants: Problems, progress, and prospects. Chin Med. 2016;11:37. doi:10.1186/s13020-016-0108-7

33. Ma'arif B, Suleman HF, Annisa R, Dianti MR, Laswati H, Agil M. Efek Antineuroinflamasi Ekstrak Etanol 96% Daun Marsilea crenata Presl. Budidaya Papda Sel Mikroglia HMC3. J Farmasi Udayana. 2020;9(2):91-9. doi:10.24843/JFU.2020.v09.i02.p04

34. Engler-Chiurazzi EB, Brown CM, Povroznik JM, Simpkins JW. Estrogens as neuroprotectants: Estrogenic actions in the context of cognitive aging and brain injury. Prog Neurobiol. 2017;157:188–211. doi:10.1016/j.pneurobio.2015.12.008

35. Muchtaridi M, Dermawan D, Yusuf M. Molecular docking, 3D structure-based pharmacophore modeling, and ADME prediction of alpha mangostin and its derivatives against estrogen receptor alpha. J Young Pharm. 2018;10(3):252–9. doi:10.5530/jyp.2018.10.58

36. Rettberg J, Yao J, Brnton R. Estrogen: A master regulator of bioenergetic systems in the brain and body. Front Neuroendocrinol. 2014;35(1):515–25. doi:10.1016/j.yfrne.2013.08.001

37. Tang Y, Le W. Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases. Mol Neurobiol. 2016;53(2):1181–94. doi:10.1007/s12035-014-9070-5

38. Papageorgiou IE, Lewen A, Galow LV, Cesetti T, Scheffel J, Regen T, et al. TLR4-activated microglia require IFN-γ to induce severe neuronal dysfunction and death in situ. Proc Natl Acad Sci U S A. 2016;113(1):212-7. doi:10.1073/pnas.1513853113

39. Cherry JD, Olschowka JA, O'Banion MK. Neuroinflammation and M2 microglia: The good, the bad, and the inflamed. J Neuroinflammation. 2014;11:98. doi:10.1186/1742-2094-11-98

40. Shih RH, Wang CY, Yang CM. NF-kappaB signaling pathways in neurological inflammation: A mini review. Front Mol Neurosci. 2015;8:77. doi:10.3389/fnmol.2015.00077

41. Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Lee DH, et al. Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. Endocr Rev. 2012;33(3):378–455. doi:10.1210/er.2011-1050

42. Lagarde F, Beausoleil C, Belcher SM, Belzunces LP, Emond C, Guerbet M, et al. Non-monotonic dose-response relationships and endocrine disruptors: A qualitative method of assessment. Environ Health. 2015;14:13. doi:10.1186/1476-069x-14-13

43. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. J Pharm Anal. 2016;6(2):71–9. doi:10.1016/j.jpha.2015.11.005

44. Lu J, Muhmood A, Czekała W, Mazurkiewicz J, Dach J, Dong R. Untargeted metabolite profiling for screening bioactive compounds in digestate of manure under anaerobic digestion. Water. 2019;11(11):2420. doi:10.3390/w11112420

45. Duarte AJ, Ribeiro D, Moreira L, Amaral O. In silico analysis of missense mutations as a first step in functional studies: Examples from two sphingolipidoses. Int J Mol Sci. 2018;19(11):3409. doi:10.3390/ijms19113409

46. Aditama APR, Ma'arif B, Mirza DM, Laswati H, Agil M. In vitro and in silico analysis on the bone formation activity of N-hexane fraction of Semanggi (Marsilea crenata Presl.). Syst Rev Pharm. 2020;11(11):837–49.

47. Kartika IGAA, Bang IJ, Riani C, Insanu M, Kwak JH, Chung KH, et al. Isolation and Characterization of Phenylpropanoid and Lignan Compounds from Peperomia pellucida [L.] Kunth with Estrogenic Activities. Molecules. 2020;25(21):4914. doi:10.3390/molecules25214914

48. Pinto VdS, Araújo JSC, Silva RC, da Costa GV, Cruz JN, Neto MFDA, et al. In silico study to identify new antituberculosis molecules from natural sources by hierarchical virtual screening and molecular dynamics simulations. Pharmaceuticals. 2019;12(1):36. doi:10.3390/ph12010036

49. Chen D, Oezguen N, Urvil P, Ferguson C, Dann SM, Savidge TC. Regulation of protein-ligand binding affinity by hydrogen bond pairing. Sci Adv. 2016;2(3):e1501240. doi:10.1126/sciadv.1501240

50. Sellami A, Montes M, Lagarde N. Predicting Potential Endocrine Disrupting Chemicals Binding to Estrogen Receptor α (ERα) Using a Pipeline Combining Structure-Based and Ligand-Based in Silico Methods. Int J Mol Sci. 2021;22(6):2846. doi:10.3390/ijms22062846

51. Vourinen A, Engeli R, Meyer A, Bachmann F, Griesser UJ, Schuster D, et al. Ligand-based pharmacophore modeling and virtual screening for the discovery of novel 17β-hydroxysteroid dehydrogenase 2 inhibitors. J Med Chem. 2014;57(14):5995-6007. doi:10.1021/jm5004914

52. Kaserer T, Beck KR, Akram M, Odermatt A, Schuster D. Pharmacophore Models and Pharmacophore-Based Virtual Screening: Concepts and Applications Exemplified on Hydroxysteroid Dehydrogenases. Molecules. 2015;20(12):22799-832. doi:10.3390/molecules201219880

53. Truong J, George A, Holien JK. Analysis of physicochemical properties of protein-protein interaction modulators suggests stronger alignment with the "rule of five". RSC Med Chem. 2021;12(10):1731-49. doi:10.1039/d1md00213a

54. Benet LZ, Hosey CM, Ursu O, Oprea TI. BDDCS, the Rule of 5 and Drugability. Adv Drug Deliv Rev. 2016;101:89-98. doi:10.1016/j.addr.2016.05.007

55. Geldenhuys WJ, Mohammad AS, Adkins CE, Lockman PR. Molecular determinants of blood-brain barrier permeation. Ther Deliv. 2015;6(8):961-71. doi:10.4155/tde.15.32

56. Daina A, Zoete V. A BOILED-Egg To Predict Gastrointestinal Absorption and Brain Penetration of Small Molecules. ChemMedChem. 2016;11(11):1117–21. doi:10.1002/cmdc.201600182

57. Chagas CM, Moss S, Alisaraie L. Drug metabolites and their effects on the development of adverse reactions: Revisiting Lipinski's Rule of Five. Int J Pharm. 2018;549(1–2):133–49. doi:10.1016/j.ijpharm.2018.07.046

58. Yang L, Wen KS, Ruan X, Zhao YX, Wei F, Wang Q. Response of plant secondary metabolites to environmental factors. Molecules. 2018;23(4):762. doi:10.3390/molecules23040762

Borneo Journal of Pharmacy
Vol 5 Issue 3 August 2022
Page 229 – 246

http://journal.umpalangkaraya.ac.id/index.php/bjop/article/view/2095
DOI: https://doi.org/10.33084/bjop.v5i3.2095
e-ISSN: 2621-4814

Review Article

Monoclonal Antibodies: A Therapeutic Option for the Treatment of Ophthalmic Diseases of the Eye Posterior Segment

Catalina Ayón [1]
Daniel Castán [1]
Adrián Mora [1]
Dunia Naranjo [1]
Francini Obando [1]
Juan José Mora [2*]

[1] Bachelor Programme of Pharmacy, Universidad de Costa Rica, San Pedro Montes de Oca, San José, Costa Rica

[2] Department of Industrial Pharmacy, Universidad de Costa Rica, San Pedro Montes de Oca, San José, Costa Rica

*email: juanjose.moraroman@ucr.ac.cr

Keywords:
Adverse effects
Eye
Intravitreal therapy
Monoclonal antibody
Posterior segment disease

Abstract

The eye is an organ that allows us to observe the outside world. Pathologies of the eye's posterior segment, such as glaucoma, macular degeneration, diabetic retinopathy, uveitis, and retinoblastoma, cause vision loss. Traditional treatments consist of applying topical medications that do not penetrate properly or using high doses that generate adverse effects. Different laser surgeries stop the pathology's progression but do not allow visual improvement. So, an alternative is to use monoclonal antibodies, proteins produced by different processes that selectively bind to metabolites associated with diseases, reducing the adverse effects of traditional treatments and improving the application of the drug in the area. The two main molecular targets are TNF (adalimumab, infliximab, and certolizumab pegol) and VEGF (bevacizumab and ranibizumab); other possibilities are under investigation.

Received: February 4th, 2021
1st Revised: June 1st, 2022
Accepted: June 19th, 2022
Published: August 31th, 2022

INTRODUCTION

The eye is the organ in charge of vision. It is a means by which the human being communicates with the outside[1]. This function is performed by converting, through photoreceptors, the energy of the visible spectrum from the periphery into action potentials that the optic nerve conducts towards the cerebral cortex[2]. On this site, it is interpreted to form an image of what is happening within the visual field[3]. The vision is a sense that people fear losing, being the target of various systemic and local pathologies[4]. Worldwide, about 2.2 billion people are visually impaired or blind. Of these cases, 1 billion could have been avoided or not yet treated[5].

Different disorders (associated with the posterior segment of the human eye) lead to visual impairment and blindness. They include glaucoma, age-related macular degeneration (AMD), diabetic retinopathy, uveitis, and retinoblastoma[6]. The World Health Organization (WHO) reported that three of the nine leading causes of visual impairment were disorders associated with the posterior segment of the eye (glaucoma, AMD, and diabetic retinopathy)[7]. Related to uveitis, it is one of the five leading causes of blindness in developed countries and represents up to 10% of all cases in the United States[8]. Retinoblastoma is also relevant, being the most common ocular cancer in childhood. Around 8,000 children per year develop this disease globally[9].

The most utilized route for pharmacological treatment is intravitreal, providing direct administration[10]. First-line treatments include corticosteroids, steroids, prostaglandin analogs, beta-blockers, diuretics, cholinergic agonists, and alpha agonists.

How to cite: Ayón C, Castán D, Mora A, Naranjo D, Obando F, Mora JJ. Monoclonal Antibodies: A Therapeutic Option for the Treatment of Ophthalmic Diseases of the Eye Posterior Segment. Borneo J Pharm. 2022;5(3):229-46. doi:10.33084/bjop.v5i3.2095

However, its pharmacokinetics is complicated. There is no uniformity because of variations in the vitreous, such as viscosity or loss of collagen fibril links[11,12]. Therefore, it has been decided to employ new therapeutic options such as monoclonal antibodies based on the comprehension of the molecular biology of these ocular diseases[13]. An example is the vascular endothelial growth factor (VEGF), related to AMD[14].

The Food and Drug Administration (FDA) approved the first monoclonal antibody in 1986. Since then, the most widely utilized therapeutic proteins are immunoglobulins G (IgGs). These products primarily work by blocking target receptors or ligands and reducing the activity of specific pathways involved in various ophthalmological diseases' pathogenesis, making them a crucial therapeutic alternative in severe eye conditions[15,16]. Given the tremendous progress in recent years, this work's objective was to check information about monoclonal antibody treatments for the most recurrent ophthalmic pathologies in the eye's posterior segment.

ANATOMIC OVERVIEW OF THE EYE

An image of the eye's anatomy is shown in **Figure 1**. The eyeball occupies approximately one-third of the orbit volume, while the other two-thirds are fat, muscles, nerves, and vasculature[17]. The organ can be divided into two segments: anterior and posterior. The former comprises cornea, conjunctiva, aqueous humor, iris, ciliary body, and crystalline lens. Together they represent one-third of the eye. The remaining two-thirds (posterior segment) include the sclera, choroid, Bruch's membrane, retinal pigment epithelium, neural retina, and vitreous humor[18].

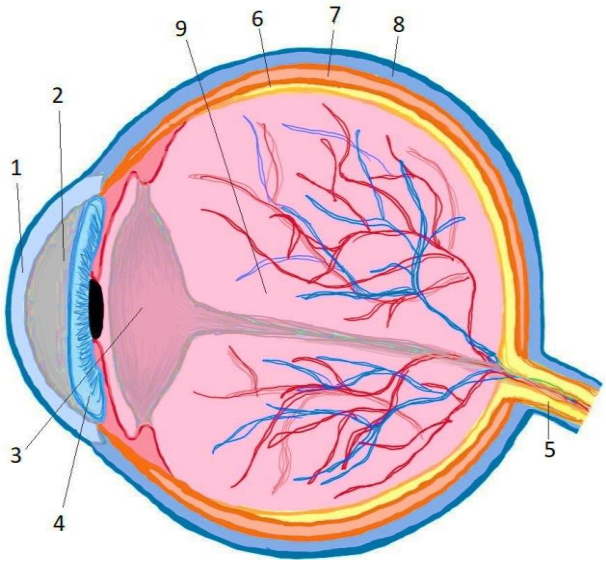


Figure 1. Section of an eyeball with its anatomical sites. (**1**) cornea; (**2**) anterior chamber; (**3**) crystalline lens; (**4**) iris; (**5**) optic nerve; (**6**) retina; (**7**) choroid; (**8**) sclera; (**9**) vitreous humor.

When making a lateral eyeball cut, three main layers are distinguished: a fibrous outer, a vascular/muscular medium, and a neural inner. The fibrous outer layer is what surrounds the organ and protects it. It includes the cornea (positioned in the anterior fraction) and the sclera (it extends back to the optic nerve). Both are formed of collagen and elastin. Their difference is the structural organization of the collagen fibers. They are arranged in very regular laminae in the cornea, allowing light rays to pass through without interference. In the sclera, they appear interwoven and extend in all directions[19]. The cornea is thin, convex, transparent, smooth, avascular, and highly innervated. Therefore, it is the most sensitive tissue in the body directly exposed to the external environment, constituting 20% of the outer layer. The sclera, commonly known as the eye's white, is a hard, avascular muscle with elastic tissue[20].

The middle or uveal layer comprises three pigmented tissue structures: choroid, ciliary body, and iris. They have a nutritional function. In the anterior part is the iris, in the form of a muscular ring. Longitudinal muscle fibers allow the pupil

to dilate when they contract at the edge. In an intermediate position is the ciliary body (formed by the ciliary muscle), in charge of adjusting the shape of the lens and by the ciliary processes, whose function is to produce aqueous humor[21]. Finally, posteriorly and in contact with the retina, the choroid is located. It has a vascular arrangement, which supplies oxygen and nutrients to the outer and inner layers[22].

As a complement, the inner or neural layer is the retina. It has photoreceptors (rods and cones), which detect light impulses from the environment. In addition, there are first- and second-order neurons (ganglion cells) and neuroglial elements in command of transmitting impulses to the visual cortex. On the outside is the pigment epithelium. It consists of a single layer of cells with adjacent nuclei joined together by tight junctions. Together, they form the retinal blood barrier[23].

Inside the eyeball are two fluid media: the aqueous humor and the vitreous body, separated by the crystalline lens and the suspensory ligament[24]. Aqueous humor is a clear liquid secreted by the ciliary epithelium. It helps form the eye's anterior and posterior chambers as a blood substitute for the lens and cornea. This element provides nutrition, eliminates excretory products of metabolism, transports neurotransmitters, stabilizes the ocular structure, and contributes to these ocular tissues' homeostasis regulation. Its main components are carbohydrates, glutathione, urea, proteins, oxygen, carbon dioxide, water, and inorganic ions[25].

For its part, the vitreous humor is a transparent gel that provides structural support. It occupies the eye's posterior segment, between the lens and the retina, and consists of 99% water. The remaining 1% is a mixture of collagen fibers, hyaluronic acid, hyalocytes, inorganic salts, lipids, and proteins (albumin being the main one, with 60 to 70% of the total protein concentration)[26].

DISEASES THAT COMMONLY AFFECT THE POSTERIOR SEGMENT OF THE EYE

The pathologies associated with this anatomical region are very diverse. Some are specific to each component or may be related to a secondary condition. The most relevant ones are detailed below:

Uveitis

It refers to inflammation of the uveal tract. It can also produce inflammation of adjacent tissues (cornea, sclera, retina, and even optic nerve)[27]. About 5 to 10% of cases appear in children. Around 30% are associated with juvenile idiopathic arthritis[28]. Furthermore, it has been linked to other autoimmune diseases such as Behcet's syndrome and sarcoidosis[29]. Common symptoms are blurred or distorted vision, pain, photophobia, floaters, photopsia, blind spots, and haloes. Cataracts, macular edema, epiretinal membrane, and glaucoma are common complications. Other signs include ciliary flush, corneal or scleral thinning, keratic precipitates, and anterior or posterior synechiae. Some chronic forms are asymptomatic)[30].

Most non-infectious uveitis is mediated by helper T lymphocytes (CD4+) through a T helper 1 (Th1) phenotype. Th1 cells induce cytotoxic cells and inflammatory reactions mediated by interleukin-2 (IL-2), interferon-gamma (INF-γ), and tumor necrosis factor-alpha (TNF-α). The primary function of IL-2 is the proliferation and activation of B and T cells[31]. Understanding the ocular inflammation pathology is limited, and most cases are indistinct (inflammatory, infectious, traumatic, genetic, neoplastic, ischemic, or drug-induced mechanisms). There is a transposition between them because there is not likely a single reason[32].

Retinoblastoma

It is a tumor located in the nuclear layer of the primary retinal photoreceptor cells. The disease originates from an alteration on chromosome 13, specifically in the q14 band. For its initiation, mutations of both alleles are necessary, usually called Knudson's "two-hit" hypothesis[33].

This malignant neoplasm is the most common in childhood, being equivalent to 10 to 15% of cancer cases that occur in one-year-old children) and 2.5 to 4% of all pediatric cancers. It develops very quickly and metastasizes if it is not treated. A good

prognosis occurs with an early diagnosis. Otherwise, the retina is destroyed within a few weeks, and the tumor spreads within the eye[34].

It should be noted that retinoblastoma was first cancer for which it was demonstrated that genetic factors influence its development, with two clinical forms. The bilateral or multifocal hereditary form occurs in 25% of events. The mean onset age is nine months earlier than in unilateral situations[35]. Germline mutations of the RB129 gene are observed. This gene is a tumor suppressor, transmitted with recessive autonomic inheritance. It encodes the Rb protein in the cell nucleus and regulates the cell cycle[36]. The mutation can be inherited from an affected person (25%) or be a new germline one (75%). Additionally, trilateral retinoblastoma corresponds to bilateral retinoblastoma association with a primary intracranial tumor (less than 10% of cases)[35].

The unilateral or unifocal form is equivalent to 75% of events[34]. The average onset age is 2 to 3 years. Usually, the illness does not develop in the other eye. Metachronous retinoblastoma occurs when a new lesion in the contralateral eye appears more than 30 days after the unilateral retinoblastoma diagnosis. This situation occurs only in 1.5 to 3% of the case[35]. It is usually discovered in two-year-old kids. Still, it can be detected from birth. The first symptoms occur in the first year of life but sometimes can be asymptomatic for a period. In the non-hereditary form, neoplastic changes can occur for up to 5 years[33].

Mostly, leukocoria is seen in children under two years. It can be noticed after a flash photo. Another common sign is strabismus (related to macular involvement). Moreover, advanced intraocular tumors can become painful due to secondary glaucoma. Common symptoms are redness, tenderness, pain in the eyeball, choroidal inflammation of the eye, and bleeding into the ocular chamber[37].

Diabetic retinopathy

It damages the retina microvasculature, a common diabetes complication derived from its increased duration and chronic hyperglycemia[38]. The disease is one of the leading causes of visual impairment, affecting around 4.2 million people worldwide[39]. As the diabetes duration augments, chronic hyperglycemia damages the retina's blood vessels, and the pericytes are lost. Consequently, involution in the microcirculation occurs. Besides, loss of regular capillary exchange and leakage of endovascular products are facilitated. The disease progresses from the nonproliferative type to the proliferative one. The first condition is aneurysms, hemorrhages, and exudation in the retinal circulation. The other implies ocular neovascularization in the iris, retina, or optic nerve[40].

This retinopathy generally does not originate symptoms significantly if only one eye is affected. The internal mechanism includes producing advanced glycated end products, creating a pro-inflammatory microenvironment, and inducing oxidative stress. Visual acuity is gradually lost because of preretinal or intraretinal hemorrhages, diabetic macular edema, and retinal detachment[41].

AMD

It is an acquired disease of the retina. It produces progressive loss of central vision through non-vascular (drusen and atrophy) and neovascular (choroidal neovascular membranes) disorders[42]. Disease evolution presents diverse stages. The early is characterized by extracellular material deposit between the retinal pigment epithelium (RPE) and Bruch's membrane (outer layer close to the choriocapillaris), allowing the passage of nutrients towards the retina while acting as a barrier) known as drusen[43]. The drusen are medium size (63 to 125 µm) at this stage. Another feature is the pigmentary changes of the retina (hyper or hypopigmentation) in the macular region. There is a slight central distortion and a reduced ability to read in low light. The stage is often asymptomatic[44].

In the intermediate one, the drusen size exceeds 125 µm in diameter (large), and there is a greater risk of progressing to the late stage[43]. In this phase, a severe and permanent visual impairment and legal blindness occur (visual acuity of 20/200 or worse)[44]. It is characterized by neovascular or atrophic AMD signs. The manifestations can coexist in the same eye or one in each organ[43].

The late stage progresses faster in the neovascular form (weeks or months) than the atrophic one (years or decades). The first symptoms may be a distorted vision when reading, driving, or watching television and difficulty recognizing faces. If only one eye is affected, the pathology may be asymptomatic until it progresses to the other[44].

Age is a risk factor. Most late cases occur in people over 60 years old. Also, non-genetic and environmental factors involve smoking and diet. The former is the most substantial modifiable risk factor, generating twice the possibility of developing the late disease. In 2017, 52 common and rare variants were identified at 34 genetic loci independently associated with late AMD[44].

Glaucoma

It is a group of eye disorders associated with damage to the retinal ganglion cells (RGCs) and optic nerve degeneration. Changes in the optic disc and progressive visual field loss are observed[45]. It is the most frequent cause of irreversible blindness worldwide[46]. Primary open-angle glaucoma (POAG) and primary angle-closure glaucoma (PACG) are common. The angle is the junction between the iris and the cornea, where the trabecular meshwork drains the aqueous humor from the anterior chamber[47].

The angle remains open in the POAG as the iris tissue unblocks the trabecular meshwork. Intraocular pressure is transmitted to the RCCs axons at the optic nerve as mechanical stress, causing cell death. Nevertheless, about 50% of cases have normal intraocular pressure when diagnosed. After losing 30% of the RGCs, visual field damage is seen in perimetric tests[47].

PACG implies that the peripheral iris obstructs the exit of aqueous humor, leading to intraocular pressure increase and optic nerve damage. Shorten eyes with a shallower anterior chamber are at higher risk. The disease can have a subacute or acute (after a sudden increase in intraocular pressure) or chronic (insidious and mostly asymptomatic) development[47].

Most patients are previously diagnosed with a chronic disease of both types and are unaware of any visual field loss. When left untreated, chronic, progressive, and irreversible loss occurs, moving to tunnel vision and the central one. Patients remain asymptomatic even as the disease progresses because the gradual loss is peripheral and asymmetric. This development generates compensation given by the other eye[47].

The main risk factor is increased intraocular pressure (greater than 21 mmHg), frequently observed in POAG. Vascular factors, oxidative stress, and elevated glutamate or nitric oxide levels are also considered. Furthermore, there is an immunologic component involved[45]. Other risk factors include advanced age, ethnic origin, positive family history of glaucoma, disease stage, high myopia, and thin central cornea[46].

PRINCIPAL CHARACTERISTICS OF MONOCLONAL ANTIBODIES

Its discovery began at the end of the 19th century from studies seeking defense mechanisms against microbial agents. These investigations found that serum produces substances capable of antagonizing different toxins[48]. Antitoxin is generated by blood cells, producing side chains that react against toxins specifically, like a key with its lock[49]. Subsequently, the term toxin was replaced by antigen and antitoxins by antibodies. These molecules come from B lymphocytes. Each one has its specificity, given by mutations in B cells' maturation[50].

Structure and isotypes

As shown in **Figure 2**, antibodies are made up of two light chains and two heavy ones, identical to each other and linked by disulfide bridges. Together, they form two binding sites for the antigen. Additionally, they have an amino-terminal end (binds and recognizes the antigen) and a carboxyl-terminal end (effector function). Both chains have variable and constant portions. The variable fragment provides the antibody specificity, and the constant determines the class and the isotype. The five classes are IgA, IgD, IgE, IgG, and IgM[50].

The light chains have two domains (each with 110 amino acids) with beta sheets, one in the variable portion and the other in the constant fragment. Heavy chains have one domain in the variable portion and three or four in the constant one,

depending on the Ig class. Between the domains of the constant portion is a hinge region, which generates flexibility and a better adaptive coupling. This area gives the antibody a Y shape[50].

The variable regions of the heavy and light chains generate the antigen-binding site. It consists of three hypervariable segments of 10 amino acids that produce space on the antibodies' surface and interact with antigens[50]. This part, and the constant region of the light chain and the heavy chain's first constant domain, are known as the antigen-binding fragment (Fab). The heavy chain's last two domains are the crystallizable fragment (Fc)[51]. This section has the immunological capacity, mainly cytotoxic functions. Therefore, it mediates antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC)[52,53].

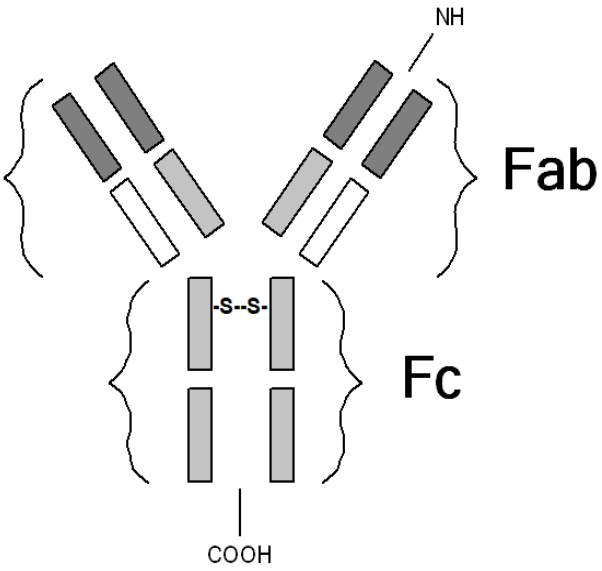


Figure 2. Structure of an antibody. The constant region of the heavy chain is shown in gray and that of the light in white. The dark gray portions are the variable regions of both chains.

Types of monoclonal antibodies

In 1975, monoclonal antibodies were discovered by Köhler and Milstein[48]. For its development, mouse myeloma cell lines and spleen cells of an immunized mouse were used to fuse the heavy and light chains of antibodies from both cell types, creating hybrid molecules capable of expressing both parents' characteristics and new ones. It resulted from the DNA translocation and the ordering during their transcription. Thus, antibodies were generated toward a specific antigen. This technique is called a hybridoma[54,55].

The procedure combines B lymphocytes from an immunized animal spleen with immortalized myeloma cells that cannot produce the enzyme hypoxanthine-guanine-phosphoribosyltransferase (HGPRT), which allows nucleotide production. Their mixture (those of the hybridoma and those that did not fuse) is cultivated in a culture media with aminopterin, inhibiting de novo nucleotide production. Because myeloma cells have a blocked nucleotide production pathway, they will not be viable. In contrast, B lymphocytes can produce them even if this pathway is useless, thus selecting hybridomas[55].

Monoclonal antibodies have a specific target and are produced from a single cellular clone[51]. They are generated to restore, imitate, or improve the immune system's attack by binding to antigens found in the body cells[56]. The first ones were made from murine proteins (they are identified with the -omab suffix). However, they generated many allergic reactions and antibodies against the drug. Furthermore, they showed weak binding to the Fc region in humans and were unsuitable for promoting ADCC and CDC. Therefore, other antibodies were developed[53,57,58]:

1. Chimeric monoclonal antibodies: chimerization is a technique related to these proteins where the murine variable region (antigen-binding), but they have the constant portions of human heavy and light chains, obtaining 65% human antibodies. They are on the market with the -ximab suffix.

2. Humanized monoclonal antibodies: are generated from a human antibody framework and the murine hypervariable region (approximately 95% human). Its suffix is -zumab.
3. Fully human antibodies: these molecules were created by animals carrying human immunoglobulin genes. These drugs are less antigenic than the others and have the -umab.

As a result of the immunogenicity decrease, the antibodies' half-life progressively increases, with entirely human ones having the most extended values[59].

The advancement for the humanization of monoclonal antibodies has been linked to various techniques. One was the creation of phage display libraries from hybridoma technology. The procedure generates clones that encode the Fab region of B lymphocytes in bacteriophage plasmid vectors. Then, the bacteria express genes from a viral capsid. The library obtained can generate new antibodies *in vitro*. Similarly, more antigens can be tested by presenting the ability to engineer and manipulate genes and quickly obtain antibodies. As a complement, the molecules are more stable since the phages can withstand adverse conditions, including temperature, pH, and others[59].

There are various antibody libraries: immune, naive, semi-synthetic, and synthetic. Immune libraries are made from IgG mRNA from infected or recovered people. They consist of specific antibodies and can be used as direct therapy or diagnosis, generally infectious agents such as viruses. Naive, "single-pot," or universal libraries are made from IgM mRNA of B cells from non-immunized healthy people and are employed to obtain antigen binders, regardless of the person's condition. The last two consists of synthetic or semi-synthetic sequences and are utilized to select antibodies against autoantibodies[59]. They can be highly defined, and natural antibodies are not required[60].

Other methods are antigen-specific single B cell sorting strategies and B cell culturing methods. Techniques with B lymphocytes present a significant impediment since they require sophisticated instrumentation and great personnel experience[59]. For its part, transgenic mice generate antibodies from the hybridoma technique. Endogenous Ig genes are silenced in rodents, and portions of human heavy and light chain genes are inserted, yielding human antibodies. These humanized mice are immunized against the antigen of interest. Later, the B cells with specificity for this antigen are isolated, generating the desired proteins[61-63].

INDUSTRIAL PRODUCTION

Cells suitable for the process must secrete the desired membrane protein for production. Mammalian cells can produce complex molecules and patterns compatible with the human immune system. Some are Chinese hamster ovary (CHO), human embryonic kidney (HEK293), mouse myeloma (NS0), and transformed human embryo retina (PER.C6) cells. These cell lines have been modified to express a specific membrane protein through transient transfection of expression vectors or stable integration of a transgene. Therefore, they can produce humanized and chimeric antibodies in large quantities. Other cells come from genetically modified plants, insects, and microorganisms. The latter offer ease of handling and modification and reproducible production[64-66].

Regarding the culture media, they should be free of any animal component. Its conditions are already established. Typically, when the temperature and pH decrease to lower values than usual, the compound's production increases. Additionally, the CHO cell line generated antibodies glycosylation by the presence of n-acetylglucosaminyltransferase III in the cell. Without this enzyme, they will have a lower ADCC[67]. Furthermore, glycosylation can affect antibody stability, receptor binding, effector functions, clearance, and half-life[68].

The current processes for monoclonal antibody production are upstream cell culture and downstream purification. These procedures are not the same for all since they have various properties. However, there is a general method to perform its production. Upstream cell culture refers to the rapid growth and high-specific-productivity manufacture of cell cultures with determined media. Thus, effective expression systems must be defined, and markers within the cell line development vectors must be previously determined. They are genes that encode dihydroxy folate reductase and glutamine synthetase, using promoters that enhance cell messenger RNA (mRNA) transcription[64].

The selection of cell lines with high specific productivity can be made by fluorescence-activated cell sorting, choosing those that produce the highest antibody levels. For its large-scale production (upstream cell culture), a bioreactor with controlled dissolved oxygen, pH, and temperature conditions must be employed[64]. The types comprise stirred tanks, airlifts, hollow fiber bioreactors, and rotatory cell culture systems. The usually chosen for antibody production is the stirred tank bioreactor[69].

One way to accomplish production is by fed-batch mode. There are two methods. First, a near-optimal basal media is added, and its concentration is maintained by putting concentrated nutrients as cell growth occurs. The second way is to incorporate concentrated nutrients into the complete media with or without standard amino acids, glucose, and glutamine, increasing antibody production at the beginning. This fed-batch technique allows the product concentration to augment and has given the best manufacture and yield results. Still, other strategies are perfusion and fed-perfusion culture[64,69].

Perfusion feeding involves retaining cells in a culture vessel while the spent culture medium is removed and an equal volume of fresh one is incorporated. As only the media is renewed, dead cells accumulate, and toxic metabolites are released. Then, a small stream containing cells is removed. In contrast, fed-perfusion culture involves replenishing depleted components and keeping nutrients constant, minimizing the toxic metabolite generation[69].

For the second part, the downstream purification is based on a filtration sequence of the bioreactor harvest through various chromatographic columns. This process depends on the components' physicochemical properties, so that the chromatography type may differ[70]. Filtration is usually done through a series of depth filters or by centrifuging the bioreactor harvest. The first step is capture chromatography, where the impurities binding is generated with their subsequent elution, increasing the product safety[71].

The column's stationary phase is protein A, for which the antibody exhibits affinity and interacts with the column. Cellular proteins, DNA, and other impurities pass through it. The pure antibody is obtained by its Fc region affinity with the protein A ligand at low pH[64,71,72]. This protein A comes from *Staphylococcus aureus*, which is highly immunogenic[73]. The process is precise. After performing the chromatography, the sample is further purified, and impurities are removed. Then, viral elimination and inactivation must be ensured by filtration. Finally, ultrafiltration/diafiltration is executed to reduce the volume[64].

Other proteins such as G and L can be considered in the stationary phase, depending on the type of antibody purified, the matrices employed, and the available culture supernatant. G is derived from *Streptococcus sp*, and L comes from *Peptostreptococcus magnus*[73].

The possibility of purification without a protein related to them should be noted because it dramatically increases production costs. They are more complicated techniques based on small-molecule ligands with similar selectivity to protein A[64,70]. They are presented as resins. Its ability to bind with the antibody depends on its density and concentration in the load material—moreover, some work by its ionic strength[74].

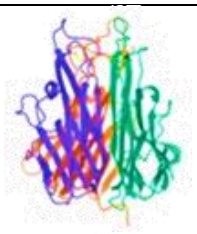
One consideration is the microheterogeneity of these molecules, which produces structural variations, affecting their biological activity and presenting more adverse effects. They could be generated by post-translational modifications during production or by physicochemical modifications during the purification, formulation, or storage process. Therefore, quality control is focused on verifying its physicochemical properties throughout the production process. Some tests to ensure drug quality are capillary electrophoresis, liquid chromatography techniques (size exclusion, ion exchange, reversed-phase), polyacrylamide-gel electrophoresis, capillary zone electrophoresis, and capillary isoelectric focusing. These methods allow the determination of the size and charge variants and antibody glycosylation through the procedure[75].

Its administration in the eye can be done by direct intravitreal, subconjunctival, or systemic intravenous injections. Each has its advantages and limitations. The intravitreal option is the most used with the most significant number of studies. This pathway reduces pro-inflammatory agents and retinal edema, preserves the retinal structure, and prevents ganglion cell neuronal death[76].

APPLICATIONS

Ophthalmic monoclonal antibody offers many advantages over traditional treatments due to a considerable reduction in side effects and a better therapeutic response. The main molecular targets are TNF and VEGF, whose three-dimensional structure and main features appear in **Table I**. Likewise, intravitreal products against these molecular targets offer better safety and efficacy in treating the previously described diseases[77]. In the first place, commercialized monoclonal antibodies, whose molecular target is TNF, will be discussed. Then, those active principles made against VEGF will be addressed. Finally, these diseases' products in different development phases will be mentioned.

Table I. Three-dimensional structure and principal characteristics of TNF and VEGF

Target	Three-dimentional structure	Characteristics
TNF		It is a pleiotropic cytokine synthesized mainly by monocytes, macrophages, and T lymphocytes and, to a lesser extent, by neutrophils, mast cells, and fibroblasts, in response to infection or immune impairment. It promotes inflammation by direct cytotoxicity and through indirect mechanisms, including the production of pro-inflammatory cytokines, arachidonic acid mediators, metalloproteinases, chemokines, adhesion molecules, and angiogenesis factors[78].
VEGF		The protein was identified as a potent promoter of vascular permeability and endothelial cell proliferation and, later, as the angiogenesis master regulator. Its increased concentration at the ocular level has been associated with diabetic retinopathy, AMD, glaucoma, and retinoblastoma. As a complement, it augments inflammation by inducing the expression of vascular cell adhesion molecule 1 (VCAM-1)[79-81]. Besides, it promotes endothelial cell proliferation with a signaling cascade mediated by tyrosine kinases. VEGF-A regulates angiogenesis and vascular permeability through the VEGF receptors 1 and 2 (VEGFR-1 and VEGFR-2), while VEGF-C/VEGF-D regulates lymphangiogenesis through the VEGFR-3[82].

Note: images were taken from the Protein Data Bank (https://www.rcsb.org)

TNF

In the case of autoimmune ocular inflammation, TNF-α is the central molecule to consider. The target receptors are TNFR-1 or p55 (involved in pro-apoptotic and inflammatory signals) and TNFR-2 or p75 (participated in cell growth and proliferation)[83]. The ophthalmic drugs used against this target are adalimumab, infliximab, certolizumab pegol, and golimumab. They are utilized for the treatment of uveitis.

Adalimumab

It is a fully human IgG1 monoclonal antibody. It interacts with TNF and prevents its binding to the p55 and p75 receptors[83,84]. This drug showed a lower risk of failure than a placebo in clinical studies. Furthermore, it controlled many uveitis aspects without glucocorticoid support. Nonetheless, the vitreous haze was the primary cause of failure in the placebo group compared to those receiving adalimumab. Likewise, chorioretinal lesions were more frequent in patients receiving a placebo concerning the management of said antibody[85].

Infliximab

Chimeric IgG1 antibody that has two murine antigen-binding sites. It neutralizes the biological activity of TNF. Therefore, the drug has been used to treat non-infectious uveitis[84,86,87]. A single intravitreal dose of infliximab (15 µg/eye) or control vehicle was applied in a preclinical investigation, and the samples were analyzed with flow cytometry. In mice who received the monoclonal antibody, a significantly reduced CD45+ infiltrate was seen on day 14, showing a decrease in CD4+ lymphocytes. In contrast, the control group presented in the same period the typical symptoms of the disease (vasculitis and choroidal lesions)[88].

Later, a clinical study with 72 patients demonstrated efficacy since 81.8 % showed clinical remission. However, 58.3 % of these patients required additional therapy with immunomodulators. The most common adverse effects were skin rash and fatigue[86].

Certolizumab pegol

Certolizumab is a humanized monoclonal antibody[89]. It does not have the Fc portion, impeding to induce of CDC, ADCC, apoptosis, or granulocyte degranulation. In addition, it has a Fab fragment conjugated to polyethylene glycol (PEG) to enhance plasma half-life[90]. The latter showed efficacy in a clinical study with 21 patients receiving either golimumab or certolizumab pegol[91]. Meanwhile, some case reports show good outcomes as a therapy against refractory, non-infectious uveitis[90].

VEGF

Drugs against this target have emerged as a tool widely utilized in intravitreal therapy in recent years. This alternative offers excellent safety, although there may be systemic absorption[92]. The main medications administered through the ophthalmic route are listed below.

Bevacizumab

It is a humanized IgG1 antibody. The concentrations required for its adequate pharmacological effect are deficient (around 1800 pM). Its intravitreal employment is considered for diabetic retinopathy and AMD[80,93]. It can cross ocular barriers and generates an inhibitory effect of VEGF in plasma (systemic effects cannot be ruled out)[94].

Preclinical studies have shown that VEGF neutralization with bevacizumab could inhibit the differentiation of retinoblastoma cells by blocking the extracellular pathway regulated by kinases. Also, it affects cell growth and differentiation *in vitro*. Although this therapeutic strategy may play a role in its clinical management, further studies and tests are required to optimize therapy for patients with this illness[95]. Moreover, safety and improved disease progress were demonstrated in a clinical trial in which 26 eyes with neovascular glaucoma were treated using intravitreal bevacizumab. The average intraocular pressure passes from 39.79 mmHg to 16.51 mmHg one week after injection[96].

Ranibizumab

It is a humanized monoclonal antibody that only has its variable fraction. This structure is endowed with activity against VEGF, binding to the active form of VEGF-A. The constant fraction absence in its structure implies the impossibility of binding to the neonatal Fc receptor and the lack of blood transport. Consequently, its systemic bioavailability is nil after intravitreal administration, avoiding effects on other human body's anatomical sites. The formulation is prepared for intraocular administration, avoiding problems derived from handling[94,97].

In a clinical study of 5496 patients with neovascular AMD who were given bevacizumab or ranibizumab intravitreally, they gained an average of 15 letters in visual acuity, and no statistically significant difference in efficacy was shown. The most frequent adverse effects were increased intraocular pressure and ocular inflammation[98]. Both antibodies showed similar efficacy in other clinical investigations, although bevacizumab reported a higher proportion of adverse effects, as it has a much longer half-life (20 versus 0.5 days). Nevertheless, bevacizumab is applied more widely for its lower cost[97].

OTHER MONOCLONAL ANTIBODIES UNDER CLINICAL TRIALS

In addition to those mentioned above, there are currently commercialized products to treat other pathologies. Their clinical studies are being performed for the ophthalmic diseases of the posterior segment of the eye.

Golimumab

The fully human monoclonal antibody of the IgG1 type selectively binds to TNF. It is approved for rheumatoid arthritis, ankylosing spondylitis, and Crohn's disease[84]. It is currently in phase II clinical studies to treat refractory Behcet's uveitis[99].

Brolucizumab

Humanized, single-chain fragment antibody that targets VEGF-A. It was approved in 2019 for the treatment of AMD. It has presented efficacy similar to aflibercept in preclinical studies and with fewer adverse effects[100,101]. The data obtained shows a higher affinity than other VEGF-A antagonists with scarce side effects, making it an excellent option to manage AMD and diabetic retinopathy. The most common adverse effects in clinical trials were conjunctival hemorrhage, eye pain, and hyperemia, which were mild in intensity and resolved within a few days without treatment[101]. It is currently in phase III clinical investigations to treat diabetic retinopathy and AMD[102].

THERAPEUTIC TARGETS UNDER CLINICAL INVESTIGATION

Tocilizumab

It is a humanized monoclonal antibody of the IgG1 type acting as an antagonist of the IL-6 receptor. It is widely utilized in rheumatic diseases such as juvenile idiopathic arthritis[103]. In a clinical study with 11 patients who presented refractory uveitis associated with Behcet's disease, the antibody treatment combined with traditional immunosuppressants significantly improved compared to the group that only received therapy with traditional immunosuppressants[104]. Phase II clinical studies have been done[105].

Ustekinumab

It is an IgG1 human monoclonal antibody that binds to the p40 subunit of IL-12 and 23. It is employed to treat Crohn's disease[106]. In studies made in humans, increased levels of IL-23 have been detected compared to control patients, doing it a relevant therapeutic target. Therefore, phase II clinical investigations are being done for uveitis treatment[107].

Faricimab

It is the first bispecific monoclonal antibody designed for intravitreal use, binding VEGF and angiopoietin-2[108]. Its good safety profile was established in phase I clinical studies, and no toxic effects were observed up to the highest dose (6 mg). Besides, all the parameters to define visual acuity improved significantly in most patients[109]. Also, in phase II clinical investigation, the efficacy of ranibizumab was compared with faricimab. The latter demonstrated greater efficacy and better gain in visual acuity[110]. Phase III clinical studies are in progress[111].

CONCLUSION

Monoclonal antibodies have been developed to treat disorders associated with the eye's posterior segment by blocking TNF (adalimumab, infliximab, and certolizumab pegol) and VEGF (bevacizumab and ranibizumab). Other options with different targets are studied through clinical trials, like golimumab, brolucizumab, tocilizumab, ustekinumab, and faricimab. Therefore, it is expected that more research will be done in the next future to find novel molecules for the treatment of these diseases.

ACKNOWLEDGMENT

None.

AUTHORS' CONTRIBUTION

All authors have an equal contribution in carrying out this study.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest in this research.

REFERENCES

1. McDougal DH, Gamlin PD. Autonomic control of the eye. Compr Physiol. 2015;5(1):439-73. doi:10.1002/cphy.c140014

2. Lankford CK, Laird JG, Inamdar SM, Baker SA. A Comparison of the Primary Sensory Neurons Used in Olfaction and Vision. Front Cell Neurosci. 2020;14:595523. doi:10.3389/fncel.2020.595523

3. Moschos MM. Physiology and psychology of vision and its disorders: a review. Med Hypothesis Discov Innov Ophthalmol. 2014;3(3):83-90.

4. Wentzel A, Mchiza ZJR. Exploring Factors Associated with Diabetic Retinopathy Treatment Compliance Behaviour in Cape Town, South Africa. Int J Environ Res Public Health. 2021;18(22):12209. doi:10.3390/ijerph182212209

5. Demmin DL, Silverstein SM. Visual Impairment and Mental Health: Unmet Needs and Treatment Options. Clin Ophthalmol. 2020;14:4229-51. doi:10.2147/opth.s258783

6. Rai BB, Morley MG, Bernstein PS, Maddess T. Pattern of vitreo-retinal diseases at the national referral hospital in Bhutan: a retrospective, hospital-based study. BMC Ophthalmol. 2020;20(1):51. doi:10.1186/s12886-020-01335-x

7. GBD 2019 Blindness and Vision Impairment Collaborators, Vision Loss Expert Group of the Global Burden of Disease Study. Causes of blindness and vision impairment in 2020 and trends over 30 years, and prevalence of avoidable blindness in relation to VISION 2020: the Right to Sight: an analysis for the Global Burden of Disease Study. Lancet Glob Health. 2021;9(2):e144-60. doi:10.1016/s2214-109x(20)30489-7

8. Hart CT, Zhu EY, Crock C, Rogers SL, Lim LL. 2019. Epidemiology of uveitis in urban Australia. Clin Exp Ophthalmol. 47(6):733-40. doi:10.1111/ceo.13517

9. Fabian ID, Sagoo MS. 2018. Understanding retinoblastoma: epidemiology and genetics. Community Eye Health. 31(101):7.

10. Awwad S, Ahmed AHAM, Sharma G, Heng JS, Khaw PT, Brocchini S, et al. Principles of pharmacology in the eye. Br J Pharmacol. 2017;174(23):4205-23. doi:10.1111/bph.14024

11. Gamalero L, Simonini G, Ferrara G, Polizzi S, Giani T, Cimaz R. Evidence-Based Treatment for Uveitis. Isr Med Assoc J. 2019;21(7):475-9.

12. Conlon R, Saheb H, Ahmed IIK. Glaucoma treatment trends: a review. Can J Ophthalmol. 2017;52(1):114-24. doi:10.1016/j.jcjo.2016.07.013

13. Quinteros DA, Bermúdez JM, Ravetti S, Cid A, Allemandi DA, Palma SD. Therapeutic use of monoclonal antibodies: general aspects and challenges for drug delivery. Nanostruct Drug Deliv. 2017:807-33. doi:10.1016/B978-0-323-46143-6.00025-7

14. Mousa SA, Mousa SS. Current status of vascular endothelial growth factor inhibition in age-related macular degeneration. BioDrugs. 2010;24(3):183-94. doi:10.2165/11318550-000000000-00000

15. Awwad S, Angkawinitwong U. Overview of Antibody Drug Delivery. Pharmaceutics. 2018;10(3):83. doi:10.3390/pharmaceutics10030083

16. Lu RM, Hwang YC, Liu IJ, Lee CC, Tsai HZ, Li HJ, et al. Development of therapeutic antibodies for the treatment of diseases. J Biomed Sci. 2020;27(1):1. doi:10.1186/s12929-019-0592-z

17. Turvey TA, Golden BA. Orbital anatomy for the surgeon. Oral Maxillofac Surg Clin North Am. 2012;24(4):525-36. doi:10.1016/j.coms.2012.08.003

18. de Andrade FA, Fiorot SHS, Benchimol EI, Provenzano J, Martins VJ, Levy RA. The autoimmune diseases of the eyes. Autoimmun Rev. 2016;15(3):258-71. doi:10.1016/j.autrev.2015.12.001

19. Sridhar MS. Anatomy of cornea and ocular surface. Indian J Ophthalmol. 2018;66(2):190-4. doi:10.4103/ijo.ijo_646_17

20. Ljubimov AV. Diabetic complications in the cornea. Vision Res. 2017;139:138-52. doi:10.1016/j.visres.2017.03.002

21. Wisely CE, Sayed JA, Tamez H, Zelinka C, Abdel-Rahman MH, Fischer AJ, et al. The chick eye in vision research: An excellent model for the study of ocular disease. Prog Retin Eye Res. 2017;61:72-97. doi:10.1016/j.preteyeres.2017.06.004

22. Rutkowski P, May CA. Nutrition and Vascular Supply of Retinal Ganglion Cells during Human Development. Front Neurol. 2016;7:49. doi:10.3389/fneur.2016.00049

23. Ptito M, Bleau M, Bouskila J. The Retina: A Window into the Brain. Cells. 2021;10(12):3269. doi:10.3390/cells10123269

24. Baino F, Kargozar S. Regulation of the Ocular Cell/Tissue Response by Implantable Biomaterials and Drug Delivery Systems. Bioengineering. 2020;7(3):65. doi:10.3390/bioengineering7030065

25. Goel M, Picciani RG, Lee RK, Bhattacharya SK. Aqueous Humor Dynamics: A Review. Open Ophthalmol J. 2010;4:52-9. doi:10.2174/1874364101004010052

26. Ankamah E, Sebag J, Ng E, Nolan JM. Vitreous Antioxidants, Degeneration, and Vitreo-Retinopathy: Exploring the Links. Antioxidants. 2019;9(1):7. doi:10.3390/antiox9010007

27. Dunn JP. Uveitis. Prim Care. 2015;42(3):305-23. doi:10.1016/j.pop.2015.05.003

28. Listing M, Mönkemöller K, Liedmann I, Niewerth M, Sengler C, Listing J, et al. The majority of patients with newly diagnosed juvenile idiopathic arthritis achieve a health-related quality of life that is similar to that of healthy peers: results of the German multicenter inception cohort (ICON). Arthritis Res Ther. 2018;20(1):106. doi:10.1186/s13075-018-1588-x

29. Angeles-Han ST, Rabinovich CE. Uveitis in children. Curr Opin Rheumatol. 2016;28(5):544–9. doi:10.1097/BOR.0000000000000316

30. Townsend WM. Canine and feline uveitis. Vet Clin North Am Small Anim Pract. 2008;38(2):323-46. doi:10.1016/j.cvsm.2007.12.004

31. Ramstein J, Broos CE, Simpson LJ, Ansel KM, Sun SA, Ho ME, et al. IFN-γ-Producing T-Helper 17.1 Cells Are Increased in Sarcoidosis and Are More Prevalent than T-Helper Type 1 Cells. Am J Respir Crit Care Med. 2016;193(11):1281-91. doi:10.1164/rccm.201507-1499oc

32. Harthan JS, Opitz DL, Fromstein SR, Morettin CE. Diagnosis and treatment of anterior uveitis: optometric management. Clin Optom. 2016;8:23-35. doi:10.2147/opto.s72079

33. Budny A, Grochowski C. Retinoblastoma. J Educ Health Sport. 2018;8(7):204-13. doi:10.5281/zenodo.1299573

34. Rodriguez-Galindo C, Orbach DB, VanderVeen D. Retinoblastoma. Pediatr Clin North Am. 2005;62(1):201-23. doi:10.1016/j.pcl.2014.09.014

35. Ortiz MV, Dunkel IJ. Retinoblastoma. J Child Neurol. 2016;31(2):227-36. doi:10.1177/0883073815587943

36. del Río NR, Gómez JMA, de la Rosa FJAG, Calvo JMP, Martín AdlH. Retinoblastoma trilateral. Correlación de las alteraciones genéticas del gen RB1 y la presencia de quistes en la glándula pineal. Arch Soc Esp Oftalmol. 2014;89(1):4-9. doi:10.1016/j.oftal.2013.07.006

37. Balmer A, Munier F. Differential diagnosis of leukocoria and strabismus, first presenting signs of retinoblastoma. Clin Ophthalmol. 2007;1(4):431-9.

38. ud Din J, Khan Z, Khan I. Diabetic Retinopathy; Prevalence of Diabetic Retinopathy in Recently Diagnosed Type 2 Diabetic Patients. A Single Center Study. Professional Med J. 2019;26(4):663-8. doi:10.29309/TPMJ/2019.26.04.3374

39. Hendrick AM, Gibson MV, Kulshreshtha A. Diabetic Retinopathy. Prim Care. 2015;42(3):451-64. doi:10.1016/j.pop.2015.05.005

40. Beltramo E, Porta M. Pericyte loss in diabetic retinopathy: mechanisms and consequences. Curr Med Chem. 2013;20(26):3218-25. doi:10.2174/09298673113209990022

41. Homme RP, Singh M, Majumder A, George AK, Nair K, Sandhu HS, et al. Remodeling of Retinal Architecture in Diabetic Retinopathy: Disruption of Ocular Physiology and Visual Functions by Inflammatory Gene Products and Pyroptosis. Front Physiol. 2018;9:1268. doi:10.3389/fphys.2018.01268

42. Mehta S. Age-Related Macular Degeneration. 2015. Prim Care. 2015;42(3):377-91. doi:10.1016/S0140-6736(12)60282-7

43. Grassmann F, Fleckenstein M, Chew EY, Strunz T, Schmitz-Valckenberg S, Göbel AP, et al. Clinical and Genetic Factors Associated with Progression of Geographic Atrophy Lesions in Age-Related Macular Degeneration. PloS One. 2015;10(5):0126636. doi:10.1371/journal.pone.0126636

44. Mitchell P, Liew G, Gopinath B, Wong TY. Age-related macular degeneration. Lancet. 2018;392(10153):1147-59. doi:10.1016/S0140-6736(18)31550-2

45. Hohenstein-Blaul NVTU, Bell K, Pfeiffer N, Grus FH. Autoimmune aspects in glaucoma. Eur J Pharmacol. 2016;787:105-18. doi:10.1016/j.ejphar.2016.04.031

46. Jonas JB, Aung T, Bourne RR, Bron AM, Ritch R, Panda-Jonas S. Glaucoma. Lancet. 2017;390(10108):2183-93. doi:10.1016/S0140-6736(17)31469-1

47. Gupta D, Chen PP. Glaucoma. Am Fam Physician. 2016;93(8):668-74.

48. Merino AG. Anticuerpos monoclonales. Aspectos básicos. Neurología. Neurología. 2011;26(5):301-6. doi:10.1016/j.nrl.2010.10.005

49. Bertelsen MB, Senissar M, Nielsen MH, Bisiak F, Cunha MV, Molinaro AL, Daines DA, et al. Structural Basis for Toxin Inhibition in the VapXD Toxin-Antitoxin System. Structure. 2021;29(2):139-50. doi:10.1016/j.str.2020.10.002

50. Herraiz CG. Anticuerpos monoclonals frente a PCSK9: del desarrollo básico a la clínica. Clín Investig Arterioscler. 2016;28(Suppl 2):14-21. doi:10.1016/S0214-9168(16)30166-8

51. Le Basle Y, Chennell P, Tokhadze N, Astier A, Sautou V. Physicochemical Stability of Monoclonal Antibodies: A Review. J Pharm Sci. 2020;109(1):169-90. doi:10.1016/j.xphs.2019.08.009

52. Ying T, Gong R, Ju TW, Prabakaran P, Dimitrov DS. Engineered Fc based antibody domains and fragments as novel scaffolds. Biochim Biophys Acta. 2014;1844(11):1977-82. doi:10.1016/j.bbapap.2014.04.018

53. Weiner LM, Surana R, Wang S. Monoclonal antibodies: versatile platforms for cancer immunotherapy. Nat Rev Immunol. 2010;10(5):317-27. doi:10.1038/nri2744

54. Morisson SL, Johnson MJ, Herzenberg LA, Oi VT. Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. Proc Natl Acad Sci U S A. 1984;81(21):6851-5. doi:10.1073/pnas.81.21.6851

55. Mitra S, Tomar PC. Hybridoma technology; advancements, clinical significance, and future aspects. J Genet Eng Biotechnol. 2021;19(1):159. doi:10.1186/s43141-021-00264-6

56. Nicholson LB. The immune system. Essays Biochem. 2016;60(3):275-301. doi:10.1042/ebc20160017

57. Bayer V. An Overview of Monoclonal Antibodies. Semin Oncol Nurs. 2019;35(5):150927. doi:10.1016/j.soncn.2019.08.006

58. Buss NA, Henderson SJ, McFarlane M, Shenton JM, de Haan L. Monoclonal antibody therapeutics: history and future. Curr Opin Pharmacol. 2012;12(5):615-22. doi:10.1016/j.coph.2012.08.001

59. Kumar R, Parray HA, Shrivastava T, Sinha S, Luthra K. Phage display antibody libraries: A robust approach for generation of recombinant human monoclonal antibodies. Int J Biol Macromol. 2019;135:907-18. doi:10.1016/j.ijbiomac.2019.06.006

60. Sidhu SS. Antibodies for all: The case for genome-wide affinity reagents. FEBS Letters. 2012;586(17):2778-9. doi:10.1016/j.febslet.2012.05.044

61. Nielsen UB, Marks JD. Internalizing antibodies and targeted cancer therapy: direct selection from phage display libraries. Pharm Sci Technol Today. 2000;3(8):282-91. doi:10.1016/s1461-5347(00)00280-7

62. Rodgers KR, Chou RC. Therapeutic monoclonal antibodies and derivatives: Historical perspectives and future directions. Biotechnol Adv. 2016;34(6):1149-58. doi:10.1016/j.biotechadv.2016.07.004

63. Lonberg N. Fully human antibodies from transgenic mouse and phage display platforms. Curr Opin Immunol. 2008;20(4):450-9. doi:10.1016/j.coi.2008.06.004

64. Shukla AA, Thömmes J. Recent advances in large-scale production of monoclonal antibodies and related proteins. Trends Biotechnol. 2010;28(5):253-61. doi:10.1016/j.tibtech.2010.02.001

65. Dodd R, Schofield DJ, Wilkinson T, Britton ZT. Generating therapeutic monoclonal antibodies to complex multi-spanning membrane targets: Overcoming the antigen challenge and enabling discovery strategies. Methods. 2020;180:111-26. doi:10.1016/j.ymeth.2020.05.006

66. Fliedl L, Grillari J, Grillari-Voglauer R. Human cell lines for the production of recombinant proteins: on the horizon. N Biotechnol. 2015;32(6):673-9. doi:10.1016/j.nbt.2014.11.005

67. Li F, Vijayasankaran N, Shen AY, Kiss R, Amanullah A. Cell culture processes for monoclonal antibody production. mAbs. 2010;2(5):466-79. doi:10.4161/mabs.2.5.12720

68. Lanter C, Lev M, Cao L, Loladze V. Rapid Intact mass based multi-attribute method in support of mAb upstream process development. J Biotechnol. 2020;314-315:63-70. doi:10.1016/j.jbiotec.2020.04.001

69. Jain E, Kumar A. Upstream processes in antibody production: Evaluation of critical parameters. Biotechnol Adv. 2008;26(1):46-72. doi:10.1016/j.biotechadv.2007.09.004

70. Chahar DS, Ravindran S, Pisal SS. Monoclonal antibody purification and its progression to commercial scale. Biologicals. 2020;63:1-13. doi:10.1016/j.biologicals.2019.09.007

71. Chon JH, Zarbis-Papastoitsis G. Advances in the production and downstream processing of antibodies. N Biotechnol. 2011;28(5):458-63. doi:10.1016/j.nbt.2011.03.015

72. Vasiljevic S, Beale EV, Bonomelli C, Easthope IS, Pritchard LK, Seabright GE, et al. Redirecting adenoviruses to tumour cells using therapeutic antibodies: Generation of a versatile human bispecific adaptor. Mol Immunol. 2015;68(2, Part A):234-43. doi:10.1016/j.molimm.2015.08.014

73. Hnasko RM, McGarvey JA. Affinity Purification of Antibodies. Methods Mol Biol. 2015;1318:29-41. doi:10.1007/978-1-4939-2742-5_3

74. Urmann M, Graalfs H, Joehnck M, Jacob LR, Frech C. Cation-exchange chromatography of monoclonal antibodies: characterisation of a novel stationary phase designed for production-scale purification. MAbs. 2010;2(4):395-404. doi:10.4161/mabs.12303

75. Ladner Y, Mas S, Coussot G, Bartley K, Montels J, Morel J, et al. Integrated microreactor for enzymatic reaction automation: An easy step toward the quality control of monoclonal antibodies. J Chromatogr A. 2017;1528:83-90. doi:10.1016/j.chroma.2017.10.066

76. Barcelona PF, Galan A, Nedev H, Jian Y, Sarunic MV, Saragovi HU. The route of administration influences the therapeutic index of an anti-proNGF neutralizing mAb for experimental treatment of Diabetic Retinopathy. PloS One. 2018;13(6):e0199079. doi:10.1371/journal.pone.0199079

77. VanderVeen DK, Cataltepe SU. Anti-vascular endothelial growth factor intravitreal therapy for retinopathy of prematurity. Semin Perinatol. 2019;43(16):375-80. doi:10.1053/j.semperi.2019.05.011

78. Franco CJV, Monsalve P, Martínez GIS, Rivera A, Zuluaga L, Duran C, et al. Uveítis y terapia anti-TNF. Rev Colomb Reumatol. 2011;18(1):42-54.

79. Tolentino M. Systemic and Ocular Safety of Intravitreal Anti-VEGF Therapies for Ocular Neovascular Disease. Surv Ophthalmol. 2011;56(2):95-113. doi:10.1016/j.survophthal.2010.08.006

80. Fogli S, Del Re M, Rofi E, Posarelli C, Figus M, Danesi R. Clinical pharmacology of intravitreal anti-VEGF drugs. Eye. 2018;32(6):1010-20. doi:10.1038/s41433-018-0021-7

81. Wu Q, Sun X, Zheng G. VEGF overexpression is associated with optic nerve involvement and differentiation of retinoblastoma: A PRISMA-compliant meta-analysis. Medicine. 2018;97(51):e13753. doi:10.1097/MD.0000000000013753

82. Shibuya M. Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis: A Crucial Target for Anti- and Pro-Angiogenic Therapies. Genes Cancer. 2011;2(12):1097-105. doi:10.1177/1947601911423031

83. Durrani K, Kempen JH, Ying GS, Kacmaz RO, Artornsombudh P, Rosenbaum JT, et al. Adalimumab for Ocular Inflammation. Ocul Immunol Inflamm. 2017;25(3):405-12. doi:10.3109/09273948.2015.1134581

84. Ono M, Horita S, Sato Y, Nomura Y, Iwata S, Nomura N. Structural basis for tumor necrosis factor blockade with the therapeutic antibody golimumab. Protein Sci. 2018;27(6):1038-46. doi:10.1002/pro.3407

85. Jaffe GJ, Dick AD, Brézin AP, Nguyen QD, Thorne JE, Kestelyn P, et al. Adalimumab in Patients with Active Noninfectious Uveitis. N Engl J Med. 2016;375(10):932-43. doi:10.1056/NEJMoa1509852

86. Kruh JN, Yang P, Suelves AM, Foster CS. Infliximab for the Treatment of Refractory Noninfectious Uveitis: A Study of 88 Patients with Long-term Follow-up. Ophthalmology. 2014;121(1):358-64. doi:10.1016/j.ophtha.2013.07.019

87. Markomichelakis N, Delicha E, Masselos S, Sfikakis PP. Intravitreal Infliximab for Sight-Threatening Relapsing Uveitis in Behçet Disease: A Pilot Study in 15 Patients. Am J Ophthalmol. 2012;154(3):534–41. doi:10.1016/j.ajo.2012.03.035

88. Khalili H, Lee RW, Khaw PT, Brocchini S, Dick AD, Copland DA. An anti-TNF-α antibody mimetic to treat ocular inflammation. Sci Rep. 2016;6:36905. doi:10.1038/srep36905

89. Nesbitt A, Fossati G, Bergin M, Stephens P, Stephens S, Foulkes R, et al. Mechanism of Action of Certolizumab Pegol (CDP870): In Vitro Comparison with Other Anti-tumor Necrosis Factor α agents. Inflamm Bowel Dis. 2007;13(11):1323-32. doi:10.1002/ibd.20225

90. Sharon Y, Chu DS. Certolizumab pegol - Tumor necrosis factor inhibitor for refractory uveitis. Am J Ophthalmol Case Rep. 2020;18:100633. doi:10.1016/j.ajoc.2020.100633

91. Tosi GM, Sota J, Vitale A, Rigante D, Emmi G, Lopalco G, et al. Efficacy and safety of certolizumab pegol and golimumab in the treatment of non-infectious uveitis. Clin Exp Rheumatol. 2019;37(4):680-3.

92. Touzani F, Geers C, Pozdzik A. Intravitreal Injection of Anti-VEGF Antibody Induces Glomerular Endothelial Cells Injury. Case Rep Nephrol. 2019;2019:2919080. doi:10.1155/2019/2919080

93. Ferrer LG, López MR, Santana YM, Hernández MC, Miniet EP, Reydmond KG. Estrategias en el tratamiento de la retinopatía diabética. Rev Cubana Olftalmol. 2018;31(1):90-9.

94. Perea JRA, Layana AG. Ranibizumab versus bevacizumab. Pharmacological considerations. Arch Soc Esp Oftalmol. 2012;87(Suppl 1):3-9. doi:10.1016/S0365-6691(12)70046-1

95. Wu AL, Wu WC. Anti-VEGF for ROP and Pediatric Retinal Diseases. Asia Pac J Ophthalmol. 2018;7(3):145-51. doi:10.22608/APO.201837

96. Ha JY, Lee TH, Sung MS, Park SW. Efficacy and Safety of Intracameral Bevacizumab for Treatment of Neovascular Glaucoma. Korean J Ophthalmol. 2017;31(6):538-47. doi:10.3341/kjo.2017.0017

97. Jiang S, Park C, Barner JC. Ranibizumab for age-related macular degeneration: a meta-analysis of dose effects and comparison with no anti-VEGF treatment and bevacizumab. J Clin Pharm Therap. 2014;39(3):234-9. doi:10.1111/jcpt.12146

98. Solomon SD, Lindsley K, Vedula SS, Krzystolik MG, Hawkins BS. Anti-vascular endothelial growth factor for neovascular age-related macular degeneration. Cochrane Database Syst Rev. 2014;8(8):CD005139. doi:10.1002/14651858.CD005139.pub3

99. Leclercq M, Desbois AC, Domont F, maalouf G, Touhami S, Cacoub P, et al. Biotherapies in Uveitis. J Clin Med. 2020;9(11):3599. doi:10.3390/jcm9113599

100. Haug SJ, Hien DL, Uludag G, Ngoc TTT, Lajevardi S, Halim MS, et al. Retinal arterial occlusive vasculitis following intravitreal brolucizumab administration. Am J Ophthalmol Case Rep. 2020;18:100680. doi:10.1016/j.ajoc.2020.100680

101. Nguyen QD, Das A, Do DV, Dugel PU, Gomes A, Holz FG, et al. Brolucizumab: Evolution through Preclinical and Clinical Studies and the Implications for the Management of Neovascular Age-Related Macular Degeneration. Ophthalmology. 2020;127(7):963-76. doi:10.1016/j.ophtha.2019.12.031

102. Karasavvidou EM, Tranos P, Panos GD. Brolucizumab for the Treatment of Degenerative Macular Conditions: A Review of Clinical Studies. Drug Des Devel Ther. 2022;16:2659-80. doi:10.2147/dddt.s378450

103. Sheppard M, Laskou F, Stapleton PP, Hadavi S, Dasgupta B. Tocilizumab (Actemra). Hum Vaccin Immunother. 2017;13(9):1972-88. doi:10.1080/21645515.2017.1316909

104. Atienza-Mateo B, Calvo-Río V, Beltrán E, Martínez-Costa L, Valls-Pascual E, Hernández-Garfella M, et al. Anti-interleukin 6 receptor tocilizumab in refractory uveitis associated with Behçet's disease: multicentre retrospective study. Rheumatology. 2018;57(5):856-64. doi:10.1093/rheumatology/kex480

105. Strohbehn GW, Heiss BL, Rouhani SJ, Trujillo JA, Yu J, Kacew AJ, et al. COVIDOSE: A Phase II Clinical Trial of Low-Dose Tocilizumab in the Treatment of Noncritical COVID-19 Pneumonia. Clin Pharmacol Ther. 2021;109(3):688-96. doi:10.1002/cpt.2117

106. Feagan BG, Sandborn WJ, Gasink C, Jacobstein D, Lang Y, Friedman JR, et al. Ustekinumab as Induction and Maintenance Therapy for Crohn´s Disease. N Engl J Med. 2016;375(20):1946-60. doi:10.1056/NEJMoa1602773

107. Pepple KL, Lin P. Targeting Interleukin-23 in the Treatment of Noninfectious Uveitis. Ophthalmology. 2018;125(12):1977-83. doi:10.1016/j.ophtha.2018.05.014

108. Shirley M. Faricimab: First Approval. Drugs. 2022;82(7):825-30. doi:10.1007/s40265-022-01713-3

109. Chakravarthy U, Bailey C, Brown D, Campochiaro P, Chittum M, Csaky K, et al. Phase I Trial of Anti-Vascular Endothelial Growth Factor/Antiangiopoietin 2 Bispecific Antibody RG7716 for Neovascular Age-Related Macular Degeneration. Ophthalmol Retina. 2017;1(6):474-85. doi:10.1016/j.oret.2017.03.003

110. Sahni J, Patel SS, Dugel PU, Khanani AM, Jhaveri CD, Wykoff CC, et al. Simultaneous Inhibition of Angiopoietin-2 and Vascular Endothelial Growth Factor-A with Faricimab in Diabetic Macular Edema: BOULEVARD Phase 2 Randomized Trial. Ophthalmology. 2019;126(8):1155-70. doi:10.1016/j.ophtha.2019.03.023

111. Sharma S, Kumar N, Kuppermann BD, Bandello F, Loewenstein A. Faricimab: expanding horizon beyond VEGF. Eye. 2020;34(5):802-4. doi:10.1038/s41433-019-0670-1

Borneo Journal of Pharmacy
Vol 5 Issue 3 August 2022
Page 247 – 254

http://journal.umpalangkaraya.ac.id/index.php/bjop/article/view/3293
DOI: https://doi.org/10.33084/bjop.v5i3.3293
e-ISSN: 2621-4814

Research Article

Antibiotics Susceptibility Profile of Gram-Positive Bacteria from Primary Health Centers in Jega, Kebbi State

Bilyaminu Garba Jega [1*]

Isa Musa Maishanu [1]

Basiru Aliyu [2]

Mohammed Ndagi Kasim [1]

[1] Department of Microbiology, Kebbi State University of Science and Technology, Aliero, Aliero, Kebbi State, Nigeria

[2] Department of Microbiology, Federal University, Birnin Kebbi, Birnin Kebbi, Kebbi State, Nigeria

*email: abunasmatu@gmail.com

Keywords:
Antibiotics susceptibility
Bacteria
HCAI
McFarland standard

Abstract

Nosocomial or healthcare-associated infection (HCAI) is an infection acquired during receiving health care that was not present during admission. The research aimed to determine the antibiotic susceptibility pattern of gram-positive bacteria isolated from Primary Health Centers in Jega Town. A total of fifty (50) swab samples were collected from 10 different health centers and analyzed using the streak plate technique. Pure bacterial isolates were maintained and characterized using biochemical tests; their percentage of occurrence show; *Staphylococcus aureus* 18 (43.9%), *Enterococcus feacalis* 8 (19.5%), *Streptococcus spp* 8 (19.5%), *Bacillus cereus* 4 (9.8%), and *Staphylococcus epidermidis* 3 (7.3%). McFarland standard solution was prepared and used to control inoculants, after which the antibiotic susceptibility pattern of the isolates was determined using the disc diffusion method. *Staphylococcus epidermidis* was resistant to Gentamycin, and other isolates were multi-drug resistant. In light of this research, there is a need for thorough disinfection and conscientious contact control procedures to minimize the spread of these pathogens in health centers where interaction between patients, HCWs, and caregivers is widespread and frequent.

Received: February 23rd, 2022
1st Revised: May 13th, 2022
Accepted: June 19th, 2022
Published: August 31th, 2022

INTRODUCTION

Nosocomial or healthcare-associated infections (HCAI) appear in a patient under medical care in the hospital or other healthcare facility which was absent at the time of admission[1]. These infections can occur during healthcare delivery for other diseases and even after the discharge of the patients. Additionally, they comprise occupational infections among the medical staff. Invasive devices such as catheters and ventilators employed in modern health care are associated with these infections[2]. With much medical equipment in hospitals coming in direct contact with healthcare workers, patients, technicians, cleaners, and sometimes caregivers, it is essential to pay close attention to their capacity to harbor potentially harmful pathogens[3].

Nosocomial infections affect many patients globally, leading to increased mortality and financial impact on healthcare systems[4]. While the actual global burden of healthcare-associated infection (HAI) remains unknown due to the lack of reliable data and surveillance systems. The endemic burden of HAI appears to be higher in developing countries[5]. A pooled analysis of data from developing countries showed an HAI prevalence of 15.5%, most of which occur as ventilator-associated pneumonia (VAP) and neonatal infections in intensive care settings[6].

According to the Extended Prevalence of Infection in Intensive Care (EPIC II) study, the proportion of infected Patients within the ICU is often as high as 51%[7]. Out of every hundred hospitalized patients, 7% in developed and 10% in developing

How to cite: Jega BG, Maishanu IM, Aliyu B, Kasim MN. Antibiotics Susceptibility Profile of Gram-Positive Bacteria from Primary Health Centers in Jega, Kebbi State. Borneo J Pharm. 2022;5(3):247-54. doi:10.33084/bjop.v5i3.3293

countries can acquire one of the healthcare-associated infections[1]. Healthcare-associated infection (HAI) complications are broad and depend on the type of infection, the severity of illness, and implicated pathogen. The most prevalent nosocomial infections were pneumonia, urinary tract infection, bloodstream infection (BSI), and meningitis/ventriculitis[8]. Significant independent associations with pneumonia included older age, poor Hunt and Hess grade, intubation/mechanical ventilation, and loss of consciousness *at ictus*[9].

Identifying common fomites and associated pathogens in any hospital setting is essential because the most critical factor in preventing disease is simply identifying what has been transferring the disease in the first place[10]. Fomites are, therefore, an opportunity to interrupt the spread of infection by recognizing them, avoiding them, disinfecting them, or cleansing the hands after touching them; the spread of many infections can be halted[11]. Ikeh and Isamade[12], in their study of bacterial flora of fomites in a Nigerian multi-disciplinary intensive care unit, reported that the majority of the isolates were Gram-positives organisms (52.2%: 12/23) as compared to the Gram-negative (47.8%: 11/23).

Staphylococci were isolated from all the fomites; *Staphylococcus epidermidis* (8.7%: 2/23) from the IVF stand, stethoscope, and *Staphylococcus aureus* (21.7%: 5/23) from the other fomites. Isolation of more Gram-positive organisms is consistent with previous reports[13,14] and agrees with Inweregbu *et al*.[15] and Ikeh and Isamade[12] that Gram-positive bacteria have overtaken the Gram-negative as the predominant bacteria isolated from fomites. Gram-positive organisms have earlier been noted by Gopinathan *et al*.[16] to be causing more severe infections than ever in surgical patients, who are increasingly aged, ill, and debilitated.

The present study necessitates investigating the constant drug resistance in the area, as the number of prevalent cases is frequently referred to the general hospital. It is also discovered that people are contracted some diseases that are not transported from their homes but within the hospital settings, where proper environmental sanitation is not practiced. This study was therefore conducted to isolate Gram-positive bacteria (more prominent for nosocomial infections) from Primary Health Centers in Jega and to determine their antibiotic susceptibility pattern to the commonly prescribed antibiotics in primary health centers.

MATERIALS AND METHODS

Research area/Sites

The research area was Jega Local Government Area (**Figure 1**), located at the latitude 12.3667°N and longitude 4.6333°E, with a total area of 891 km^2 and a population of 193,352 as of the 2006 Nigeria census estimate. The area has one General Hospital and 11 Primary Health Cares (PHC) that work daily to safeguard the health status of the inhabitants, predominantly Gimbanawa, with minority groups of Kambari, Zamfarawa, and Zabarmawa. The samples were collected at PHC Birnin Yari, PHC Tudun Wada, PHC GRA, PHC Town Council, PHC Jandutsi, PHC Basaura, PHC Bumbegu, PHC Gindi, PHC Nassarawa, and PHC Kimba.

Sample collection

A total of 50 samples from fomites were collected from ten distinct Primary Health Centers (five samples each); this was aseptically performed using sterile swab sticks as described in the report of Olise and Simon-Oke[17].

Isolation of bacterial isolates

The swab samples were cultured on Mannitol Salt Agar medium using the streak plate technique to select only Gram-positive bacteria. The incubations were demonstrated at 37°C for 18-24 hours; isolates were sub-cultured and further maintained on Nutrient Agar slants[18].

Biochemical identification of bacterial isolates

Citrate utilization, Indole, Methyl red, Voges–Proskauer, Triple sugar ion, Catalase, and Coagulase tests were employed to determine the biochemical reaction of the isolates[19].

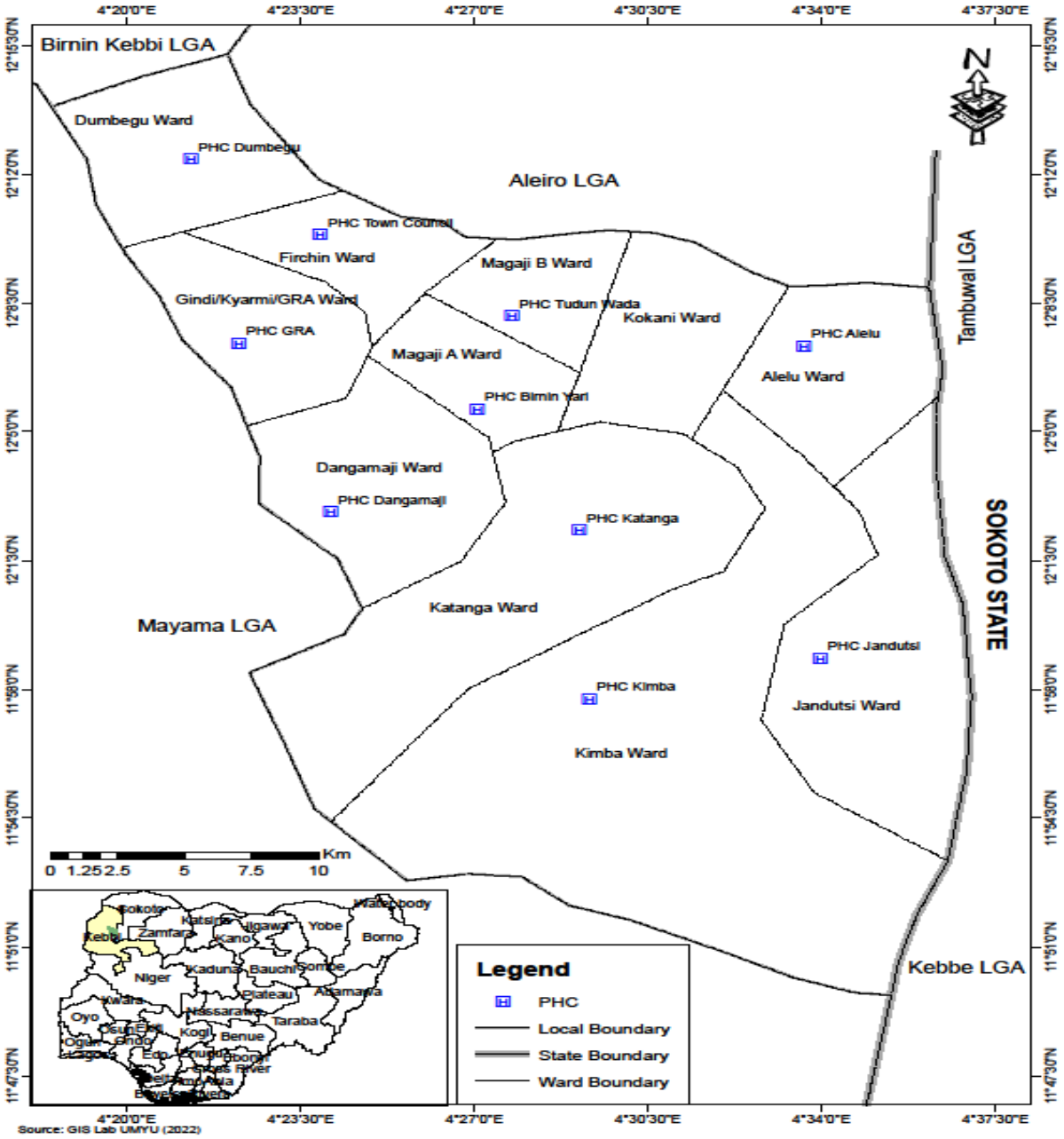


Figure 1. Map of the research area indicating different sampling sites.

Antibiotic susceptibility testing

The antibiotics susceptibility profile of the isolates was determined using the disc diffusion method, the antibiotic discs used were those of Maxi disc; Septrin (30 µg), chloramphenicol (30 µg), sparfloxacin (10 µg), ciprofloxacin (30 µg), amoxicillin (30 µg), augmentin (10 µg), gentamycin (30 µg), pefloxacin (30 µg), Tarivid (10 µg), streptomycin (30 µg). The McFarland standard was prepared by adding 1 mL of sulphuric acid into 99 mL of water and 0.5 g of dehydrated barium chloride into 50 mL of distilled water, each in a separate conical flask mixed. About 0.6 mL of barium chloride solution and mixed. The small volume of the solution was transferred into the Bijou bottle and was used to control inoculants. Aliquots of 100 µL from each suspension were spread-plated on Mueller-Hinton Agar plates and incubated at 37°C for 6 hours. Antibiotic discs were applied to the plates using sterile forceps, and the plates were incubated at 37°C for 24 hours. The zone of inhibitions of the plates was measured and classified as resistant (R), intermediate (I), and or sensitive (S) to a particular antibiotic using standard reference values according to the Clinical Laboratory Standards Institute (CLSI)[20].

Data analysis

All the experiments were performed in triplicates, and the data obtained were analyzed as mean plus standard deviation. The statistical analysis was performed using the analysis of variance (ANOVA), and the least significant difference between means and standard deviation was expressed using SPSS version 20.0.

RESULTS AND DISCUSSION

Isolation and identification of bacteria

The biochemical identification of the isolates and their occurrence (**Figure 2**) revealed five bacterial genera; *S. aureus* 18 (43.9%), *Enterococcus feacalis* 8 (19.5%), *Streptococcus sp* 8(19.5%), *Bacillus cereus* 4 (9.8%) and *S. epidermidis* 3 (7.3%). The occurrence of the bacteria could probably be because they are members of the body flora of both asymptomatic carriers and sick persons. These organisms can be spread by the hand, expelled from the respiratory tract, or transmitted by animate or inanimate objects[21]. Their primary source(s) of colonization on the fomites might likely be nasal carriage by hospital personnel[22], facilitated by hand-to-mouth or hand-to-nose contact while using these fomites, and poor hand-washing habits[23]. This finding is consistent with the reports of Neely and Maley[13] as well as Chikere *et al*.[24] and agrees with Inweregbu *et al*.[15] that Gram-positive bacteria have overtaken the Gram-negative as the predominant bacteria isolated from hospital fomites. Gram-positive organisms have earlier been noted to be causing more severe infections than ever in surgical patients, who are increasingly aged, ill, and debilitated[25].

The highest occurrence of *S. aureus* from almost all the samples shows its ubiquity in nature and that they can be sources of infection in patients, as previously noted by previous research[12,15]. Although the strains of the isolated *S. aureus* were not determined in this study, methicillin-resistant *Staphylococcus aureus* (MRSA) strains are transmissible from many fomites to the skin. For example, earlier studies showed that one in three stethoscopes tested to harbor *S. aureus* and that 15% of all stethoscopes tested were contaminated with MRSA[26]. *Staphylococcus epidermidis* was isolated with the lowest frequency in this study. Though these strains are known to be non-pathogenic to the body, when they harbor antimicrobial resistance genes, they constitute a severe health hazard. This *S. epidermidis* has been isolated from keyboards on multiple user computers[27], and increased virulence of this organism resulting from the acquisition of methicillin-resistance has been recognized[28].

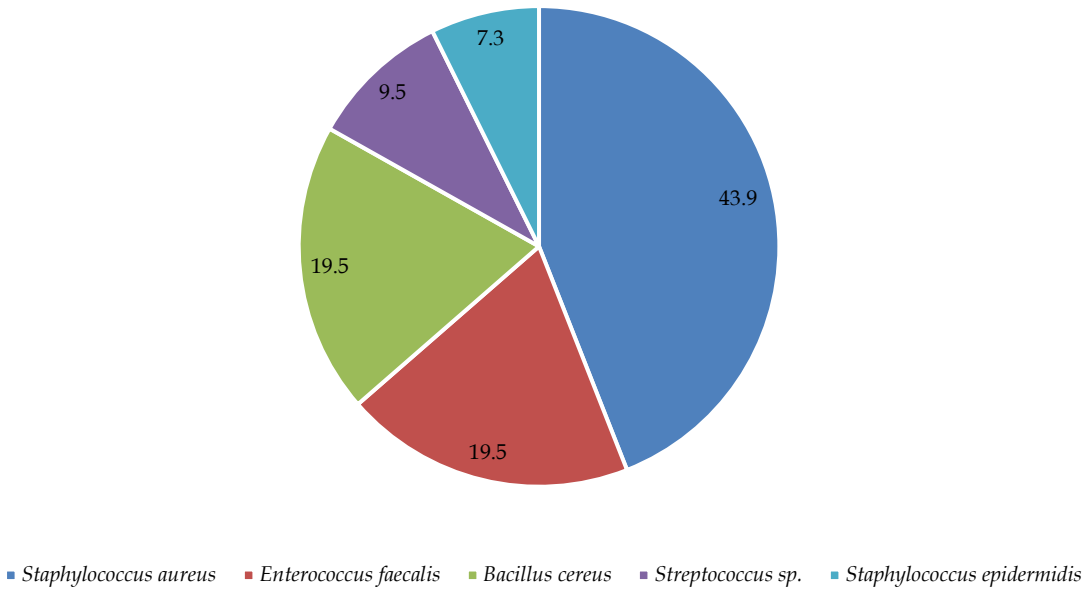


Figure 2. Percentage occurrence of the identified bacteria.

Antibiotic susceptibility profile of the bacterial isolates

The diameter of the zone of inhibition of the antibiotics (**Table I**) revealed that all the bacterial isolates were susceptible to streptomycin (S) and ciprofloxacin (CPX) at ≥25.0±0.03 mm, with the intermediate pattern on sparfloxacin (SP), amoxicillin (AM) and Tarivid (OFD) at ≤ 24.0±0.0 mm, while all the bacterial isolates exhibit resistance to augmentin (AU) and gentamicin (CN) at ≤ 16.0±0.05 mm, with amoxicillin (AM) showing resistance to *S. aureus*, *E. feacalis*, and *Strep. sp*. at ≤ 16.0±0.05mm. The susceptibility of bacterial isolates to antibiotics (**Figure 3**) indicates the ineffectiveness of the AU, CN, and AM in treating HAIs that might result from infection with these pathogens.

This implies that these fomites might act as vehicles for transferring these pathogens. Many studies have shown uniforms to be potential reservoirs for hospital organisms, potentially reinfecting the hands of HCWs, thereby causing resistance patterns to antibiotics[23]. Treakle *et al*.[29] showed a large proportion of HCWs' white coats to be contaminated with *S. aureus*, including MRSA and postulated that white coats might be an essential vector for patient-to-patient transmission of *S. aureus*. Potential pathogens such as *S. aureus*, *Acinetobacter* spp., and enterococci have been recently isolated from hands that were used to touch uniforms[23].

The resistance of the isolates is in line with the findings by Sani *et al*.[30] and Ogunshe *et al*.[31], who reported gram-positive isolates to demonstrate a low-to-moderate sensitivity to gentamicin (20.0–57.1%), streptomycin (21.7–57.1%), and ciprofloxacin (20.0–42.9%); a moderate sensitivity to ceftriaxone (57.1–60.9%); and high sensitivity to imipenem (85.7–87.0%) except for *S. epidermidis* which showed a low sensitivity (20.0%) to imipenem. The Gram-positive cocci were least resistant to imipenem and ceftriaxone, with the isolates from acute wounds demonstrating slightly higher resistance.

Chikere *et al*.[24] reported a similar weakness and activity of some antibiotics against bacteria from clinical specimens; as more bacteria become resistant to antibiotics, the ability to control the spread of these bacteria with antibiotic treatments decreases. These findings also align with the investigation of Lee *et al*.[32], who found a strong correlation between oxacillin resistance and co-resistance to non–β-lactam antimicrobials such as gentamicin, erythromycin, and ciprofloxacin ($p > .01$). Such high rates of co-resistance suggest the presence of multidrug-resistant MRSA strains. The strong resistance of the augmentin (AU), gentamicin (CN), and amoxicillin (AM) obtained agreed with the findings of Sani *et al*.[30] and Ogunshe *et al*.[31]. in their studies on antibiotic susceptibility of wound swab isolates in a tertiary hospital in southwest Nigeria and microbiological evaluation of antibiotic resistance in bacterial flora from skin wounds respectively.

Table I. Mean ± standard deviation of the zone of inhibition (mm) of the antibiotics to the bacterial isolates

Antibiotic	Potency (µg)	*S. aureus*	*E. feacalis*	*B. cereus*	*Strep. sp*	*S. epidermidis*
SXT	30	26.0^{a}±0.0	21.0^{b}±0.3	24.0^{ab}±0.0	30.0^{a}±0.3	28.0^{a}±0.3
CH	30	30.0^{a}±0.3	28.0^{a}±0.4	22.0^{b}±0.02	28.0^{a}±0.0	30.0^{a}±0.3
SP	10	25.0^{a}±0.0	28.0^{a}±0.4	25.0^{a}±0.03	26.0^{a}±0.1	25.0^{a}±0.2
CPX	30	30.0^{a}±0.4	30.0^{a}±0.3	30.0^{a}±0.3	30.0^{a}±0.3	31.0^{a}±0.3
AM	30	14.0^{a}±0.0	2.0^{b}±0.0	20.0^{c}±0.0	16.0^{ac}±0.05	25.0^{d}±0.1
AU	10	5.0^{a}±0.0	00.0^{b}±0.0	00.0^{b}±0.0	5.0^{a}±0.0	00.0^{b}±0.0
CN	30	11.0^{a}±0.0	8.0^{ab}±0.01	5.0^{b}±0.0	00.0^{c}±0.0	6.0^{b}±0.0
PEF	30	31.0^{a}±0.2	30.0^{a}±0.3	28.0^{ab}±0.1	26.0^{b}±0.1	25.0^{b}±0.1
OFD	10	21.0^{a}±0.5	26.0^{b}±0.1	25.0^{b}±0.2	21.0^{a}±0.3	22.0^{a}±0.02
S	30	32.0^{a}±0.6	30.0^{a}±0.0	30.0^{a}±0.3	32.0^{a}±0.03	30.0^{a}±0.0

Mean values with different alphabet appearing on the same row are significantly different otherwise they are the same

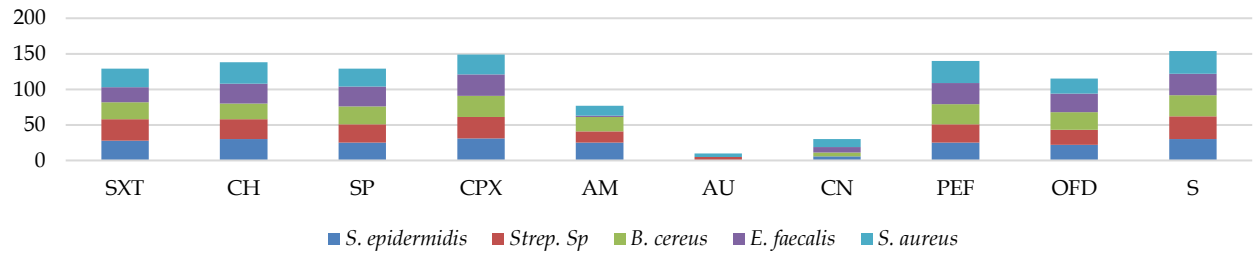


Figure 3. Antibiotic susceptibility pattern of the bacterial isolates. SXT= Septin, CH= chloramphenicol, SP= sparfloxacin, CPX= ciprofloxacin, AM= amoxicillin, AU= augmentin, CN= gentamycin, PEF= pefloxacin, OFD= Tarivid, S= streptomycin.

CONCLUSION

Based on the study findings, it is concluded that the hospital equipment (Benches, beds, stethoscopes, door handles, tables, cupboards, and chairs) harbored infectious pathogens with the potential of causing hospital-acquired infections (HAIs), which may eventually lead to diseases like urinary tract infections (UTIs), gastroenteritis, meningitis, pneumonia, septicemia, endocarditis and or wound infections. In light of this, there is a need for thorough disinfection and conscientious contact control procedures to minimize the spread of these pathogens in health centers where interaction between patients, HCWs, and caregivers is ubiquitous and frequent.

ACKNOWLEDGMENT

The authors acknowledge the consent of the entire health centers covered and all that have contributed to the success of this study.

AUTHORS' CONTRIBUTION

All authors have an equal contribution in carrying out this study.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

No conflict of interest among the study participants exist.

REFERENCES

1. Haque M, Sartelli M, McKimm J, Bakar MA. Health care-associated infections - an overview. Infect Drug Resist. 2018;11:2321-33. doi:10.2147/idr.s177247

2. Khan HA, Baig FK, Mehboob R. Nosocomial infections: Epidemiology, prevention, control and surveillance. Asian Pac J Trop Biomed. 2017;7(5):478-82. doi:10.1016/j.apjtb.2017.01.019

3. Ssekitoleko RT, Oshabaheebwa S, Munabi IG, Tusabe MS, Namayega C, Ngabirano BA, et al. The role of medical equipment in the spread of nosocomial infections: a cross-sectional study in four tertiary public health facilities in Uganda. BMC Public Health. 2020;20(1):1561. doi:10.1186/s12889-020-09662-w

4. Voidazan S, Albu S, Toth R, Grigorescu B, Rachita A, Moldovan I. Healthcare Associated Infections-A New Pathology in Medical Practice? Int J Environ Res Public Health. 2020;17(3):760. doi:10.3390/ijerph17030760

5. Maki G, Zervos M. Health Care-Acquired Infections in Low- and Middle-Income Countries and the Role of Infection Prevention and Control. Infect Dis Clin North Am. 2021;35(3):827-39. doi:10.1016/j.idc.2021.04.014

6. Rangelova VR, Raycheva RD, Kevorkyan AK, Krasteva MB, Kalchev YI. Ventilator-Associated Pneumonia in Neonates Admitted to a Tertiary Care NICU in Bulgaria. Front Pediatr. 2022;10:909217. doi:10.3389/fped.2022.909217

7. Vincent JL, Sakr Y, Singer M, Martin-Loeches I, Machado FR, Marshall JC, et al. Prevalence and outcomes of infection among patients in intensive care units in 2017. JAMA. 2020;323(15):1478-87. doi:10.1001/jama.2020.2717

8. Ghashghaee A, Behzadifar M, Azari S, Farhadi Z, Bragazzi N, Behzadifar M, et al. Prevalence of Nosocomal Infections in Iran: Asystematic Review and Meta-analysis. Med J Islam Repub Iran. 2018;32:48. doi:10.14196/mjiri.32.48

9. Winters ME, Hu K, Martinez JP, Mallemat H, Brady WJ. The critical care literature 2018. Am J Emerg Med. 2020;38(3):670-80. doi:10.1016/j.ajem.2019.11.032

10. Li S, Xu Y, Cai J, Hu D, He Q. Integrated environment-occupant-pathogen information modeling to assess and communicate room-level outbreak risks of infectious diseases. Build Environ. 2021;187:107394. doi:10.1016/j.buildenv.2020.107394

11. Yen MY, Schwartz J, King CC, Lee CM, Hsueh PR, Society of Taiwan Long-term Care Infection Prevention and Control. Recommendations for protecting against and mitigating the COVID-19 pandemic in long-term care facilities. J Microbiol Immunol Infect. 2020;53(3):447-53. doi:10.1016/j.jmii.2020.04.003

12. Ikeh EI, Isamade ES. Bacterial flora of fomites in a Nigerian multi-disciplinary intensive care unit. Lab Med. 2011;42(7):411-3. doi:10.1309/LMTVPU3PMWAWL0IG

13. Neely AN, Maley MP. Survival of enterococci and staphylococci on hospital fabrics and plastic. J Clin Microbiol. 2000;38(2):724-6. doi:10.1128/jcm.38.2.724-726.2000

14. Maryam A, Hadiza US, Aminu UM. Characterization and determination of antibiotics susceptibility pattern of bacteria from some fomites in a teaching hospital in northern Nigeria. Afr J Microbiol Res. 2014;8(8):814-8. doi:10.5897/AJMR2013.6512

15. Inweregbu K, Dave J, Pittard A. Nosocomial infections. Contin Educ Anaesth Crit Care Pain. 2005;5(1):4-17. doi:10.1093/bjaceaccp/mki006

16. Gopinathan U, Sharma S, Garg P, Rao GN. Review of Epidemiological Feature, Microbiological Diagnosis and Treatment Outcome of Microbial Keratitis: experience of over a decade. Indian J Ophthalmol. 2009;57(4):273-9. doi:10.4103/0301-4738.53051

17. Olise CC, Simon-Oke IA. Formites: Possible Vehicle of Nosocomial Infections. J Public Health Nutr. 2018;1(1):16. doi:10.35841/public-health-nutrition.1.1.11-16

18. Sathish KSR, Kokati VBR. In-vitro antimicrobial activity of marine actinobacteria against multidrug resistance Staphylococcus aureus. Asian Pac J Trop Biomed. 2012;2(10):787-92. doi:10.1016/s2221-1691(12)60230-5

19. Bhutia MO, Thapa N, Tamang JP. Molecular Characterization of Bacteria, Detection of Enterotoxin Genes, and Screening of Antibiotic Susceptibility Patterns in Traditionally Processed Meat Products of Sikkim, India. Front Microbiol. 2021;11:599606. doi:10.3389/fmicb.2020.599606

20. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. J Pharm Anal. 2016;6(2):71-9. doi:10.1016/j.jpha.2015.11.005

21. Alphons KS, Fortune TV, Haindongo E, Guillaume AY. Bacterial contamination and antimicrobial susceptibility from the hands of health care workers (HCWs) and inanimate surfaces in the neonatal intensive care unit (NICU) at the Windhoek Central Hospital (WCH). Microbiol Nat. 2020;1(3):83-95. doi:10.26167/x34y-rn94

22. Kim MW, Greenfield BK, Snyder RE, Steinmaus CM, Riley LW. The association between community-associated Staphylococcus aureus colonization and disease: a meta-analysis. BMC Infect Dis. 2018;18(1):86. doi:10.1186/s12879-018-2990-3

23. Munoz-Price LS, Arheart KL, Mills JP. Associations between bacterial contamination of health care workers' hands and contamination of white coats and scrubs. Am J Infect Control. 2012;40(9):e245-8. doi:10.1016/j.ajic.2012.03.032

24. Chikere CB, Omoni VT, Chikere BO. Distribution of potential nosocomial pathogens in a hospital environment. Afr J Biotechnol. 2008;7(20):3535-8.

25. Yahav D, Eliakim-Raz N, Leibovici L, Paul M. Bloodstream infections in older patients. Virulence. 2016;7(3):341-52. doi:10.1080/21505594.2015.1132142

26. Vasudevan R, Shin JH, Chopyk J, Peacock WF, Torriani FJ, Maisel AS, et al. Aseptic barriers allow a clean contact for contaminated stethoscope diaphragms. Mayo Clin Proc Innov Qual Outcomes. 2020;4(1):21-30. doi:10.1016/j.mayocpiqo.2019.10.010

27. Koscova J, Hurnikova Z, Pistl J. Degree of bacterial contamination of mobile phone and computer keyboard surfaces and efficacy of disinfection with chlorhexidine digluconate and triclosan to its reduction. Int J Environ Res Public Health. 2018;15(10):2238. doi:10.3390/ijerph15102238

28. Lee AS, de Lencastre H, Garau J, Kluytmans J, Malhotra-Kumar S, Peschel A, et al. Methicillin-resistant Staphylococcus aureus. Nat Rev Dis Primers. 2018;4:18033. doi:10.1038/nrdp.2018.33

29. Treakle AM, Thom KA, Furuno JP, Strauss SM, Harris AD, Perencevich EN. Bacterial contamination of health care workers' white coats. Am J Infect Control. 2009;37(2):101-5. doi:10.1016/j.ajic.2008.03.009

30. Sani RA, Garba SA, Oyewole OA, Ibrahim A. Antibiotic resistance profile of gram positive bacteria isolated from wound infections in Minna, Bida, Kontagora and Suleja area of Niger State. J Health Sci. 2012;2(3):19-22. doi:10.5923/j.health.20120203.01

31. Ogunshe AA, Niemogha MT, Azum GN, Odikagbue AN. Microbiological evaluation of antibiotic resistance in bacterial flora from skin wounds. J Pharm Biomed Sci. 2012;22(6):1-7.

32. Lee NY, Song JH, Kim S, Peck KR, Ahn KM, Lee SI. Carriage of antibiotic-resistant pneumococci among Asian children: A multinational surveillance by the Asian Network for Surveillance of Resistant Pathogens (ANSORP). Clin Infect Dis. 2001;32(10):1463-9. doi:10.1086/320165

Borneo Journal of Pharmacy
Vol 5 Issue 3 August 2022
Page 255 – 267

http://journal.umpalangkaraya.ac.id/index.php/bjop/article/view/3367
DOI: https://doi.org/10.33084/bjop.v5i3.3367
e-ISSN: 2621-4814

Review Article

Anti-inflammatory and Immunostimulant Therapy with *Lactobacillus fermentum* and *Lactobacillus plantarum* in COVID-19: A Literature Review

Harry Noviardi [1,2]
Dyah Iswantini [1,3*]
Sri Mulijani [1]
Setyanto Tri Wahyudi [3,4]
Tatik Khusniati [5]

[1] Department of Chemistry, IPB University, Bogor, West Java, Indonesia

[2] Department of Pharmacy, Sekolah Tinggi Teknologi Industri dan Farmasi Bogor, Bogor, West Java, Indonesia

[3] Tropical Biopharmaca Research Center, IPB University, Bogor, West Java, Indonesia

[4] Department of Physics, IPB University, Bogor, West Java, Indonesia

[5] Research Center for Biology, National Research and Innovation Agency Republic of Indonesia, Cibinong, West Java, Indonesia

*email: dyahis@apps.ipb.ac.id

Keywords:
Anti-inflammatory
COVID-19
Immunostimulant
Lactobacillus fermentum
Lactobacillus plantarum

Abstract

Inflammatory diseases are diseases characterized by inflammatory symptoms. Acute inflammatory disease can cause dysregulation of the inflammatory immune response, thereby inhibiting the development of protective immunity against infection. Among the acute inflammatory disease is COVID-19. The initial viral infection causes the antigen-presenting cells to detect the virus through a phagocytosis mechanism in the form of macrophage and dendritic cells. *Lactobacillus fermentum* and *L. plantarum* are gram-positive bacteria potentially serving as immunomodulators caused by inflammation and immune system response. Short-chain fatty acids (SCFA) produced by *Lactobacillus* can induce immune response through tolerogenic dendritic cells. This probiotic bacterium can induce the production of different cytokines or chemokines. Following the results of *in vitro* and *in vivo* tests, *L. fermentum* and *L. plantarum* can induce IL-10 release to activate regulatory T-cell and inhibit tumor necrosis factor-α (TNF-α) binding activity of nuclear factor kappa B (NF-κB). Literature review showed that dysregulation of inflammatory immune response disorders due to inflammatory disease could be treated using probiotic bacteria *L. fermentum* and *L. plantarum*. Therefore, it is necessary to conduct further studies on the potential of indigenous Indonesian strains of these two bacteria as anti-inflammatory and immunostimulants.

Received: March 30th, 2022
1st Revised: April 30th, 2022
2nd Revised: June 20th, 2022
Accepted: June 22nd, 2022
Published: August 31th, 2022

INTRODUCTION

Inflammation is a defense process of the body's system due to infections from bacteria and viruses and can also be caused by damage to body tissues[1]. Acute inflammation is the first line of defense due to infection. Coronavirus 2019 (COVID-19) is an acute inflammatory disease that can cause an impaired inflammatory immune response[2]. COVID-19 disease is a clinical syndrome caused by SARS-CoV-2. Originally discovered in China in December 2019, this disease has spread worldwide and was declared a pandemic by WHO on 11 March 2020. This disease causes human acute respiratory system like other betacoronavirus types such as human coronavirus 229E, NL63, OC43, HKU1, Middle-East respiratory syndrome (MERS), dan Severe Acute Respiratory Syndrome (SARS)[3-5].

How to cite: Noviardi H, Iswantini D, Mulijani S, Wahyudi ST, Khusniati T. Anti-inflammatory and Immunostimulant Therapy with Lactobacillus fermentum and Lactobacillus plantarum in COVID-19: A Literature Review. Borneo J Pharm. 2022;5(3):255-67. doi:10.33084/bjop.v5i3.3367

SARS-CoV-2 is transmissible through respiratory droplets, with a viral incubation period around 4-5 before initial symptoms emerge. About 97.5% of patients were reported to exhibit symptoms in 11.5 days[6]. The symptoms include fever, dry cough, breathing difficulty, muscle soreness, headache, and diarrhea. SARS-CoV-2 infections can turn into Acute Respiratory Distress Syndrome (ARDS) approximately 8-9 days after the first symptoms[7]. Severe ARDS in COVID-19 patients can be indicated by breathing difficulty and low blood oxygen level[8]. ARDS is known to cause respiratory failure leading to death in 70% of COVID-19 cases. Viral infection or secondary infection in patients is known to cause cytokine storm and sepsis symptoms, which result in death in 28% of the patients[9]. Uncontrolled inflammation in COVID-19 disease is reported to lead to multiorgan damage, eventually resulting in organ failure, especially heart, liver, and kidney failures[10]. However, this inflammation can be treated using probiotic bacteria.

Probiotic bacteria are the potential to treat diseases caused by inflammation and immune system responses[11]. Probiotic bacteria play roles in humoral immunity by interacting with intestinal epithelial cells and lamina propria-related cells through toll receptors. The probiotic bacteria are reported to lower cytokines that produce inflammatory cells and immune system decline through NF-KB transcription factor pathways[12]. Immune response and inflammation in the cell can be affected by NF-KB. In this regard, NF-KB has become the object of developing a treatment for diseases caused by inflammation[13]. Inflammatory response and immune system can be stimulated using *Lactobacillus* strain probiotic[14].

Lactobacillus is a gram-positive, non-spore-forming, lactic acid bacteria. This bacterium generates lactic acid as its primary product through carbohydrate fermentation. Morphologically, *Lactobacillus* can be in the form of a non-shortening bar in the chain form. *Lactobacillus* is a part of microbiota colonizing the mouth and digestive tract[15]. *Lactobacillus* colony species commonly found in the digestive tract are *Lactobacillus plantarum* and *L. fermentum*[16]. *Lactobacillus plantarum* and *L. fermentum* exhibit high probiotic potentials and become potential anti-inflammatory and immune responses by modulating pro-inflammatory cytokines[17]. This paper reviews the anti-inflammatory and immunostimulant potentials of *L. plantarum* and *L. fermentum* reported *in vitro*, *in vivo*, and in clinical studies. This paper also provides information about the metabolite compounds of *L. plantarum* and *L. fermentum* as anti-inflammatory and immunostimulants in treating COVID-19.

INFLAMMATION AND IMMUNE RESPONSE

The virus is attached to the host through a receptor. Angiotensin 2 (ACE2) and TMPRSS2[18] are known to be the host receptor used by SARS-CoV-2 to infect the cell. This target receptor can be found in the respiratory tract, such as epithelial cells, alveolar epithelial cells, vascular endothelial cells, and macrophages in the lungs[19-21]. Viral replication and release may cause pyroptosis in the host cell and damage the associated molecular pattern, including ATP, nucleic acid, and ASC oligomer. The virus is recognized by epithelial cells, endothelial cells, and alveolar macrophages, triggering the formation of pro-inflammatory cytokine and chemokine (including IL-6, IP-10, macrophage inflammatory protein 1α (MIP1α), MIP1β, and MCP1). This protein attracts monocyte, macrophage, and T cell to the infected area and promotes further inflammation by adding interferon-γ (IFN-γ) produced by T cells.

The damaged immune response can cause further accumulation of immune cells in the lungs, leading to excessive pro-inflammatory cytokines and eventually damaging the lungs. The produced cytokine storm circulates to other organs, causing multiorgan damage. Bronchoalveolar fluid (BALF) patients with COVID-19 symptoms are reported to contain Chemokine CCL2 and CCL7. Both chemokines are responsible for recruiting Cc-chemokine receptor 2-positive (CCR2+)[22]. Several cytokine and chemokine monocytes are reported to play roles in the inflammatory process in COVID-19 patients[15,23-25]. The inflammation severity is indicated by the increase in cytokine and chemokine levels. Macrophage activation due to the viral infection can cause increased cytokine IL-6, IL-7, TNF-α, and inflammatory chemokine, including Cc-chemokine 2 (CCL2), CCL3, CXC-chemokine 10 (CXCL10), and IL2. The irregularity of mononuclear phagocyte activation may cause hyperinflammation in COVID-19 patients. Some hypotheses exist on the mechanism contributing to monocyte hyperactivity due to macrophage in COVID-19 patients[26,27].

The delayed type 1 interferon production leads to the increased cytopathic effect. The increased microbial threat may enhance the chemoattractant by alveolar epithelial cells, macrophages, and stromal cells, increasing the number of monocytes in the lungs. The monocytes then differentiate into pro-inflammatory macrophages through Janus-activated kinase (JAK)-signal transducer and activator of transcription (STAT). The T-cell will induce the monocyte-derived macrophages by producing granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α, and IFNγ.

Oxidized phospholipids (OxPLs) deposit in the lungs' infected area and activate monocyte-macrophage through Toll-like receptors 4 (TLR4), TRAF6, and NF-κB. The virus infection can trigger the TLR7 activation through single-stranded RNA virus recognition. The virus enters the macrophage cytoplasm through the type-1 interferon receptor. The virus activates the NLRP3 inflammasome and causes mature IL-1β and IL-18 secretions. The IL-1β cytokine can increase macrophage activation in autocrine or paracrine. It can also decrease interferon type I production in the infected lungs. Macrophage-activated monocyte contributes to the formation of cytokine storm of COVID-19 by releasing many pro-inflammatory cytokines[28].

SARS-CoV-2 hampers the body's normal immune response, causing immune system damage and uncontrolled inflammatory response in severe COVID-19 patients. COVID-19 patients are reported to exhibit lymphopenia, lymphocyte activation and dysfunction, granulocyte and monocyte disorder, high cytokine levels, increased immunoglobulin G (IgG), and a total of antibodies[29]. Immune response patterns in Covid-19 patients are depicted in **Figure 1**.

Lymphopenia is the primary marker of severe COVID-19 patients. Patients will likely exhibit declined CD4+ T, CD8+ T, and B cell levels[30]. T cell activation due to the virus infection may increase the IFN-γ, TNF-α, and IL-2 levels. In addition, lymphocytes are reported to release phenotypic programmed cell death protein-1 (PD1), T-cell immunoglobulin domain, mucin domain-3 (TIM3), and killer cell lectin-like receptor subfamily C member 1 (NKG2A). COVID-19 patients will likely exhibit increased neutrophil and decreased eosinophil, basophil, and monocyte. They also exhibit increased cytokine production, especially IL-1β, IL-6, dan IL-10. A higher IgG and total antibody titers are also observed in COVID-19 patients[29].

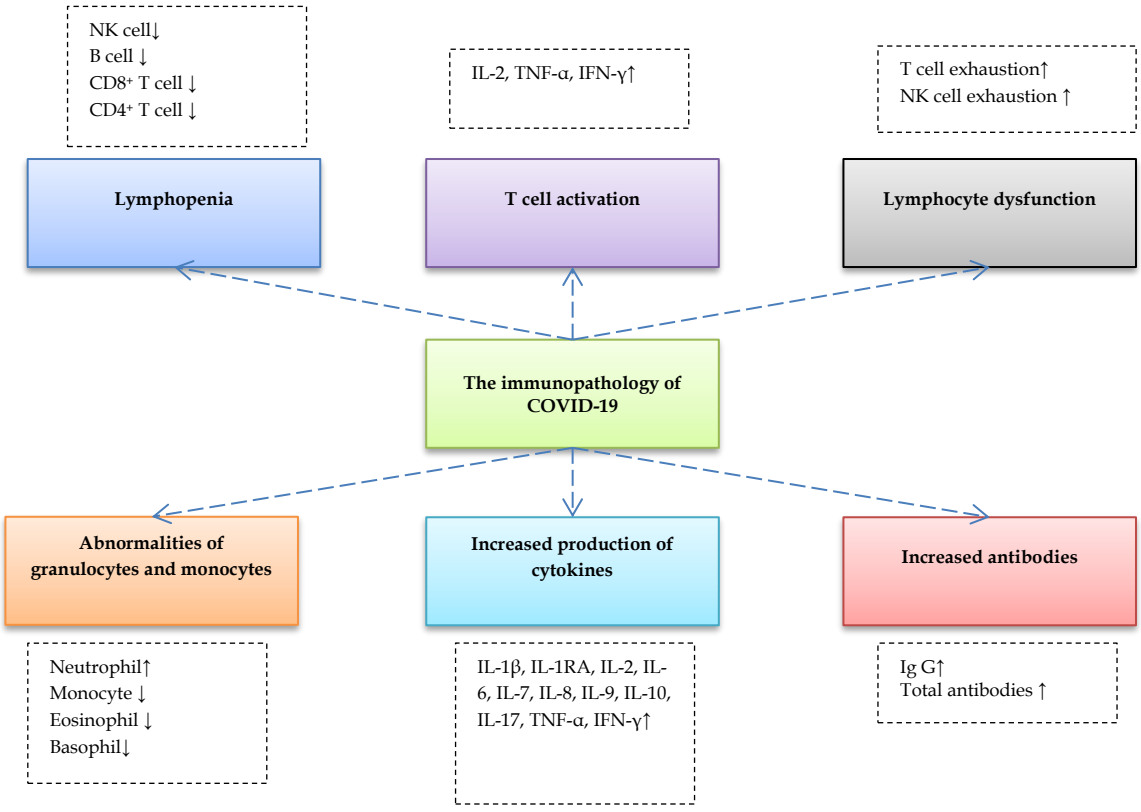


Figure 1. COVID-19 Immunopathological mechanism[29].

Lactobacillus METABOLITE COMPOUND

Lactobacillus produces intracellular and extracellular metabolism. The produced metabolite can provide information regarding the potential of bacteria on nutrition and its toxicity effect on the disease. Some metabolites are reported to be defrosting agents, antioxidants, antimicrobial agents, natural diet additives, and anti-inflammatory agents[31].

The fingerprint analysis of metabolite compounds can be performed using gas chromatography to determine *Lactobacillus*'s intracellular and extracellular metabolite analysis[32]. Gas chromatography-Mass spectrometry (GC-MS) is a chromatography with high-resolution separation results and good sensitivity and specificity. This instrument can analyze metabolic products such as carbohydrates, fatty acids, organic acids, and amino acids[33]. The sample derivatization is necessary before performing GC-MS analysis[34]. Chaudary *et al*.[35] identified 40 metabolites and five bacteria isolations, including *L. plantarum* DB-2, *L. fermentum* J-1, *Pediococcus acidilactici* M-3, *L. plantarum* SK- 3 dan *P. pentosaceus* SM-234. Metabolite compounds generated by *Lactobacillus* are presented in **Table I**.

Table I. Identification of metabolite compounds of *L. plantarum* and *L. fermentum*[35]

***Lactobacillus plantarum* DB-2**	***Lactobacillus fermentum* J-1**	***Lactobacillus plantarum* SK-3**
2-ethoxyethylamine (PubChem ID: 66970)	2-propanol,1-hydrazino (PubChem ID: 236167)	2-propanol,1-hydrazino (PubChem ID: 236167)
2-hydrazino ethanol (PubChem ID: 8017)	(Z)-9-octadecenamide (PubChem ID: 5283387)	4-amino-1-butanol (PubChem ID: 25868)
2-propanol,1-hydrazino (PubChem ID: 236167)	2,4,dimethylbenzaldehyde (PubChem ID: 61814)	(Z)-9-octadecenamide (PubChem ID: 5283387)
(Z)-9-octadecenamide (PubChem ID: 5283387)	benzoic acid (PubChem ID: 243)	2,4-dimethylbenzaldehyde (PubChem ID: 61814)
acetic acid, acetic formic anhydride (PubChem ID: 75269)	decane (PubChem ID: 15600)	benzoic acid (PubChem ID: 243)
2,4-dimetilbenzaldehyde (PubChem ID: 61814)	dodecane (PubChem ID: 8182)	decane (PubChem ID: 15600)
benzoic acid (PubChem ID: 243)	dodecanoic acid (PubChem ID: 236167)	dodecane (PubChem ID: 8182)
decane (PubChem ID: 15600)	eicosanoid acid (PubChem ID: 10467)	dodecanoic acid (PubChem ID: 236167)
dl-2,3–butanediol (PubChem ID: 225936)	Isovaleric geraniol (PubChem ID: 5362830)	2-propoxy-ethanamine (PubChem ID: 111878)
dodecane dodecane (PubChem ID: 8182)	Hexadecane (PubChem ID: 11006)	2-(2-propenyloxy)- ethanol (PubChem ID: 8116)
dodecanoic acid (PubChem ID: 236167)	2,6,11,15-tetramethylhexadecane (PubChem ID: 136331)	isovaleric geraniol (PubChem ID: 5362830)
ethylamine (PubChem ID: 6341)	1-methylhexyl hydroperoxide (PubChem ID: 12981)	Hexadecane (PubChem ID: 11006)
formamide (PubChem ID: 713)	isopropyl alcohol (PubChem ID: 3776)	2,6,11,15-tetramethylhexadecane (PubChem ID: 136331)
isovaleric geraniol (PubChem ID: 5362830)	isopropyl myristate (PubChem ID: 8042)	pentyl hydroperoxide (PubChem ID: 135961)
hexadecane (PubChem ID: 11006)	lactic acid (PubChem ID: 107689)	isopropyl alcohol (PubChem ID: 3776)
2,6,11,15-tetramethylhexadecane (PubChem ID: 136331)	hexadecanoic acid (PubChem ID: 985)	isopropyl myristate (PubChem ID: 8042)
isopropyl alcohol (PubChem ID: 3776)	phenol,2,4-bis- (1,1dimethylethyl) (PubChem ID: 7311)	lactic acid (PubChem ID: 107689)
lactic acid (PubChem ID: 107689)	propinoic acid, 2-hydroxymethyl ester (PubChem ID: 126674963)	nitrosomethane (PubChem ID: 70075)
nitrosomethane (PubChem ID: 70075)	hexahydro-3-(2- methylpropyl) pirolo[1,2-a]pirazin-1,4-dion (PubChem ID: 102892)	hexadecanoic acid (PubChem ID: 985)
hexadecanoic acid (PubChem ID: 985)	tetracosane (PubChem ID: 12592)	phenol,2,4-bis- (1,1dimethylethyl) (PubChem ID: 7311)
phenol,2,4-bis- (1,1dimethylethyl) (PubChem ID: 7311)	tetradecane (PubChem ID: 12389)	propylene glicol (PubChem ID: 1030)
propylene glicol (PubChem ID: 1030)	tetradecanoic acid (PubChem ID: 11005)	hexahydro-3-(2- methylpropyl) pirolo[1,2-a]pirazin-1,4-dion (PubChem ID: 102892)
hexahydro-3-(2- methylpropyl) pirolo[1,2-a]pirazin-1,4-dion (PubChem ID: 102892)	undecane (PubChem ID: 14257)	(R)-1,2-propanediol (PubChem ID: 259994)
(R)-1,2-propanediol (PubChem ID: 259994)		tetradecane (PubChem ID: 12389)
tetracosane (PubChem ID: 12592)		undecane (PubChem ID: 14257)
tetradecane (PubChem ID: 12389)		
undecane (PubChem ID: 14257)		

The identified metabolites, such as isopropyl alcohol, dodecane, hexadecane, tetradecane, hexahydro-3-(2-methyl propyl) pirolo[1,2-a], pyrazine-1,4-dion, 2,4-dimethyl benzaldehyde, isovaleric geraniol, phenol, 2,4 bis (1,1-dimethyl); 2,6,11,15-tetramethyl-hexadecanoic acid, (Z)-9-octadecenamide, are reported to be potential defrosting, antioxidant, antimicrobial, and anti-inflammatory agents[31]. In addition, short-chain fatty acids (SCFA) produced by probiotic bacteria, such as acetate,

butyrate, and propionate, play roles in decreasing nitric oxide (NO)[36,37]. Inflammation causes an immune response to activating cytokine in producing NO, resulting in increased NO. SCFA produced by Lactobacillus can induce immune response through tolerogenic dendritic cells (**Figure 2**). Fatty acid compounds can have an inhibitory effect on inflammation, especially omega-6 fatty acids. However, the interaction mechanism of omega-6 fatty acids and their lipid mediators in inflammation is still not well understood[38].

The tolerogenic process of dendritic cells makes the T-cell (CD4+) differentiate into T-cell regulators (Treg) and inhibits cytokine production by neutrophils and macrophages. Tolerogenic dendritic cells produce anti-inflammatory cytokines, interleukin-10 (IL-10), and transforming growth factor-β (TGF-β). A tolerogenic dendritic cell is a potential candidate for specific immunotherapy[37].

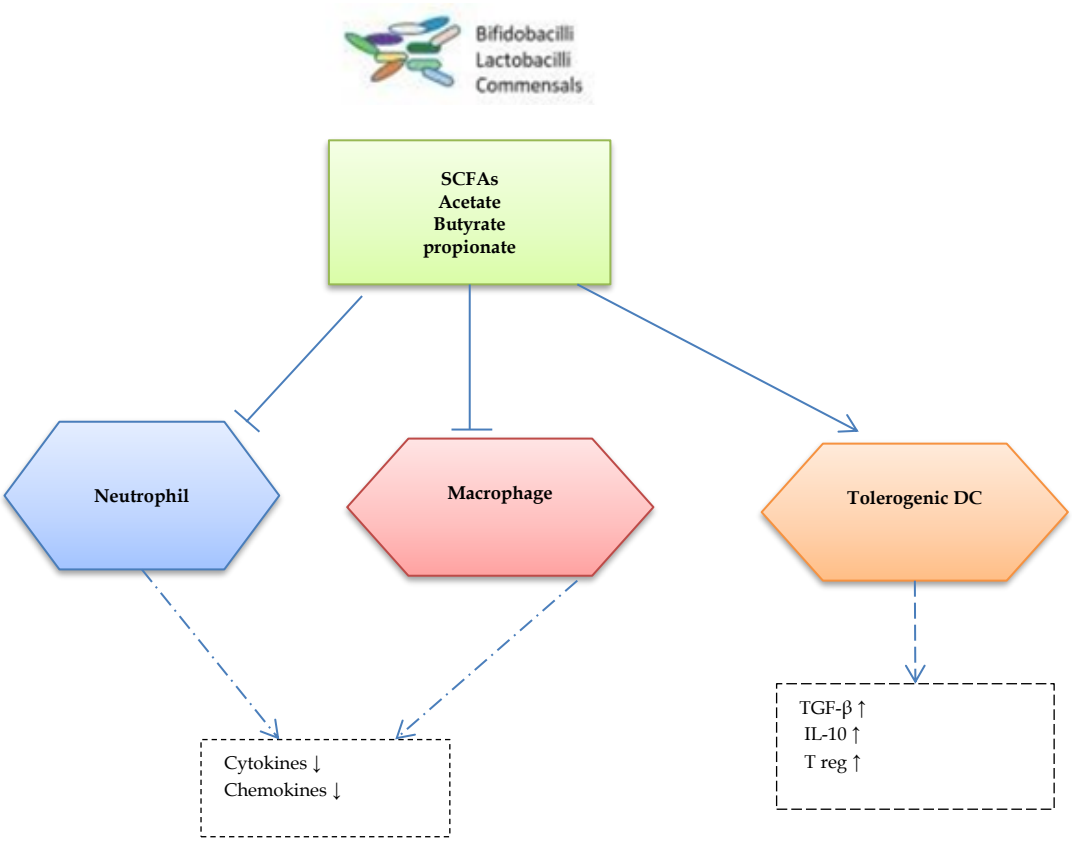


Figure 2. SCFA's work mechanism in decreasing inflammatory activities[37].

ANTI-INFLAMMATORY AND IMMUNOSTIMULANT ACTIVITIES OF *Lactobacillus*

Anti-inflammatory and immunostimulant activities of *Lactobacillus* have been widely studied through *in vitro* and *in vivo* research. **Table II** displays several studies on the anti-inflammatory and immunostimulant activities of *L. plantarum* with different strains. *Lactobacillus'* immunostimulant activities can occur through the increase in cytokine IL-10 production in mononuclear cells (macrophage and T-cell) in the intestine[39]. A study shows that *L. plantarum* CM can inhibit the binding activity of NF-κB in response to TNF-α. This response weakens the release of monocyte chemotactic protein 1 (MCP-1), pro-inflammatory chemokine, and NF-κB gene and inhibits the proteasome functions. *Lactobacillus plantarum* CM inhibits the activation of NF-κB from TNF through MyD88-dependent and MyD88-independent pathways. *Lactobacillus plantarum* can also inhibit TNF-α-induced MCP-1 production in Caco-2 cells and lower NF-κB, mitogen protein kinase, and production of TNF-α or IL-1β[40-42].

In vivo studies report that *L. plantarum* and *L. fermentum* possess inflammatory activities[43,44]. The effective dose of probiotic bacteria to treat inflammation is reported to be 1x10^8 – 10^9 CFU/mL[44,45]. *Lactobacillus fermentum* is reported to significantly lower malondialdehyde levels, TNF-α, IL-6, and resistin in mouse blood serum. *Lactobacillus* bacteria is also reported to increase catalase, superoxide dismutase, glutathione peroxidase, and adiponectin activities, suppressing the inflammation-inducing- oxidative stress. Most studies show that *L. plantarum* induces IL-10 secretion in splenocytes and mesenteric lymphocytes, blocking the expression of pro-inflammatory cytokines, IL-1β, IL-6, TNF-α, COX-2, forkhead box P3 (Foxp3), suppressor of cytokine signaling 3 (SOCS3). *In vivo* study shows a decline in mucose IL-12, IFN-γ, and immunoglobulin G2a in mice[46]. The treatment using L. plantarum Biocenol™ LP96 was reported to lower the expression of IL-1α, and IL-8 genes increase the IFN-γ and cytokine IL-10 secretion[47]. This paper reviewed *in vitro* and *in vivo* studies to show *Lactobacillus'* metabolite product potential in inhibiting inflammatory activities.

Table II. Results of *in vitro* and *in vivo* studies on anti-inflammatory and immunostimulant activities of *L. plantarum* and *L. fermentum*.

Bacterial Strain	Method	Animal/cell	Dose	Inhibitory effect	Reference
L. plantarum APsulloc 331261	*in vitro*	THP1 cell	-	Inducing the expression of macrophage cytokine, IL-1β, inflammatory cytokine, and IL- 10,	48
L. plantarum L15	*in vitro*	Caco- 2	-	Lowering the expression of TLR4 and MyD88 genes and genes associated with NF-κB signalling pathways.	49
L. plantarum M2 and *L. plantarum* KO9	*in vitro*	Caco- 2	-	Inhibiting TNF-α production	50
L. plantarum MYL26	*in vitro*	Caco- 2	-	Inhibiting NF- κB, MAPK, TOLLIP, SOCS1, SOCS3, and IκBα expression	51
L. plantarum Lp62	*in vitro*	Intestinal epithelial cell HT-29, macrophage J774	-	Inhibiting production of IL-8, TNF-α, IL1-β, and IL-17.	52
L. plantarum CAU1055	*in vitro*	RAW264.7 cells	-	Inhibiting production NO, TNF-α, IL-6.	53
L. plantarum K8	*in vitro*	Intestinal epithelial cell HT-29	-	Inhibiting NF- κB and MAPK,	41
L. plantarum A41 and *L. fermentum* SRK414	*in vitro*	Intestinal epithelial cell HT-29	-	Decreasing the regulation of mRNA expression from proinflammatory cytokine TNF- α, IL1β, and IL-8 and enhancing intestinal barrier integrity by increasing protein ZO-1 expression	17
L. plantarum K8	*in vitro*	Monocytic THP-1 cell human	-	Inhibiting TNF- α , IL-1, NF-κB Increasing MAPK, Inhibiting NOD2 production	42
L. fermentum MCC 2760	*in vitro*	Caco- 2, intestinal epithelial cell HT-29	-	Increasing cytokine IL-10 production and inhibiting IL-6 production	54
L. fermentum CECT5716	*in vitro*	RAW 264.7 cells	-	Decreasing the proinflammatory cytokine TNF- α, IL1β, and IL-6	55
L. plantarum CGMCC1258	*in vivo*	Mouse without IL- 10	10^9 CFU/mL	Decreasing IFN-γ, TNF-α, and MPO production	56
L. plantarum Lp91	*in vivo*	Mouse without IL- 10	10^9 CFU/mL	Reducing expression of TNF-α and COX-2, Increasing the production of IL-10	57
L. plantarum OLL2712	*in vivo*	obese and type 2 diabetic KKAy mice	-	Increasing cytokine IL-10, suppressed proinflammatory cytokine level	58
L. fermentum DALI02	*in vivo*	Mouse hyperlipidemia	10^9 CFU/mL	Decreasing expression of TNF-α, IL- 6, and resistin and significantly increase APPN level	43
L. fermentum SNR1	*in vivo*	Wistar Albino Rats	10^8 CFU/mL	Increasing IL- 10, IL-6	59
L. fermentum and *L. salivarius*	*in vivo*	DSS mouse colitis	5×10^8 CFU/mL	Improving the colonic expression of markers in immune response	60
L. fermentum KBL374 and *L. fermentum* KBL375	*in vivo*	Female mouse C57BL/6N	10^9 CFU/mL	Increasing cytokine level associated with Th1, Th2-, and Th17, Increasing IL- 10, and increasing CD4+CD25+Foxp3 +Treg	61
L. plantarum LP-Onlly	*in vivo*	Mouse without IL- 10	10^9 CFU/mL	Lowering the inflammation and histological injury value; increasing the number of bifidobacteria and lactobacili	62

				good bacteria, decreasing the number of pathogenic bacteria of eterococci and *Clostridium perfringens*	
L. plantarum LP3457	*in vivo*	Mouse ZDF	10^8 CFU/mL	Decreasing IL-1β, IL-6, and CRP expression, Increasing IL-10 level	63
L. plantarum K8	*in vivo*	Healthy mouse	10^9 CFU/mL	Reducing expression of TNF-α and IL-6	64
L. plantarum ZS2058 (ZS2058) and *L. rhamnosus GG*	*in vivo*	Specific pathogen-free mice	5×10^9 CFU/mL	Changing in the levels of tissue necrosis factor (TNF)-α, IL-10 and myeloperoxidase (MPO)	65
L. fermentum XY18	*in vivo*	Gastric injury model group mice	1×10^9 CFU/kg	Reducing expression of TNF-α, IL-12 and IL-6	66
L. fermentum MCC2760	*in vivo*	Hypercholesterolemic C57BL6 Mice	0.95 log CFU/mL	Increasing cytokine IL-10, suppressed proinflammatory cytokine level TNF-α, IL-12 and IL-6	67
L. fermentum DALI02	*in vivo*	Hyperlipidemic mouse	10^9 CFU/mL	Reducing expression of TNF-α and IL-6	43
L. fermentum CQPC07	*in vivo*	Obessed mouse	10^9 CFU/kg	Decreasing the number of inflammatory cytokine interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), IL-6, and interferon- γ (IFN-γ), and increasing the production of cytokine IL- 10 and IL-4.	45

Lactobacillus IN THE TREATMENT OF COVID-19

Probiotics have an essential role in the eubiosis of the human microbiota[68]. Patients with COVID-19 symptoms had lower intestinal bacteria counts than normal patients[69]. These gut bacteria can enhance the immune response[70]. Probiotics and their metabolites can be used as a complementary strategy other than vaccines that can inhibit COVID-19[71]. *Lactobacillus* can inhibit the development of viruses through various mechanisms, direct interaction between probiotics and viruses; stimulation of the immune system; and virus-inhibiting metabolites[72]. The metabolites produced by lactic acid bacteria can inhibit the development of pathogenic bacteria and viruses[73]. These metabolites include amino acid derivatives (indolelactic acid, phenyllactic acid 2-hydroxy-4-,2-hydroxy-4-methylpentanoic acid, and 2-hydroxy-4-methylthio butanoic acid), fatty acids (3-hydroxy-5-cis-dodecanoic acid and 3-hydroxydodecanoic acid), organic compounds (acetic acid, lactic acid, propionic acid, succinic acid, and benzoate acid), cyclic peptides (cyclo(L-Phe-L-Pro) reutericyclin), and other groups of chemical compounds (δ-dodecalactone)[74].

Clinical trials showed that 75.61% of patients treated with probiotic bacteria had a shorter treatment time than those not treated with probiotics. These bacteria can reduce secondary infections and moderate the patient's immune system based on the analytical parameters of IL-6, CRP, total T lymphocytes, NK cells, B lymphocytes, CD4 + T cells, CD8 + T cells, and CD4/CD8 ratio[75]. In another study, patients receiving probiotic bacteria *L. plantarum* (KABP022, KABP023, and KAPB033) with a combination of *P. acidilactici* KABP021 for 30 days showed inhibition against the COVID-19 virus[76]. *In silico* studies have also carried molecular docking on the metabolite *L. plantarum* Probio-88 to the SARS-CoV-2 helicase. The high binding affinity and hydrogen bonding suggests that the association of PlnE and PlnF on the helicase of SARS-COV-2 may inhibit virus replication[77].

Indonesia abounds in biodiversity, including microorganisms. *Lactobacillus plantarum* and *L. fermentum* indigenous strains of Indonesian have potential as anti-inflammatory and immunostimulant. Our preliminary research showed that the superior candidate bacteria from the two strains had antibacterial activity and could withstand acidic conditions and high temperatures. Therefore, further study is needed to determine the anti-inflammatory and immunostimulant activities to be used as an immunomodulator for COVID-19.

CONCLUSION

Based on the results of experimental and clinical research data, *L. plantarum* and *L. fermentum* have activities as anti-inflammatory and immunostimulants in COVID-19 patients. *Lactobacillus* can reduce the activity of inflammatory cytokines IL-1β, IL-6, TNF-, COX-2, Foxp3, SOCS3 suppressor, and increase IL-10. Patients treated with probiotics had a faster recovery time than those not treated with *Lactobacillus*. *Lactobacillus* can reduce secondary infection and increase immune response in COVID-19 patients. Bioactive compounds from these bacteria can also cause anti-inflammatory and immunostimulant activities.

ACKNOWLEDGMENT

We would like to thank LPPM IPB University for supporting this research in the "*Konektivitas Pembelajaran Mahasiswa Pascasarjana*" Program.

AUTHORS' CONTRIBUTION

All authors are the main contributors in carrying out the research and writing this review article.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

REFERENCES

1. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, Li Y, et al. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 2017;9(6):7204-18. doi:10.18632/oncotarget.23208
2. Schultze JL, Aschenbrenner AC. COVID-19 and the human innate immune system. Cell. 2021;184(7):1671-92. doi:10.1016/j.cell.2021.02.029
3. Chen Y, Liu Q, Guo D. Emerging coronaviruses: Genome structure, replication, and pathogenesis. J Med Virol. 2020;92(4):418-23. doi:10.1002/jmv.25681
4. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. N Engl J Med. 2020;382(8):727-33. doi:10.1056/nejmoa2001017
5. Fehr AR, Perlman S. Coronaviruses: an overview of their replication and pathogenesis. Methods Mol Biol. 2015;1282:1-23. doi:10.1007/978-1-4939-2438-7_1
6. Lauer SA, Grantz KH, Bi Q, Jones FK, Zheng Q, Meredith HR, et al. The incubation period of coronavirus disease 2019 (CoVID-19) from publicly reported confirmed cases: Estimation and application. Ann Intern Med. 2020;172(9):577-82. doi:10.7326/M20-0504
7. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical Characteristics of 138 Hospitalized Patients with 2019 Novel Coronavirus-Infected Pneumonia in Wuhan, China. JAMA. 2020;323(11):1061-9. doi:10.1001/jama.2020.1585

8. Zhang B, Zhou X, Qiu Y, Song Y, Feng F, Feng J, et al. Clinical characteristics of 82 cases of death from COVID-19. PLoS One. 2020;15(7):e0235458. doi:10.1371/journal.pone.0235458

9. Ye Q, Wang B, Mao J. The pathogenesis and treatment of the `Cytokine Storm' in COVID-19. J Infect. 2020;80(6):607-13. doi:10.1016/j.jinf.2020.03.037

10. Zaim S, Chong JH, Sankaranarayanan V, Harky A. COVID-19 and Multiorgan Response. Curr Probl Cardiol. 2020;45(8):100618. doi:10.1016/j.cpcardiol.2020.100618

11. Plaza-Díaz J, Ruiz-Ojeda FJ, Vilchez-Padial LM, Gil A. Evidence of the Anti-Inflammatory Effects of Probiotics and Synbiotics in Intestinal Chronic Diseases. Nutrients. 2017;9(6):555. doi:10.3390/nu9060555

12. Kim YA, Keogh JB, Clifton PM. Probiotics, prebiotics, synbiotics and insulin sensitivity. Nutr Res Rev. 2018;31(1):35-51. doi:10.1017/S095442241700018X

13. Freitas RHCN, Fraga CAM. NF-κB-IKKβ Pathway as a Target for Drug Development: Realities, Challenges and Perspectives. Curr Drug Targets. 2018;19(16):1933-42. doi:10.2174/1389450119666180219120534

14. Rocha-Ramírez LM, Pérez-Solano RA, Castañón-Alonso SL, Guerrero SSM, Pacheco AR, Garibay MG, et al. Probiotic Lactobacillus Strains Stimulate the Inflammatory Response and Activate Human Macrophages. J Immunol Res. 2017;2017:4607491. doi:10.1155/2017/4607491

15. Yang Y, Shen C, Li J, Yuan J, Wei J, Huang F, et al. Exuberant elevation of IP-10, MCP-3 and IL-1ra during SARS-CoV-2 infection is associated with disease severity and fatal outcome. J Allergy Clin Immunol. 2020;146(1):119-27. doi:10.1016/j.jaci.2020.04.027

16. Tajabadi N, Mardan M, Saari N, Mustafa S, Bahreini R, Manap MYA. Identification of Lactobacillus plantarum, Lactobacillus pentosus and Lactobacillus fermentum from honey stomach of honeybee. Braz J Microbiol. 2014;44(3):717-22. doi:10.1590/s1517-83822013000300008

17. Lee CS, Kim SH. Anti-inflammatory and Anti-osteoporotic Potential of Lactobacillus plantarum A41 and L. fermentum SRK414 as Probiotics. Probiotics Antimicrob Proteins. 2020;12(2):623-34. doi:10.1007/s12602-019-09577-y

18. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. Cell. 2020;181(2):271-80.e8. doi:10.1016/j.cell.2020.02.052

19. Jia HP, Look DC, Shi L, Hickey M, Pewe L, Netland J, et al. ACE2 Receptor Expression and Severe Acute Respiratory Syndrome Coronavirus Infection Depend on Differentiation of Human Airway Epithelia. J Virol. 2005;79(23):14614-21. doi:10.1128/jvi.79.23.14614-14621.2005

20. Xu H, Zhong L, Deng J, Peng J, Dan H, Zeng X, et al. High expression of ACE2 receptor of 2019-nCoV on the epithelial cells of oral mucosa. Int J Oral Sci. 2020;12:8. doi:10.1038/s41368-020-0074-x

21. Hamming I, Timens W, Bulthuis MLC, Lely AT, Navis GJ, van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. J Pathol. 2004;203(2):631-7. doi:10.1002/path.1570

22. Tay MZ, Poh CM, Rénia L, MacAry PA, Ng LFP. The trinity of COVID-19: immunity, inflammation and intervention. Nat Rev Immunol. 2020;20(6):363-74. doi:10.1038/s41577-020-0311-8

23. Chen G, Wu D, Guo W, Cao Y, Huang D, Wang H, et al. Clinical and immunological features of severe and moderate coronavirus disease 2019. J Clin Invest. 2020;130(5):2620-9. doi:10.1172/JCI137244

24. Gong J, Dong H, Xia QS, Huang ZY, Wang DK, Zhao Y, et al. Correlation analysis between disease severity and inflammation-related parameters in patients with COVID-19: a retrospective study. BMC Infect Dis. 2020;20(1):963. doi:10.1186/s12879-020-05681-5

25. Rabaan AA, Al-Ahmed SH, Muhammad J, Khan A, Sule AA, Tirupathi R, et al. Role of Inflammatory Cytokines in COVID-19 Patients: A Review on Molecular Mechanisms, Immune Functions, Immunopathology and Immunomodulatory Drugs to Counter Cytokine Storm. Vaccines. 2021;9(5):436. doi:10.3390/vaccines9050436

26. Mehta P, McAuley DF, Brown M, Sanchez E, Tattersall RS, Manson JJ. COVID-19: consider cytokine storm syndromes and immunosuppression. Lancet. 2020;395(10229):1033-4. doi:10.1016/S0140-6736(20)30628-0

27. Schulert GS, Grom AA. Pathogenesis of macrophage activation syndrome and potential for cytokine- directed therapies. Annu Rev Med. 2015;66:145-59. doi:10.1146/annurev-med-061813-012806

28. Merad M, Martin JC. Author Correction: Pathological inflammation in patients with COVID-19: a key role for monocytes and macrophages. Nat Rev Immunol. 2020;20(7):448. doi:10.1038/s41577-020-0353-y

29. Yang L, Liu S, Liu J, Zhang Z, Wan X, Huang B, et al. COVID-19: immunopathogenesis and Immunotherapeutics. Signal Transduct Target Ther. 2020;5(1):128. doi:10.1038/s41392-020-00243-2

30. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020;395(10223):497-506. doi:10.1016/S0140-6736(20)30183-5

31. Sulijaya B, Takahashi N, Yamazaki K. Lactobacillus-Derived Bioactive Metabolites for the Regulation of Periodontal Health: Evidences to Clinical Setting. Molecules. 2020;25(9):2088. doi:10.3390/molecules25092088

32. Villas-Bôas SG, Mas S, Akesson M, Smedsgaard J, Nielsen J. Mass spectrometry in metabolome analysis. Mass Spectrom Rev. 2005;24(5):613-46. doi:10.1002/mas.20032

33. Park SE, Yoo SA, Seo SH, Lee KI, Na CS, Son HS. GC–MS based metabolomics approach of Kimchi for the understanding of Lactobacillus plantarum fermentation characteristics. LWT Food Sci Technol. 2016;68:313-21. doi:10.1016/j.lwt.2015.12.046

34. Schummer C, Delhomme O, Appenzeller BMR, Wennig R, Millet M. Comparison of MTBSTFA and BSTFA in derivatization reactions of polar compounds prior to GC/MS analysis. Talanta. 2009;77(4):1473-82. doi:10.1016/j.talanta.2008.09.043

35. Chaudhary A, Verma K, Saharan BS. A GC-MS based metabolic profiling of probiotic lactic acid bacteria isolated from traditional food products. J Pure Appl Microbiol. 2020;14(1):657-72. doi:10.22207/JPAM.14.1.68

36. Vinolo MAR, Rodrigues HG, Hatanaka E, Sato FT, Sampaio SC, Curi R. Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. J Nutr Biochem. 2011;22(9):849-55. doi:10.1016/j.jnutbio.2010.07.009

37. Liu Y, Alookaran JJ, Rhoads JM. Probiotics in autoimmune and inflammatory disorders. Nutrients. 2018;10(10):1537. doi:10.3390/nu10101537

38. Innes JK, Calder PC. Omega-6 fatty acids and inflammation. Prostaglandins Leukot Essent Fatty Acids. 2018;132:41-8. doi:10.1016/j.plefa.2018.03.004

39. Pathmakanthan S, Li CKF, Cowie J, Hawkey CJ. Lactobacillus plantarum 299: beneficial in vitro immunomodulation in cells extracted from inflamed human colon. J Gastroenterol Hepatol. 2004;19(2):166-73. doi:10.1111/j.1440-1746.2004.03181.x

40. Borthakur A, Anbazhagan AN, Kumar A, Raheja G, Singh V, Ramaswamy K, et al. The probiotic Lactobacillus plantarum counteracts TNF-{alpha}-induced downregulation of SMCT1 expression and function. Am J Physiol Gastrointest Liver Physiol. 2010;299(4):G928-34. doi:10.1152/ajpgi.00279.2010

41. Kim H, Jung BJ, Jung JH, Kim JY, Chung SK, Chung DK. Lactobacillus plantarum lipoteichoic acid alleviates TNF-α-induced inflammation in the HT-29 intestinal epithelial cell line. Mol Cells. 2012;33(5):479-86. doi:10.1007/s10059-012-2266-5

42. Kim HG, Lee SY, Kim NR, Lee HY, Ko MY, Jung BJ, et al. Lactobacillus plantarum lipoteichoic acid down-regulated Shigella flexneri peptidoglycan-induced inflammation. Mol Immunol. 2011;48(4):382-91. doi:10.1016/j.molimm.2010.07.011

43. Huang Y, Qu H, Liu D, Wa Y, Sang J, Yin B, et al. The effect of Lactobacillus fermentum DALI02 in reducing the oxidative stress and inflammatory response induced by high-fat diet of rats. RSC Adv. 2020;10(57):34396-402. doi:10.1039/d0ra05694d

44. Li S, Guo L, Si X, Dai Z, Zhou Z, Wu Z. Lactobacillus plantarum WCFS1 alleviates Aeromonas hydrophila NJ-1-induced inflammation and muscle loss in zebrafish (Danio rerio). Aquaculture. 2022;548(1):737603. doi:10.1016/j.aquaculture.2021.737603

45. Wu Y, Li X, Tan F, Zhou X, Mu J, Zhao X. Lactobacillus fermentum CQPC07 attenuates obesity, inflammation and dyslipidemia by modulating the antioxidant capacity and lipid metabolism in high-fat diet induced obese mice. J Inflamm. 2021;18(1):5. doi:10.1186/s12950-021-00272-w

46. Schultz M, Veltkamp C, Dieleman LA, Grenther WB, Wyrick PB, Tonkonogy SL, et al. Lactobacillus plantarum 299V in the treatment and prevention of spontaneous colitis in interleukin-10-deficient mice. Inflamm Bowel Dis. 2002;8(2):71-80. doi:10.1097/00054725-200203000-00001

47. Chytilová M, Mudroňová D, Nemcová R, Gancarčíková S, Buleca V, Koščová J, et al. Anti-inflammatory and immunoregulatory effects of flax-seed oil and Lactobacillus plantarum - BiocenolTM LP96 in gnotobiotic pigs challenged with enterotoxigenic Escherichia coli. Res Vet Sci. 2013;95(1):103-9. doi:10.1016/j.rvsc.2013.02.002

48. Kim W, Lee EJ, Bae IH, Myoung K, Kim ST, Park PJ, et al. Lactobacillus plantarum-derived extracellular vesicles induce anti-inflammatory M2 macrophage polarization in vitro. J Extracell Vesicles. 2020;9(1):1793514. doi:10.1080/20013078.2020.1793514

49. Yu P, Ke C, Guo J, Zhang X, Li B. Lactobacillus plantarum L15 Alleviates Colitis by Inhibiting LPS-Mediated NF-κB Activation and Ameliorates DSS-Induced Gut Microbiota Dysbiosis. Front Immunol. 2020;11:575173. doi:10.3389/fimmu.2020.575173

50. Kostelac D, Gerić M, Gajski G, Markov K, Domijan AM, Čanak I, et al. Lactic acid bacteria isolated from equid milk and their extracellular metabolites show great probiotic properties and anti-inflammatory potential. Int Dairy J. 2021;112:104828. doi:10.1016/j.idairyj.2020.104828

51. Chiu YH, Lu YC, Ou CC, Lin SL, Tsai CC, Huang CT, et al. Lactobacillus plantarum MYL26 induces endotoxin tolerance phenotype in Caco-2 cells. BMC Microbiol. 2013;13:190. doi:10.1186/1471-2180-13-190

52. Dos Santos TF, Melo TA, Almeida ME, Rezende RP, Romano CC. Immunomodulatory Effects of Lactobacillus plantarum Lp62 on Intestinal Epithelial and Mononuclear Cells. Biomed Res Int. 2016;2016:8404156. doi:10.1155/2016/8404156

53. Choi SH, Lee SH, Kim MG, Lee HJ, Kim GB. Lactobacillus plantarum CAU1055 ameliorates inflammation in lipopolysaccharide-induced RAW264.7 cells and a dextran sulfate sodium–induced colitis animal model. J Dairy Sci. 2019;102(8):6718-25. doi:10.3168/jds.2018-16197

54. Archer AC, Kurrey NK, Halami PM. In vitro adhesion and anti-inflammatory properties of native Lactobacillus fermentum and Lactobacillus delbrueckii spp. J Appl Microbiol. 2018;125(1):243-56. doi:10.1111/jam.13757

55. Rodríguez-Sojo MJ, Ruiz-Malagón AJ, Rodríguez-Cabezas ME, Gálvez J, Rodríguez-Nogales A. Limosilactobacillus fermentum CECT5716: Mechanisms and Therapeutic Insights. Nutrients. 2021;13(3):1016. doi:10.3390/nu13031016

56. Liu Z, Zhang P, Ma Y, Chen H, Zhou Y, Zhang M, et al. Lactobacillus plantarum prevents the development of colitis in IL-10-deficient mouse by reducing the intestinal permeability. Mol Biol Rep. 2011;38(2):1353-61. doi:10.1007/s11033-010-0237-5

57. Duary RK, Bhausaheb MA, Batish VK, Grover S. Anti-inflammatory and immunomodulatory efficacy of indigenous probiotic Lactobacillus plantarum Lp91 in colitis mouse model. Mol Biol Rep. 2012;39(4):4765-75. doi:10.1007/s11033-011-1269-1

58. Toshimitsu T, Mochizuki J, Ikegami S, Itou H. Identification of a Lactobacillus plantarum strain that ameliorates chronic inflammation and metabolic disorders in obese and type 2 diabetic mice. J Dairy Sci. 2016;99(2):933-46. doi:10.3168/jds.2015-9916

59. Ayyanna R, Ankaiah D, Arul V. Anti-inflammatory and Antioxidant Properties of Probiotic Bacterium Lactobacillus mucosae AN1 and Lactobacillus fermentum SNR1 in Wistar Albino Rats. Front Microbiol. 2018;9:3063. doi:10.3389/fmicb.2018.03063

60. Rodríguez-Nogales A, Algieri F, Garrido-Mesa J, Vezza T, Utrilla MP, Chueca N, et al. Differential intestinal anti-inflammatory effects of Lactobacillus fermentum and Lactobacillus salivarius in DSS mouse colitis: impact on microRNAs expression and microbiota composition. Mol Nutr Food Res. 2017;61(11):1700144. doi:10.1002/mnfr.201700144

61. Jang YJ, Kim WK, Han DH, Lee K, Ko G. Lactobacillus fermentum species ameliorate dextran sulfate sodium-induced colitis by regulating the immune response and altering gut microbiota. Gut Microbes. 2019;10(6):696-711. doi:10.1080/19490976.2019.1589281

62. Xia Y, Chen HQ, Zhang M, Jiang YQ, Hang XM, Qin HL. Effect of Lactobacillus plantarum LP-Onlly on gut flora and colitis in interleukin-10 knockout mice. J Gastroenterol Hepatol. 2011;26(2):405-11. doi:10.1111/j.1440-1746.2010.06498.x

63. Vilahur G, López-Bernal S, Camino S, Mendieta G, Padró T, Badimon L. Lactobacillus plantarum CECT 7315/7316 intake modulates the acute and chronic innate inflammatory response. Eur J Nutr. 2015;54(7):1161-71. doi:10.1007/s00394-014-0794-9

64. Ahn YS, Park MY, Shin JH, Kim JY, Kwon O. Lysate of Probiotic Lactobacillus plantarum K8 Modulate the Mucosal Inflammatory System in Dextran Sulfate Sodium-induced Colitic Rats. Korean J food Sci Anim Resour. 2014;34(6):829-35. doi:10.5851/kosfa.2014.34.6.829

65. Liu J, Gu Z, Song F, Zhang H, Zhao J, Chen W. Lactobacillus plantarum ZS2058 and Lactobacillus rhamnosus GG Use Different Mechanisms to Prevent Salmonella Infection in vivo. Front Microbiol. 2019;10:299. doi:10.3389/fmicb.2019.00299

66. Wang R, Zhou K, Xiong R, et al. Pretreatment with Lactobacillus fermentum XY18 Relieves Gastric Injury Induced by HCl/Ethanol in Mice via Antioxidant and Anti-Inflammatory Mechanisms. Drug Des Devel Ther. 2020;14:5721-34. doi:10.2147/DDDT.S280429

67. Kumar MKP, Halami PM, Peddha MS. Effect of Lactobacillus fermentum MCC2760-Based Probiotic Curd on Hypercholesterolemic C57BL6 Mice. ACS omega. 2021;6(11):7701-10. doi:10.1021/acsomega.1c00045

68. Hemarajata P, Versalovic J. Effects of probiotics on gut microbiota: mechanisms of intestinal immunomodulation and neuromodulation. Therap Adv Gastroenterol. 2013;6(1):39-51. doi:10.1177/1756283X12459294

69. Gu S, Chen Y, Wu Z, Chen Y, Gao H, Lv L, et al. Alterations of the Gut Microbiota in Patients with Coronavirus Disease 2019 or H1N1 Influenza. Clin Infect Dis. 2020;71(10):2669-78. doi:10.1093/cid/ciaa709

70. Dang AT, Marsland BJ. Microbes, metabolites, and the gut-lung axis. Mucosal Immunol. 2019;12(4):843-50. doi:10.1038/s41385-019-0160-6

71. Nguyen QV, Chong LC, Hor YY, Lew LC, Rather IA, Choi SB. Role of Probiotics in the Management of COVID-19: A Computational Perspective. Nutrients. 2022;14(2):274. doi:10.3390/nu14020274

72. Menni C, Valdes AM, Freidin MB, Sudre CH, Nguyen LH, Drew DA, et al. Real-time tracking of self-reported symptoms to predict potential COVID-19. Nat Med. 2020;26(7):1037-40. doi:10.1038/s41591-020-0916-2

73. Siedler S, Balti R, Neves AR. Bioprotective mechanisms of lactic acid bacteria against fungal spoilage of food. Curr Opin Biotechnol. 2019;56:138-46. doi:10.1016/j.copbio.2018.11.015

74. Suryani S, Purnawati Y, Putri SG, Rahmawati R, Yustitia A, Yusra Y. New Probiotic Isolation of Coconut Water's Helpful Lactic Acid Bacteria Cure Covid-19 Patients. ARRUS J Eng Technol. 2022;2(1):1-11. doi:10.35877/jetech724

75. Li Q, Cheng F, Xu Q, Su Y, Cai X, Zeng F, et al. The role of probiotics in coronavirus disease-19 infection in Wuhan: A retrospective study of 311 severe patients. Int Immunopharmacol. 2021;95:107531. doi:10.1016/j.intimp.2021.107531

76. Gutiérrez-Castrellón P, Gandara-Martí T, Abreu AT, Nieto-Rufino CD, López-Orduña E, Jiménez-Escobar I, et al. Probiotic improves symptomatic and viral clearance in Covid19 outpatients: a randomized, quadruple-blinded, placebo-controlled trial. Gut Microbes. 2022;14(1):2018899. doi:10.1080/19490976.2021.2018899

77. Rather IA, Choi SB, Kamli MR, Hakeem KR, Sabir JSM, Park YH, et al. Potential Adjuvant Therapeutic Effect of Lactobacillus plantarum Probio-88 Postbiotics against SARS-COV-2. Vaccines. 2021;9(10):1067. doi:10.3390/vaccines9101067

Borneo Journal of Pharmacy
Vol 5 Issue 3 August 2022
Page 268 – 278

http://journal.umpalangkaraya.ac.id/index.php/bjop/article/view/3156
DOI: https://doi.org/10.33084/bjop.v5i3.3156
e-ISSN: 2621-4814

Research Article

Taro (*Colosia esculenta*) Leaves Extract Inhibits *Streptococcus mutans* ATCC 31987

Ayu Nala El Muna Haerussana*
Angreni Ayuhastuti
Siti Fira Yuniar
Hana Alifah Bustami
Widyastiwi

Department of Pharmacy, Poltekkes Kemenkes Bandung, Bandung, West Java, Indonesia

*email: ayunalaelmh@gmail.com

Keywords:
Antibacterial activity
Colocasia esculenta
Streptococcus mutans
Talas
Taro leaves extract

Abstract

Dental caries was the most common disease in both adults and children. *Streptococcus mutans* is the main bacteria causing plaque formation and was the initiator of dental caries. Antibacterials derived from plants can be used to prevent plaque formation. Taro (*Colosia esculenta*) has been used in traditional medicine. Antibacterial compounds have been discovered in *C. esculenta* leaves. This study aimed to determine the ability of *C. esculenta* leaf ethanol extract to inhibit the growth of *S. mutans* ATCC 31987. Simplicia preparation, extract preparation, and phytochemical screening was carried out. Then, the antibacterial activity test was performed using the disc diffusion method to determine the zone of inhibition at various concentrations of 10, 20, 30, 40, 50, 60, and 70%. *Colosia esculenta* leaf ethanol extract contains alkaloids, flavonoids, triterpenoids, saponins, and produces an inhibition zone at each concentration variation. Very strong antibacterial activity was produced at a concentration of 70% at 21.11±0.46 mm, which was higher than the positive control.

Received: January 19th, 2022
1st Revised: July 7th, 2022
Accepted: July 20th, 2022
Published: August 31th, 2022

INTRODUCTION

The Global Burden of Disease Study reported that oral diseases afflicted about 2.3 billion people worldwide. Dental caries was the most common cause, accounting for 65% of infected permanent teeth and 15% of infected primary teeth[1]. Caries are caused by bacterial metabolism as a biofilm or plaque that changes sugars to acid, demineralizing the hard tissues of the teeth (enamel and dentine)[2]. Bacterial acid decreased the pH of tooth surfaces, causing demineralization from calcium ions and phosphate dentine release, which resulted in cavities[3]. The early stages of dental caries are frequently asymptomatic, but advanced stages can cause discomfort, infections, abscesses, and even sepsis. A severe condition in adults causes pain and infection, which may necessitate tooth extraction[4]. This caries might make children prone to infections in other parts of their bodies. The pain can make chewing meals excruciating, and getting enough nutrition can be problematic[5].

The most prevalent bacteria that cause caries were *Streptococci* and *Lactobacilli*. *Streptococci* lead to caries, and *Lactobacilli* contribute to further infection[6]. *Streptococcus mutans* was not only the basic bacterium engaged in the development of plaque but also in the commencement of dental caries. *Streptococcus mutans* have been linked to other extraoral pathologies such as cerebral microbleeds, IgA nephropathy, and atherosclerosis as a human pathogens[7,8].

Plaque treatment can be done mechanically or chemically. Brushing and flossing are mechanical techniques of plaque control, whereas mouthwash is a chemical plaque control treatment[9,10]. Mouthwash contains chemical compounds with antiseptic or antibacterial characteristics that aid in preventing plaque formation. On the other hand, regular utilization may cause tooth discoloration and temporary gustatory problems[11]. Pizzo *et al.*[12] stated another negative impact was that it induces mouth dryness, burning, and is harmful if ingested. Hence, plant components can be used as antibacterial agents.

How to cite: Haerussana ANEM, Ayuhastuti A, Yuniar SF, Bustami HA, Widyastiwi. Taro (Colosia esculenta) Leaves Extract Inhibits Streptococcus mutans ATCC 31987. Borneo J Pharm. 2022;5(3):268-78. doi:10.33084/bjop.v5i3.3156

According to a World Health Organization (WHO) report, approximately 80% of the world's population was treated using plants or plant-derived products[13]. Plants have long been employed as the primary human therapy source and have helped address the world's healthcare demands[14]. Medicinal plants have proven to be a remarkable source of newer and potent therapeutic agents and have taken the central stage in most research centers worldwide[15].

Colocasia esculenta, also known as taro (*talas* = Indonesian), was a nutritious plant widely consumed by the locals[16]. *Colocasia esculenta* tuber is used as ethnomedicine to treat diabetes, ringworm, cough, sore throat, wounds, asthma, arthritis, diarrhea, internal bleeding, neurological disorders, skin disorders, and it has been reported to have antihelminthic and anticancer properties[17,18]. Agyare *et al*.[19] stated that a decoction of the leaves was drunk to promote menstruation and was used to treat stomach problems and cysts. In addition, *C. esculenta* leaf extract has anti-inflammatory and antioxidant properties.

Colocasia esculenta biological properties were derived from natural products such as alkaloids, flavonoids, tannins, phytates, and minerals[17]. Elmosallamy *et al*.[20] showed that the significant constituents of *C. esculenta* leaves are flavonoids β-sitosterol and steroids. Anthocyanins, isoschaftoside, vicenin, apigenin, catechin, anthraquinones, β-amyrin, α-amyrin, riboflavin, and niacin were also reported in leaves[21,22]. The flavonoids in *C. esculenta* include orientin, isoorientin, vitexin, isovitexin, and luteolin-7-O-sophoroside[23]. Orientin flavonoids are reported to have antibacterial activity[24].

Wang *et al*.[25], in previous research, reported the presence of antimicrobial activity in tests using *C. esculenta*. The research of Singh *et al*.[26] stated that the water extract of *C. esculenta* leaves showed the diameter of the inhibition zone of *Staphylococcus aureus* MTCC 96 and *S. mutans* MTCC 890 at a concentration of 400 mg/mL was 8.50±0.09 mm and 15.33±0.13 mm, respectively. A different solvent produced a more significant inhibition zone, ethanol extract of *C. esculenta* leaves against *S. aureus* of 14.3±1.45 mm at a similar concentration[27].

The selection of the solvent and methods used for extraction was an important factor, so it must be fully considered[28]. The maceration extraction method has the advantage of being easy to use and requires less equipment. Since the maceration method was used at room temperature, thermolabile flavonoid compounds are appropriate[29]. Antimicrobial compounds are most commonly found in the phenolic compounds terpenoids but also found in flavonoids, saponins, and alkaloids classes[30-32]. Orientin was a flavonoid compound with low polarity, making it easily soluble in semipolar solvents. Since 96% ethanol was a semipolar solvent, it was chosen as the extraction solvent[33]. Based on the prior studies of 96% ethanol extract against *S. mutans* ATCC 31987, researchers intend to investigate the antibacterial activity of *C. esculenta* leaf ethanol extract against the growth of the primary bacteria responsible for dental caries.

MATERIALS AND METHODS

Materials

The materials used in this study were old fresh *C. esculenta* leaves, collected from Balai Penelitian Tanaman Sayuran (Research Center of Agriculture Ministry) Lembang, West Java, Indonesia. *Streptococcus mutans* ATCC 31987 isolates from Biology Department Universitas Indonesia, 96% ethanol, dimethyl sulfoxide (DMSO), chlorhexidine, 0.9% sterile physiological NaCl, Mueller Hinton Agar (MHA), blood agar, H_2SO_4, $BaCl_2$, 70% ethanol, HCl, acetic acid, *n*-hexane, Mg metal, Mayer reagent, Wagner reagent, aquadest, chloroform, $FeCl_3$, and blank disc. The instruments used in this study were an autoclave (Hirayama®), incubator (Memmert®), blender (Philips®), vacuum rotary evaporator (Buchi®), biological safety cabinet (Thermo Scientific®), oven (Memmert®), microscope (Leica®), spectrophotometer (Shimadzu®), analytic scale (Mettler Toledo®), waterbath (Electro-mag®), micropipette (Effendorf®), refrigerator, caliper, inoculation loop, and laboratory glassware.

Methods

Preparation of ethanolic extraction

Preparations of simplicia adapted from Arnida *et al*.[34] including wet sorting, washing, chopping, drying, and dry sorting (**Figure 1**). The drying was done in an oven at 50±2°C. Dry leaves or simplicia could be crushed by hand into small pieces

with a water content of 10% or less. Gravimetric methods are used to determine the water content. **Equation 1** was used to calculate the yield extract:

$$\text{Water content (\%)} = \frac{(W1-W0)-(W2-W0)}{(W1-W0)} x100\% \quad \ldots [1]$$

W0 = weight of container (g)
W1 = weight of container + weight of moist simplicia (g)
W2 = weight of container + weight of dry simplicia (g)

Simplicia was ground to a powder with a blender and sieved at 60 mesh. *Colocasia esculenta* leaves simplicia were macerated in 96% ethanol. Simplicia powder was soaked in ethanol (1 : 10) for 24 hours at room temperature, stirring occasionally. This process was repeated until the pellucidity was achieved. Following filtration of the suspension through filter paper, *C. esculenta* leaf extract was evaporated at 50°C using a rotary vacuum evaporator and a waterbath (**Figure 1**). The crude extract was kept at 40°C until it was analyzed. **Equation 2** was used to calculate yield extract:

$$\text{Yield extract (\%)} = \frac{\text{crude extract weight (g)}}{\text{simplicia weight (g)}} x\ 100\% \quad \ldots [2]$$

a **b** **c**

Figure 1. *Colocasia esculenta* leaves before chopping (fresh) (**a**), drying process in the oven (**b**), and thickening extract process to evaporate the remaining solvent (**c**).

Phytochemical analysis[35,36]

Phytochemical compounds were identified by qualitative analysis. The extract's content of flavonoids, alkaloids, saponins, tannins, steroids, and terpenoids was determined qualitatively. The extraction solvent affects the secondary metabolite content of the extract.

Alkaloid (Wagner and Mayer test): About 5 mg extract was dissolved in chloroform, then 0.5 mL of 1 M sulfuric acid was added and slowly shaken. The mixture was allowed to stand for a few moments until two layers formed. The transparent top layer was divided into two parts, one receiving 2-3 drops of Wagner's and the other receiving 2-3 drops of Mayer's reagent. The brown precipitate indicated the alkaloid compounds by Wagner's and the white precipitate by Mayer's reagent.

Flavonoid (Wilstater test): About 5 mg of extract was dissolved in 5 mL of hot water, boiled for 5 minutes, and then filtered. The filtrate was mixed with Mg metal, 1 mL of concentrated sulfuric acid, and 2 mL of 70% ethanol. The mixture was shaken vigorously and set aside. The presence of flavonoid compounds was indicated by the formation of a red, yellow, or orange color on the ethanol layer.

Tannins and Polyphenol ($FeCl_3$ test): About 0.5 g of the extract was dissolved in 2 mL of 70% ethanol, boiled in 10 mL of distilled water, and filtered in a test tube. Three drops of 0.1 % $FeCl_3$ were added, and a brownish-green or blue-black color formed indicating the presence of tannins and phenols.

Saponin (Foaming test): *Colocasia esculenta* extract was dissolved in 10 mL of hot water and allowed to cool. Once cool, the mixture was vigorously shaken vertically for 10 seconds. The presence of saponin compounds was indicated by a stable foam as high as 1 cm if the foam remains stable after adding one drop of 1% HCl.

Triterpenoids and Steroids: In a test tube, 0.5 mg of extract was placed. The mixture was then treated with 2 mL of anhydrous acetic acid and 0.5 mL of concentrated sulfuric acid. A blue or green color indicated the presence of steroids, while a brownish or purple ring indicated the presence of triterpenoids.

Bacterial preparations

The bacteria were obtained, and then Gram stained to examine cell morphology. Gram staining was tested by dripping the reagents in a specific order. The first reagent was gentian violet as a primary stain, followed by iodine solution as a mordant, alcohol as a decolorizer, and safranin as a counterstain. Gram-positive bacteria retain the first color, making them appear violet under a 100x magnification microscope. The bacteria were recultured by streaking a loop of bacteria in blood agar media and incubating it at 37°C for 18-24 hours before using the bacteria to make suspensions.

Antibacterial activity

McFarland standard 0.5 was made by combining 0.05 mL of 1% $BaCl_2$ with 9.95 mL of 1% H_2SO_4. The turbidity produced in the test tube was equivalent to $1.5x10^8$ CFU/mL of bacteria. The mixture of the two solutions was attempted for no more than 15 minutes to serve as a standard[37,39].

Recultured *S. mutans* in a sterile inoculation loop were suspended in a tube containing 10 mL of 0.9% physiological NaCl. The turbidity was then compared to McFarland 0.5 standard. Turbidity was measured with a spectrophotometer equal to 0.1 at 625 nm. The too high turbidity could be reduced by adding 0.9% physiological NaCl or bacterial colonies[40].

The test bacteria were planted in the solidified MHA media using the spread plate method. The prepared bacterial suspension was taken 100 µL with a micropipette and dripped on the surface of the solidified MHA media. The suspension droplets were spread using a sterile L-rod, with repeated rotation of the petri dish, to ensure that the test bacteria spread evenly. *Colocasia esculenta* leaf ethanol extract was prepared in various concentrations of 10, 20, 30, 40, 50, 60, and 70% dissolved in DMSO. The positive control was chlorhexidine, the negative control was DMSO solution, and the growth control was suspension only. Blank discs with a diameter of 6 mm were immersed in that test solution for 10 minutes to make sample discs. Sample discs were given to solid media mixed with the test bacteria. The samples were incubated at 37°C for 24 hours in an inverted position to prevent condensation from dripping onto the media[41].

Furthermore, the presence or absence of the formed inhibition zone was observed. The apparent diameter of the clear zone around the disc was measured using a caliper diagonally, vertically, and horizontally and averaged. The diameter of the clear zone was reduced by the diameter of the disc[37].

Statistical analysis

The inhibition zone values were calculated using IBM SPSS Statistic 25 for Windows and expressed as mean (n=3) per plate of three repetitions±standard deviations (SD). Suppose the data had a normal distribution ($p < 0.05$), the normality test was performed using Analysis of Variance (ANOVA) and Kruskal-Wallis if not. Then a Post Hoc ($p < 0.05$) analysis was performed.

RESULTS AND DISCUSSION

Ethanol extract preparations

The water content of *C. esculenta* leaves was 7.07±0.05%, less than 10%. Microbes will easily overgrow in water with content greater than 10%. The simplicia was then mashed to facilitate the extraction process. A large surface area will increase the effectiveness of the solvent in breaking down plant cell walls, so it was expected that the extracted compounds would be maximal[42].

Antibacterial compounds such as orientin in *C. esculenta* leaves were extracted with 96% ethanol. Other compounds such as luteolin, apigenin, isoorientin, vitexin, and isovitexin have similar solubility. Previous studies show ethanol was the best choice because it contains the tremendous variety and extent of bioactive components in *C. esculenta* extract[20]. The resulting yield extract was 20.53%, which was calculated to determine the adsorption value of the solvent in extracting the extract.

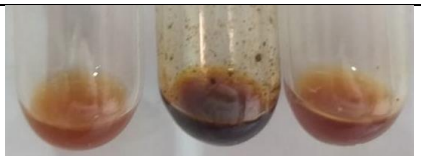
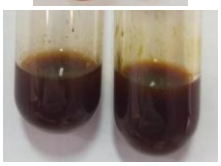
Phytochemical analysis

Qualitative identification of compound groups was performed to determine the content of compound groups in *C. esculenta* leaf extract that may have antibacterial properties. The presence of alkaloids, flavonoids, triterpenoids, and saponins was detected in the qualitative test on *C. esculenta* leaf extract shown in **Table I**. Eddy[43] stated that the water extract of *C. esculenta* leaves contains saponins and tannins. Meanwhile, the ethanol extract of *C. esculenta* leaves, apart from containing saponins and tannins, also contains terpenoids, anthraquinones, flavonoids, and alkaloids. Different solvents can lead to the extraction of various phytochemical compounds. The precipitate formed in the alkaloid test was potassium-alkaloid because the alkaloid compound contains a nitrogen atom with a lone pair of electrons that can be used to form coordinate covalent bonds with metal ions. The alkaloids' nitrogen will react with the metal ions K^+ from potassium tetraiodomercurate (II) to form a precipitated potassium-alkaloid complex[44].

Flavonoid testing was carried out by adding HCl and Mg metal, which reduced the benzopyrone core contained in flavonoid compounds, resulting in the formation of red color in these compounds[45]. Meanwhile, the saponin test was performed by adding hot water to the ethanol extract. Saponins are polar in that they can dissolve in solvents such as water, though they are also non-polar in that they contain a hydrophobic group, an aglycone (sapogenin). The presence of glycosides, which can form foam in water and hydrolyze into glucose and other compounds, causes the foam produced in the saponin test[46].

Triterpenoid testing was carried out by adding acetic acid and sulfuric acid, which can produce a color change reaction to a brownish or purple ring. This can occur due to condensation or release of water and incorporation with carbocations. This reaction begins with the acetylation of the hydroxyl group using anhydrous acetic acid. Furthermore, the release of the hydrogen group and its electrons causes the double bond to move and experience resonance which acts as an electrophile or carbocation. The presence of a carbocation causes electrophilic addition, followed by the release of hydrogen. Then the hydrogen group and its electrons are removed. As a result, the compound undergoes conjugation extension, which shows the appearance of a brownish ring[47].

Table I. Phytochemical analysis of *C. esculenta* leaves extract

Phytochemical analysis	Description	Result	Conclusion
Alkaloid	The color changed, but no white precipitate or white turbidity was formed in the Mayer's reagent, whereas a brick-red precipitate was formed in the Wagner's reagent.		+
Flavonoid	Formation of red color on the top layer when compared to the blank		+
Tannin	Same as blank, no brownish-green or blue-black color was formed.		-
Triterpenoid	A concentrated dark color resulted, and the color of the blank was lighter than the test results.		+
Saponin	The foam was formed and the foam that was formed was stableIya		+

Note: (+) presence; (-) absence

Antibacterial activity

The extract diffuses on the agar and inhibits the test microbes' growth, resulting in a clear zone formation. The zone of inhibition can be seen as the formation of a clear zone around the disc, with the visible diameter of the zone concluding as the extract's inhibition zone against the test microbes. Outside the zone, turbidity indicates the growth of microbes that are not inhibited by the extract. **Table II** shows the results of testing a 96 % ethanol extract of *C. esculenta* leaves against the caries-causing *S. mutans*. Each variation of the concentration of 96% ethanol extract of *C. esculenta* leaves has antibacterial activity, and a clear zone around the disc indicates an inhibition zone.

Tests revealed that a concentration of 70% can provide inhibition of 21.11±0.46 mm, indicating that it has a very strong antibacterial ability. Ponce *et al*.[48] classified the diameter of the inhibition zone into four categories: weak inhibition zone of 8 mm, moderate inhibition zone of 8-14 mm, strong inhibition zone of 15-19 mm, and very strong inhibition zone of >20 mm. The lowest concentration was in the weak category, and the highest was in the very strong category, according to the above categories. This could occur because the lower the concentration of an extract, the more dilute the extract produced and the smaller the inhibition zone formed to inhibit *S. mutans*.

The test revealed that chlorhexidine inhibited the microbes by 9.37±0.27 mm. Chlorhexidine was known to have bacteriostatic and bactericidal properties, and it can effectively inhibit and kill *S. mutans*. Chlorhexidine was an effective mouthwash in the treatment of dental caries[49]. The mechanism of chlorhexidine causes changes in the permeability of the bacterial cell membrane, resulting in the release of cytoplasm and cell components from within the cell through the cell membrane, resulting in bacterial death[50]. The 0.2% chlorhexidine concentration was chosen because it was the concentration commonly used as a mouthwash. Furthermore, increasing the concentration of chlorhexidine will make it toxic to dental cells.

The negative control showed no inhibition zones against the *S. mutans*. The negative control (10% DMSO), a solvent used in extract dilution, does not inhibit bacterial growth. DMSO was a neutral solvent capable of dissolving both polar and non-polar substances[51]. According to Trisia *et al*.[52], who examined the antibacterial activity test of the ethanolic extract using the disc diffusion method, DMSO had no inhibitory effect on the growth of *S. aureus* (Kirby-Bauer).

Previous research by Dutta and Aich[27] demonstrated that the diffusion of ethanol extract of *C. esculenta* leaves at a concentration of 400 mg/mL inhibited *S. aureus* by 14.3±1.45 mm. Our research yielded an inhibition of 7.33±0.64 mm at the same concentration (40%). Meanwhile, at a concentration of 20 mg/mL, the inhibition zones formed by *C. esculenta* leaves chloroform and methanol extract against *S. aureus* were 16.23±1.53 and 20±1.00 mm, respectively[53]. Singh *et al*.[26] tested the antibacterial activity of *C. esculenta* leaves aqueous extract against *S. mutans* and found an inhibition zone of 15.33±0.10 mm at 400 mg/mL.

The extract's phytochemical content influenced the antibacterial activity of *C. esculenta* leaf extract. Flavonoids, saponins, alkaloids, and terpenoids are found in *C. esculenta* leaves ethanol extract. Alkaloid group compounds can function as antibacterials by interfering with the peptidoglycan components in bacterial cells, causing the cell wall layer not to form entirely and causing the bacterial cell to die[54]. Furthermore, flavonoid compounds inhibit cell membrane function, DNA gyrase, and bacterial metabolism[55]. Other groups of compounds that are positive in the extract and have an antibacterial function are saponins and triterpenoids. Saponins can work as an antibacterial by disrupting the permeability of cell membranes so that the membrane becomes unstable and causes lysis[56]. Meanwhile, the mechanism of action of triterpenoids by damaging the bacterial cell membrane. Cell membrane damage can occur when active antibacterial compounds react with the active site of the membrane[54].

Table II showed that the test solution has a more significant zone of inhibition against *S. mutans* than the positive control (60 and 70%). The greatest concentration of 70% was twice as strong as the positive control, which had a concentration of 0.2%. According to the results of the tests, *C. esculenta* leaf extract can be utilized as an alternative to *S. mutans* inhibition.

Table II. Inhibition zone of *C. esculenta* leaves extract and control against *S. mutans*

Sample	Inhibition zone (mm)			Average±SD (mm)
	P1	P2	P3	
C. esculenta leaves extract (10%)	3.97	3.73	3.37	3.69±0.3
C. esculenta leaves extract (20%)	5.6	4.87	4.57	5.01±0.53
C. esculenta leaves extract (30%)	6.3	5.07	6.70	6.02±0.85
C. esculenta leaves extract (40%)	8.07	6.9	7.03	7.33±0.64
C. esculenta leaves extract (50%)	9.1	7.07	8.46	8.21±1.04
C. esculenta leaves extract (60%)	12.13	12.97	11.13	12.13±0.93
C. esculenta leaves extract (70%)	20.93	21.63	20.77	21.11±0.46
Chlorhexidine (0.2%)	9.67	9.13	9.33	9.37±0.27
DMSO (10%)	-	-	-	-

Note: The diameter of the inhibition zone was reduced with the disc diameter (6 mm)

Statistical analysisis

All data on the antibacterial activity of the ethanol extract of *C. esculenta* leaves against *S. mutans* were analyzed using statistical tests. This study's variables were categorical and unpaired numeric variables and had more than two groups. Therefore, the statistical test that can be used is the ANOVA test with the condition that the data obtained must be normally distributed and have homogeneous data variances. The data obtained were first analyzed using the normality test, which aims to see whether the resulting data were normally distributed or not. The normality test results have two results: the Kolmogorov-Smirnov and the Shapiro-Wilk test. The normality value was seen in the Shapiro-Wilk test because the data obtained was less than 50. The normality value of each treatment was more than 0.05, so it can be concluded that the data obtained are normal.

After the normality test, the data from the antibacterial activity test results should be tested for homogeneity, which aims to see the homogeneity of the data variants. The homogeneity of variances test shows a p-value of >0.05. Therefore, it can be concluded that there are at least two groups that have significantly different data variances. Based on the normality and homogeneity test results, we used the ANOVA test because the data obtained were normally distributed, and the data's variance was homogeneous. Analysis was conducted Post hoc to find out which groups had significantly different data. **Table III** showed that the negative control significantly differed from the positive control. The overall activity data was significantly different from the negative control. Furthermore, the test group was significantly different from the positive control, significantly smaller or larger, with a p-value of <0.05.

Table III. Statistical analysis of antibacterial activity of *C. esculenta* leaves extract

Samples	Negative control	Positive control	10%	20%	30%	40%	50%	60%	70%
Negative control	-	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
Positive control		-	0.000*	0.000*	0.000*	0.001*	0.040*	0.000*	0.000*
C. esculenta 10%			-	0.022*	0.000*	0.000*	0.000*	0.000*	0.000*
C. esculenta 20%				-	0.071	0.000*	0.000*	0.000*	0.000*
C. esculenta 30%					-	0.023*	0.000*	0.000*	0.000*
C. esculenta 40%						-	0.113	0.000*	0.000*
C. esculenta 50%							-	0.000*	0.000*
C. esculenta 60%								-	0.000*
C. esculenta 70%									-

Note: (*) a significant difference with a 95% confidence level

CONCLUSION

The results showed that the best inhibition zone was 21.11±0.46 mm at a 70% concentration of *C. esculenta*, which was more significant than the control. *Colosia esculenta* leaves had very strong antibacterial activity against *S. mutans*.

ACKNOWLEDGMENT

The author would like to express gratitude to the Poltekkes Kemenkes Bandung for financial funding through the PDP research grant scheme.

AUTHORS' CONTRIBUTION

Ayu Nala El Muna Haerussana: concept and design, research team leader and coordinator, validation, antibacterial assay, and article writing. **Angreni Ayuhastuti**: concept and design, validation, and article writing. **Siti Fira Yuniar**: sampling and simplicia preparation. **Hana Alifah Bustami**: extraction and phytochemical screening. **Widyastiwi**: data analysis.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest to report regarding the present study.

REFERENCES

1. Dye BA. The Global Burden of Oral Disease: Research and Public Health Significance. J Dent Res. 2017;96(4):361-3. doi:10.1177/0022034517693567

2. Chen X, Daliri EBM, Kim N, Kim JR, Yoo D, Oh DH. Microbial Etiology and Prevention of Dental Caries: Exploiting Natural Products to Inhibit Cariogenic Biofilms. Pathogens. 2020;9(7):569. doi:10.3390/pathogens9070569

3. Neel EAA, Aljabo A, Strange A, Ibrahim S, Coathup M, Young AM, et al. Demineralization-remineralization dynamics in teeth and bone. Int J Nanomedicine. 2016;11:4743-63. doi:10.2147/ijn.s107624

4. Siqueira Jr JF, Rôças IN. Microbiology and treatment of acute apical abscesses. Clin Microbiol Rev. 2013;26(2):255-73. doi:10.1128/cmr.00082-12

5. Colak H, Dülgergil CT, Dalli M, Hamidi MM. Early childhood caries update: A review of causes, diagnoses, and treatments. J Nat Sci Biol Med. 2013;4(1):29-38. doi:10.4103/0976-9668.107257

6. Aas JA, Griffen AL, Dardis SR, Lee AM, Olsen I, Dewhirst FE, et al. Bacteria of dental caries in primary and permanent teeth in children and young adults. J Clin Microbiol. 2008;46(4):1407-17. doi:10.1128/jcm.01410-07

7. Naka S, Wato K, Misaki T, Ito S, Matsuoka D, Nagasawa Y, et al. Streptococcus mutans induces IgA nephropathy-like glomerulonephritis in rats with severe dental caries. Sci Rep. 2021;11(1):5784. doi:10.1038/s41598-021-85196-4

8. Lemos JA, Palmer SR, Zeng L, Wen ZT, Kajfasz JK, Freires IA, et al. The Biology of Streptococcus mutans. Microbiol Spectr. 2019;7(1):10.1128/microbiolspec.GPP3-0051-2018. doi:10.1128/microbiolspec.gpp3-0051-2018

9. Vyas T, Bhatt G, Gaur A, Sharma C, Sharma A, Nagi R. Chemical plaque control - A brief review. J Family Med Prim Care. 2021;10(4):1562-8. doi:10.4103/jfmpc.jfmpc_2216_20

10. James P, Worthington HV, Parnell C, Harding M, Lamont T, Cheung A, et al. Chlorhexidine mouthrinse as an adjunctive treatment for gingival health. Cochrane Database Syst Rev. 2017;3(3):CD008676. doi:10.1002/14651858.cd008676.pub2

11. Brookes ZLS, Bescos R, Belfield LA, Ali K, Roberts A. Current uses of chlorhexidine for management of oral disease: a narrative review. J Dent. 2020;103:103497. doi:10.1016/j.jdent.2020.103497

12. Pizzo G, Guiglia R, Imburgia M, Pizzo I, D'Angelo M, Giuliana G. The Effects of Antimicrobial Sprays and Mouthrinses on Supragingival Plaque Regrowth: A Comparative Study. J Periodontol. 2006;77(2):248–56. doi:10.1902/jop.2006.050116

13. Ekor M. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. Front Pharmacol. 2014;4:177. doi:10.3389/fphar.2013.00177

14. Sofowora A, Ogunbodede E, Onayade A. The role and place of medicinal plants in the strategies for disease prevention. Afr J Tradit Complement Altern Med. 2013;10(5):210-29. doi:10.4314/ajtcam.v10i5.2

15. Anand U, Jacobo-Herrera N, Altemimi A, Lakhssassi N. A Comprehensive Review on Medicinal Plants as Antimicrobial Therapeutics: Potential Avenues of Biocompatible Drug Discovery. Metabolites. 2019;9(11):258. doi:10.3390/metabo9110258

16. Ladeska V, Am RA, Hanani E. Colocasia esculanta L. (Talas): Kajian Farmakognosi, Fitokimia dan Aktivitas Farmakologi. J Sains Kesehatan. 2021;3(2):351-8. doi:10.25026/jsk.v3i2.441

17. Eleazu CO. Characterization of The Natural Products in Cocoyam (Colocasia esculenta) using GC–MS. Pharm Biol. 2016;54(12):2880–5. doi:10.1080/13880209.2016.1190383

18. Al-Kaf AG, Al-Deen AMT, ALhaidari SAA, Al-Hadi FA. Phytochemical Analysis and Antimicrobial Activity of Colocasia esculenta (Taro) Medicinal Plant Leaves Used in Folk Medicine for Treatment of Wounds and Burns in Hufash District Al Mahweet Governorate–Yemen. Univers J Pharm Res. 2019;4(2):29-33. doi:10.22270/ujpr.v4i2.254

19. Agyare C, Boakye YD. Antimicrobial and Anti-Inflammatory Properties of Anchomanes difformis (Bl.) Engl. and Colocasia esculenta (L.) Schott. Biochem Pharmacol. 2015;5:1. doi:10.4172/2167-0501.1000201

20. Elmosallamy A, Eltawil N, Ibrahim S, Hussein SAA. Phenolic Profile: Antimicrobial Activity and Antioxidant Capacity of Colocasia esculenta (L.) Schott. Egypt J Chem. 2021;64(4):2165–72. doi:10.21608/ejchem.2021.56495.3213

21. Pawar HA, Choudhary PD, Kamat SR. An Overview of Traditionally Used Herb, Colocasia esculenta, as a Phytomedicine. Med Aromat Plants. 2018;7:4. doi:10.4172/2167-0412.1000317

22. Alam S, Rashid MA, Sarker MMR, Emon NU, Arman M, Mohamed IN, et al. Antidiarrheal, Antimicrobial and Antioxidant Potentials of Methanol Extract of Colocasia gigantea Hook. f. Leaves: Evidenced from In Vivo and In Vitro Studies Along with Computer-aided Approaches. BMC Complement Med Ther. 2021;21:119. doi:10.1186/s12906-021-03290-6

23. Li HM, Hwang SH, Kang BG, Hong JS, Lim SS. Inhibitory Effects of Colocasia esculenta (L.) Schott Constituents on Aldose Reductase. Molecules. 2014;19(9):13212–24. doi:10.3390/molecules190913212

24. Lam KY, Ling APK, Koh RY, Wong YP, Say YH. A Review on Medicinal Properties of Orientin. Adv Pharmacol Sci. 2016;2016:4104595. doi:10.1155/2016/4104595

25. Wang S, Yao J, Zhou B, Yang J, Chaudry MT, Wang M, et al. Bacteriostatic Effect of Quercetin as An Antibiotic Alternative In Vivo and Its Antibacterial Mechanism In Vitro. J Food Prot. 2018;81(1):67–78. doi:10.4315/0362-028x.jfp-17-214

26. Singh B, Namrat, Kumar L, Dwivedi SC. Antibacterial and Antifungal Activity of Colocasia esculenta Aqueous Extract: An Edible Plant. J Pharm Res. 2011;4(5):1459–60.

27. Dutta S, Aich B. A Study of Antibacterial and Antifungal Activity of The Leaves of Colocasia esculenta Linn. Int J Pharm Sci Res. 2017;8(3):1184–7.

28. Zhang QW, Lin LG, Ye WC. Techniques for Extraction and Isolation of Natural Products: A Comprehensive Review. Chinese Med. 2018;13:20. doi:10.1186/s13020-018-0177-x

29. Tiwari P, Kumar B, Kaur M, Gurpreet K, Kaur H. Phytochemical screening and Extraction: A Review. Int Pharm Sci. 2011;1:98-106.

30. Tungmunnithum D, Thongboonyou A, Pholboon A, Yangsabai A. Flavonoids and Other Phenolic Compounds from Medicinal Plants for Pharmaceutical and Medical Aspects: An Overview. Medicines. 2018;5(3):93. doi:10.3390/medicines5030093

31. Guimarães AC, Meireles LM, Lemos MF, Guimarães MCC, Endringer DC, Fronza M, et al. Antibacterial Activity of Terpenes and Terpenoids Present in Essential Oils. Molecules. 2019;24(13):2471. doi:10.3390/molecules24132471

32. Patra AK. An Overview of Antimicrobial Properties of Different Classes of Phytochemicals. Dietary Phytochem Microbes. 2012;1-32. doi:10.1007/978-94-007-3926-0_1

33. Othman L, Sleiman A, Abdel-Massih RM. Antimicrobial Activity of Polyphenols and Alkaloids in Middle Eastern Plants. Front Microbiol. 2019;10:911. doi:10.3389/fmicb.2019.00911

34. Arnida A, Maulidia M, Khairunnisa A, Sutomo S, Faisal F. Standardization of Simplicia and Ethanol Extract of Purun Danau (Lepironia articulata (Retz.) Domin) Rhizome. Borneo J Pharm. 2021;4(4):273–82. doi:10.33084/bjop.v4i4.2794

35. Praptiwi P, Wulansari D, Fahoni A, Harnoto N, Novita R, Alfridsyah, et al. Phytochemical Screening, Antibacterial and Antioxidant Assessment of Leuconotis eugenifolia Leaf Extract. Nusantara Biosci. 2020;12(1):79–85. doi:10.13057/nusbiosci/n120114

36. Ngibad K. Phytochemical Screening of Sunflower Leaf (Helianthus annuus) and Anting-Anting (Acalypha indica Linn) Plant Ethanol Extract. Borneo J Pharm. 2019;2(1):24–30. doi:10.33084/bjop.v2i1.689

37. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in Vitro Evaluating Antimicrobial Activity: A Review. J Pharm Anal. 2016;6(2):71–9. doi:10.1016/j.jpha.2015.11.005

38. Díez-Aguilar M, Martínez-García L, Cantón R, Morosini MI. Is a New Standard Needed for Diffusion Methods for In Vitro Susceptibility Testing of Fosfomycin against Pseudomonas aeruginosa? Antimicrob Agents Chemother. 2015;60(2):1158-61. doi:10.1128/aac.02237-15

39. Saifudin A, Raharjo S, Eso A, Uji Aktivitas Antibakteri Ekstrak Metanol Rumput Laut (Kappaphycus alvarezii) pada Berbagai Tingkat Konsentrasi terhadap Pertumbuhan Bakteri Streptococcus mutans. Medula. 2015;3(1):185–91. doi:10.46496/medula.v3i1.2541

40. MaiaMRG, Marques S, Cabrita ARJ, Wallace RJ, Thompson G, Fonseca AJM, et al. Simple and Versatile Turbidimetric Monitoring of Bacterial Growth in Liquid Cultures Using a Customized 3D Printed Culture Tube Holder and a Miniaturized Spectrophotometer: Application to Facultative and Strictly Anaerobic Bacteria. Front Microbiol. 2016;7:1381. doi:10.3389/fmicb.2016.01381

41. Haerussana ANEM, Dwiastuti WP, Sukowati CA. Antibacterial Activity of Salam (Syzygium polyanthum) Leaves 70% Ethanolic Extract on Staphylococcus aureus and Staphylococcus epidermidis. J Trop Pharm Chem. 2021;5(4):375-80. doi:10.25026/jtpc.v5i4.352

42. Dewijanti ID, Mangunwardoyo W, Dwianti A, Hanafi M, Artanti N, Mozef T, et al. Antimicrobial Activity of Bay Leaf (Syzygium polyanthum (Wight) Walp) extracted using various solvent. AIP Conf Proceed. 2019;2175(1):020021. doi:10.1063/1.5134585

43. Eddy NO. Inhibitive and Adsorption Properties of Ethanol Extract of Colocasia esculenta Leaves for The Corrosion of Mild Steel in H2SO4. Int J Phys Sci. 2009;4(4):165–71. doi:10.5897/IJPS.9000319

44. Parbuntari H, Prestica Y, Gunawan R, Nurman MN, Adella F. Preliminary Phytochemical Screening (Qualitative Analysis) of Cacao Leaves (Theobroma cacao L.). Eksakta. 2018;19(2):40–5. doi:10.24036/eksakta/vol19-iss2/142

45. Zarmouh NO, Eyunni SK, Soliman KFA. The Benzopyrone Biochanin-A as a reversible, competitive, and selective monoamine oxidase B inhibitor. BMC Complement Altern Med. 2017;17(1):34. doi:10.1186/s12906-016-1525-y

46. Cheok CY, Salma HAK, Sulaiman R. Extraction and quantification of saponins: A review. Food Res Int. 2014;59:16-40. doi:10.1016/j.foodres.2014.01.057

47. Kareru PG, Keriko JM, Gachanja AN, Kenji GM. Direct detection of triterpenoid saponins in medicinal plants. Afr J Tradit Complement Altern Med. 2007;5(1):56-60. doi:10.4314/ajtcam.v5i1.31257

48. Ponce AG, Fritz R, Del Valle C, Roura SI. Antimicrobial Activity of Essential Oils on The Native Microflora of Organic Swiss Chard. LWT Food Sci Technol. 2003;36(7):679–84. doi:10.1016/S0023-6438(03)00088-4

49. Rajendiran M, Trivedi HM, Chen D, Gajendrareddy P, Chen L. Recent Development of Active Ingredients in Mouthwashes and Toothpastes for Periodontal Diseases. Molecules. 2021;26(7):2001. doi:10.3390/molecules26072001

50. Cheung HY, Wong MMK, Cheung SH, Liang LY, Lam YW, Chiu SK. Differential actions of chlorhexidine on the cell wall of Bacillus subtilis and Escherichia coli. PLoS One. 2012;7(5):e36659. doi:10.1371/journal.pone.0036659

51. Mi H, Wang D, Xue Y, Zhang Z, Niu J, Hong Y, et al. Dimethyl Sulfoxide Protects Escherichia coli from Rapid Antimicrobial-Mediated Killing. Antimicrob Agents Chemother. 2016;60(8):5054-8. doi:10.1128/aac.03003-15

52. Trisia A, Philyria R, Toemon AN. Uji Aktivitas Antibakteri Ekstrak Etanol Daun Kalanduyung (Guazuma ulmifolia Lamk.) terhadap Pertumbuhan Staphylococcus aureus dengan Metode Difusi Cakram (Kirby-Bauer). Anterior J. 2018;17(2):136–43. doi:10.33084/anterior.v17i2.12

53. Kubde MS, Khadabadi SS, Saboo SS, Ghorpade DS, Modi AJ. In Vitro Antimicrobial Activity of The Crude Extracts of Colocasia esculenta Leaves (Araceae). Int J Pharm Sci Res. 2010;1(8):88-91.

54. Khameneh B, Iranshahy M, Soheili V, Bazzaz BSF. Review on plant antimicrobials: a mechanistic viewpoint. Antimicrob Resist Infect Control. 2019;8:118. doi:10.1186/s13756-019-0559-6

55. Cushnie TPT, Lamb AJ. Antimicrobial activity of flavonoids. Int J Antimicrob Agents. 2005;26(5):343-56. doi:10.1016/j.ijantimicag.2005.09.002

56. Sudji IR, Subburaj Y, Frenkel N, García-Sáez AJ, Wink M. Membrane Disintegration Caused by the Steroid Saponin Digitonin Is Related to the Presence of Cholesterol. Molecules. 2015;20(11):20146-60. doi:10.3390/molecules201119682

Borneo Journal of Pharmacy
Vol 5 Issue 3 August 2022
Page 279 – 287

http://journal.umpalangkaraya.ac.id/index.php/bjop/article/view/3692
DOI: https://doi.org/10.33084/bjop.v5i3.3692
e-ISSN: 2621-4814

Research Article

Isolation of Endophytic Fungus from Leaves of *Uncaria cordata* (Lour.) Merr and Antibacterial Activity Against *Propionibacterium acnes* and *Escherichia coli*

Melzi Octaviani*
Winda Yusma Ameliah
Neni Frimayanti
Meiriza Djohari
Haiyul Fadhli

Department of Pharmacy, Sekolah Tinggi Ilmu Farmasi Riau, Pekanbaru, Riau, Indonesia

*email: melzioctaviani@stifar-riau.ac.id

Keywords:
Antibacterial
Endophyte fungi
Isolation
Uncaria cordata

Abstract

Uncaria cordata (Lour). Merr (*akar kaik-kaik*) is one of the medicinal plants used as antibacterial because it contains bioactive compounds that can inhibit the growth of microorganisms. The plant is one of the sources of endophyte fungal isolates that can be developed as an alternative to producing antibacterial compounds. This research aimed to isolate the endophytic fungus from the leaves of *U. cordata* and know the antibacterial activity against *Propionibacterium acnes* and *Escherichia coli* by disc diffusion. The Fungi that were isolated from the leaves of *U. cordata* were 17 isolates. The isolates were continued for antibacterial activity testing: IFED 1 (*Nigrospora* sp.), IFED 2 (*Aspergillus* sp.), IFED 3 (*Fusarium* sp.), and IFED 4, whose genus was unknown. The results obtained were fungal isolates IFED 1 to IFED 4 had activity in inhibiting the growth of *P. acnes* with moderate category (18.16 mm) and weak categories (6.21, 6.16, and 6.68 mm) and in *E. coli* with moderate category (14.56 mm) and weak categories (6.53, 6.71, and 7.23 mm). The results of One-Way ANOVA and Tukey's test showed a significant difference ($p < 0.05$) between the diameter of the inhibition zone with the type of endophytic fungus supernatant isolated from the leaves of *U. cordata*. The best isolate of endophytic fungi inhibiting *P. acnes* and *E. coli* bacteria was IFED 1 (*Nigrospora* sp.).

Received: June 29th, 2022
1st Revised: August 4th, 2022
Accepted: August 14th, 2022
Published: August 31th, 2022

INTRODUCTION

Infectious diseases are disorders caused by organisms such as bacteria, viruses, fungi, or parasites. Many organisms live in and on our bodies[1]. They are generally harmless or even helpful. However, certain organisms that may cause disease are called pathogens under certain conditions[2]. Pathogenic bacteria consist of Gram-positive bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Propionibacterium acnes*, while Gram-negative bacteria, for example, *Escherichia coli*, *Salmonella typhi*, and *Pseudomonas aeruginosa*[3,4].

Bacterial infections can be treated with antibiotics[5]. However, excessive use of antibiotics can cause drug side effects and even antibiotic resistance[6]. Therefore, people return to using natural materials such as medicinal plants[7]. Medicinal plants have certain parts that can be used, including roots, rhizomes, stems, leaves, and fruit[8]. The lack of side effects from natural ingredients is an alternative for treatment for the community[9].

Bioactive compounds from medicinal plants can be used by extracting certain parts[10]. However, this method can make the existence of plants even more scarce if bioactive compounds are taken directly from rare medicinal plants[11]. One efficient way to overcome this is to use endophytic fungi[12].

How to cite: Octaviani M, Ameliah WY, Frimayanti N, Djohari M, Fadhli H. Isolation of Endophytic Fungus from Leaves of Uncaria cordata (Lour.) Merr and Antibacterial Activity Against Propionibacterium acnes and Escherichia coli. Borneo J Pharm. 2022;5(3):279-87. doi:10.33084/bjop.v5i3.3692

Endophytic fungi are microorganisms with a living habitat in organs such as seeds, roots, stems, and leaves for a certain period, colonizing plant tissues without harming the host plant[13]. Endophytic fungi can live in symbiotic mutualism with their host plants and produce secondary metabolites that have bioactivity, such as antimicrobial, antifungal, anticancer, antiviral, and antiparasitic[14]. Endophytic fungi produce secondary metabolites similar to those of the host plant. Therefore, efforts have been made to identify endophytic fungi isolated from medicinal plants so that they do not have to be extracted from their host plants[15].

Based on the description above, in plants of the *Uncaria* genus, endophytic fungi have antibacterial activity. Therefore, researchers are interested in researching the leaves of *Uncaria cordata* (Lour.) Merr or *akar kaik-kaik* (Indonesian). This study aimed to isolate the endophytic fungus from the leaves of the *U. cordata* and determine the antibacterial activity against *P. acnes* and *E. coli* using the disc diffusion method.

MATERIALS AND METHODS

Materials

The materials used include 70% ethanol, sterile distilled water, 5.3% sodium hypochlorite, chloramphenicol antibiotic disc, Nutrient agar (NA) (Merck), Potato Dextrose agar (PDA) (Merck), Potato Dextrose yeast (PDY) (Merck), Potato Dextrose broth (PDB) (Himedia), 0.9% NaCl solution, 2N sulfuric acid, concentrated hydrochloric acid, 1% iron (III) chloride, chloroform, 0.005N ammonia chloroform, magnesium metal, activated carbon, Liebermann-Burchard reagent, Dragendorff's reagent, and Mayer's reagent. The main instruments used in this study were an autoclave (GEA Model YX-280B), oven (Memmert), incubator (Memmert), incubator shaker (Selecta), analytical balance (Shimadzu), microscope (Shimadzu), UV-Vis spectrophotometer (Shimadzu). The sample used in this study was the leaves of *U. cordata* taken in Special Purpose Forest Area (*Kawasan Hutan dengan Tujuan Khusus*; KHDTK) Bukit Suligi, Rokan Hulu Regency, Riau, Indonesia. Endophytic fungi isolated from leaves of *U. cordata*.

Methods

Identification of the sample

The sample was identified at the Botanical Laboratory of the Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Riau, Pekanbaru, with the letter 88/UN19.5.1.1.3-4.1/EP/2021. The *U. cordata* plants used in this study are shown in **Figure 1**.

Figure 1. *Uncaria cordata* plants.

Phytochemical screening

A phytochemical screening test was carried out on fresh leaf samples and endophytic fungus supernatants. The screening test included alkaloids, flavonoids, saponins, tannins, steroids, and terpenoids[16].

Isolation and purification of endophytic fungi

Endophytic fungi were isolated from the leaves of the *U. cordata*. The leaves were washed with running water to remove dirt on the surface and then cut into 1-1.5 cm sizes. The clean leaves were surface sterilized by soaking successively in 70% ethanol for a minute, 5.3% sodium hypochlorite for five minutes, and 70% ethanol for 30 seconds, then rinsed with distilled water three times. The sterilized leaves were dried on filter paper, and then the leaves were split using a sterile scalpel on a sterile slide, after that the pieces of the leaf halves were placed on PDA media that had been given 25 mg of chloramphenicol and then incubated at 28°C for seven days[17].

Purification of endophytic fungi

Purification of endophytic fungi was carried out to obtain pure endophytic fungal cultures. The medium used to purify endophytic fungi was the new Potato Dextrose agar chloramphenicol (PDAC) media. Endophytic fungi growing on PDAC media were purified each on new PDAC media, then incubated at 28ºC for seven days. After incubation, observations were made on the shape and color of the colonies on PDAC media. Each colony with different shapes and colors was subcultured again on new PDAC media.

Identification of fungus isolates

Identification of fungi was carried out by observing the morphological characteristics and characteristics macroscopically and microscopically from fungal colonies grown on PDAC media at 28°C. Macroscopically the observed characters included the color of the colony surface, the color at the bottom of the Petri dish, the shape of the surface, concentric circles, and diameter. Microscopic observations include septal hyphae or not. Hyphae are hyaline (colorless) or dark pigmented. Hyphae are spiral-shaped, nodular, or rhizoid in shape[18].

Fermentation of endophytic fungi

Fermentation of endophytic fungi was carried out by liquid fermentation using PDY media. Seven days old on PDAC media in a Petri dish, endophytic fungi were taken using a sterile scalpel. A total of four pieces of mushrooms were put into 50 mL of PDY liquid fermentation medium. Furthermore, shaken fermentation was carried out using an incubator shaker at a temperature of 28ºC at a speed of 100 rpm for 14 days. Centrifugation was carried out for 20 minutes at a speed of 2000 rpm to obtain the fermentation results. The endophytic fungal supernatant was filtered using Whatman No.1 filter paper. The supernatant obtained was used to test the antibacterial activity[19].

Antibacterial activity test of endophytic fungus

The method of testing the antibacterial activity of agar diffusion method using NA medium. Antibacterial activity test of the endophytic fungus supernatant against *P. acnes* and *E. coli* bacteria was carried out using the Kirby-Bauer test method using disc paper. Disc paper was made from Whatman filter paper with a pore size of 0.22 m by cutting with a paper punch to obtain a paper disc with a diameter of 6 mm.

The suspension of the test bacteria aged 24 hours was transferred to a Petri dish containing the test medium at a temperature of 40-50°C, then homogenized and allowed to solidify. Sterile disc paper was dripped aseptically with 20 L of endophytic fungus supernatant and allowed to stand for 15 minutes, and then the disc paper was placed aseptically on the surface of the media that had been inoculated with the test bacteria. Petri dishes were then incubated at 37°C for 24 hours. The diameter of the clear zone formed was measured using a caliper. Paper disc chloramphenicol 30 g was used as a positive control, while 10 L sterile distilled water was used as a negative control. The test was carried out with three repetitions[20].

Statistical analysis

The data were analyzed statistically using the One-Way ANOVA method with the SPSS program if the data distribution was normal and homogeneous. If the result of One-Way ANOVA is $p < 0.05$, there is a significant difference between each

endophytic fungal supernatant and the inhibition diameter of the tested bacteria. Further testing can be carried out using Tukey for One-Way ANOVA to find out which treatments are significantly different in providing inhibition.

RESULTS AND DISCUSSION

Phytochemical screening of fresh samples aims to determine the content of secondary metabolites contained in fresh samples. The secondary metabolites contained in the fresh samples were alkaloids, flavonoids, terpenoids, saponins, and phenolics. The results of the observations can be seen in **Table I**.

Table I. Results of phytochemical screening of fresh samples of leaves of *U. cordata*

Secondary metabolites	Reagent	Description	Result
Alkaloid	Mayer	White precipitate	+
Flavonoid	Mg + HCl	Red color	+
Phenolic	$FeCl_3$	Green color	+
Saponin	Foam test	Produce foam	+
Steroid	Liebermann-Burchard	No green color	-
Terpenoid	Liebermann-Burchard	Red color	+

The leaves of the *U. cordata* plant that have been taken are washed using running water until they are clean of stains attached to the leaf surface. Then the leaves were cut into small fragments with an area of 1 cm^2 using a sterile scalpel. Then the leaf pieces were sterilized using 5.3% NaOCl solution and 70% alcohol to kill and inhibit epiphytic microbes during the incubation of endophytic fungi. The sterile leaf pieces were then cut in half using a sterile scalpel, placed into a Petri dish containing PDAC media, and then incubated at 28ºC for seven days. The isolates of endophytic fungi from the leaves of *U. cordata* were purified and incubated for seven days at 28ºC to obtain pure isolates based on different morphological characteristics. The purification results obtained as many as 17 isolates of endophytic fungi that showed different macroscopic morphological characteristics with isolate codes IFEDs 1 to 17.

Macroscopic observation IFED 1 had the characteristics of culture growth within 3-4 days, the colonies were white and gray, the color at the bottom of the Petri dish was brown to black, the colony size was medium, the texture was rough, had concentric circles, and the growth diameter was 5 cm. Microscopic observation of hyphae insulated, conidia black. Based on the macroscopic and microscopic characteristics described and compared with the literature, IFED 1 belongs to the genus *Nigrospora*[21-23]. *Nigrospora* has white and then gray colonies; the color on the bottom of the Petri dish is brown to black; the hyphae are hyaline and insulated, and the conidiophores are simple and round.

Macroscopic observations of IFED 2 had the characteristics of colony growth within 3-5 days, white colonies then rapidly growing black, the color at the bottom of the Petri dish was black, the surface was downy, had concentric circles, and the growth diameter was 9 cm. Microscopic observations of hyphae are insulated and branched; conidiophores are hyaline in color, and conidia are round and oval. Based on the macroscopic and microscopic characteristics described and compared with the literature indicating that IFED 2 belongs to the genus *Aspergillus*[21-23]. *Aspergillus* has a white colony color, then quickly grows black and has concentric circles. Insulated hyphae and conidiophores are hyaline in color and round in shape. The mycelium is initially white, and then the sporangium becomes yellowish brown, green, or blackish.

Macroscopic observations of IFED 3 had colony growth characteristics within four days, and colonies were white like cotton and round in shape; the color at the bottom of the Petri dish was cream and had concentric circles and a growth diameter of 3.6 cm. Microscopic observations of hyphae are not insulated and are hyaline in color, branched conidiophores. Based on the macroscopic and microscopic characteristics that have been described and compared with the literature, IFED 3 is included in the genus *Fusarium* and has the characteristics of white colony color, short, simple, or branched conidiophores[21-23].

The observation results of the morphological characteristics of IFED 4 had the characteristics of white-green colonies. The color at the bottom of the Petri dish was dark green yellow, had concentric circles, and a growth diameter of 7.7 cm. Microscopic observations of the hyphae are hairline and insulated. The macroscopic and microscopic characteristics

described and compared with the literature indicate that IFED 4 is not yet known[21-23]. Isolates of endophytic fungus leaves of *U. cordata* can be seen in **Table II** and **Figure 2**.

Table II. Identification results of isolates of endophytic fungus of leaves of *U. cordata*

Fungal isolates code	IFED 1	IFED 2	IFED 3	IFED 4
Colony color	Grayish white	white	white	white
The color of the bottom of the Petri dish	Yellow dark brown	blackish green	White cream	White yellow
Diameter	5 cm	9 cm	3.6 cm	7.7 cm
Concentric circle	Yes	Yes	No	Yes
Texture	Coarse	Smooth	Cotton	Smooth
Hyphae	Insulated hyphae	Insulated hyphae	Insulated hyphae	Insulated hyphae
Hyphae color	Hyaline	Hyaline	Hyaline	Hyaline
Genus	*Nigrospora* sp.	*Aspergillus* sp.	*Fusarium* sp.	Unknown

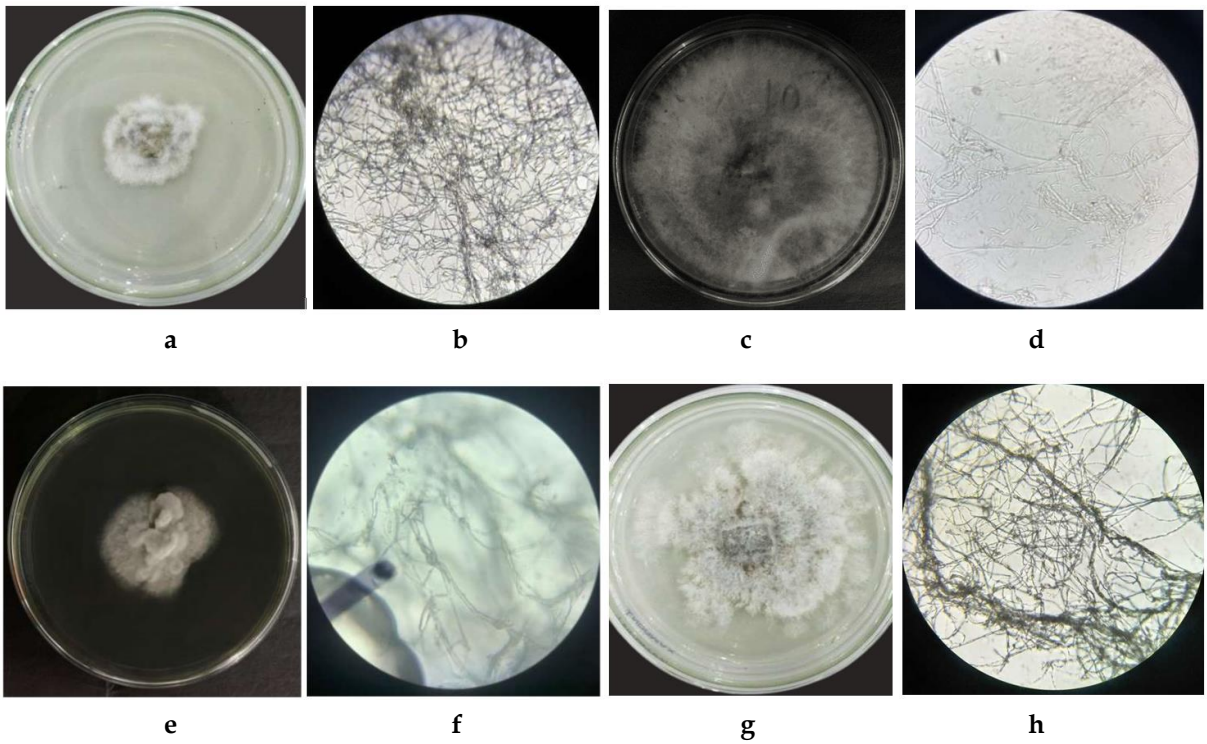


Figure 2. Isolates of endophytic fungus leaves of *U. cordata*. Respectively, the following are macroscopic and microscopic views of IFED 1 (**a** and **b**); IFED 2 (**c** and **d**); IFED 3 (**e** and **f**); and IFED 4 (**g** and **h**).

In this study, the IFEDs 1 to 4 has been identified by macroscopic and microscopic stages so that these isolates could be tested for antibacterial activity. As for the other isolates, the identification stages have not been carried out yet. The antibacterial activity test was used as supernatant of fermented endophytic fungi. Fermentation of endophytic fungi was carried out for 14 days by shaking using an incubator shaker. The fermentation process aims to remove secondary metabolites contained in endophytic fungal colonies. The formation of secondary metabolites occurs in the stationary phase, namely the phase when the rate of cell division and the rate of microbial death reach equilibrium, starting when the nutrients in the growth medium of microorganisms have been exhausted. The limitation of nutrients in the medium can cause the accumulation of secondary metabolite enzymes and secondary metabolite genes, which are thought to increase the production of secondary metabolites[24].

The fermentation process of endophytic fungi uses liquid media because fermentation with liquid media is more effective in producing biomass and bioactive compounds than fermentation in solid media. Fermentation of endophytic fungi using PDY media because this medium contains carbon from potatoes, dextrose, and yeast as nitrogen sources. Fermentation media must contain nutrients for growth, energy source, a constituent of cell substances, and biosynthesis of fermentation products. The most important media components are carbon and nitrogen sources because microbial cells and fermentation

products are mainly composed of carbon and nitrogen elements. In addition, it also contains organic salts as well as several vitamins and minerals[24].

The formation of microbial fermentation products can be influenced by several factors, such as substrates and nutrients. In fermentation, substrates are needed that are cheap, easy to obtain, and efficient in their use. Some substrates that can be used as carbon sources are molasses and starch. Meanwhile, ammonium salts, urea, nitrates, and soybean flour can be used as nitrogen sources. The second factor is pH; pH measurements are carried out to maintain the medium at the optimum pH during fermentation. Molds have an optimum pH between 5 and 7, and can grow in the pH range of 3-8.5[24,25].

The third factor is temperature; the fermentation temperature is carried out at a temperature where cell growth or metabolite production is highest. Most microorganisms can only grow in a temperature range of 20-30ºC. Based on the optimum growth temperature, the microorganisms used in the fermentation were classified as mesophiles with an optimum temperature of 20-45ºC and thermophiles with an optimum temperature of 45ºC. Microorganisms with a reasonable growth rate below 20ºC are classified as psychrophiles. The fourth factor is aeration and agitation; aeration aims to provide adequate oxygen supply, maintain aerobic conditions and remove carbon dioxide gas produced during fermentation. Agitation also aims to even out the spread of microorganisms, nutrients, and oxygen in the medium[24,26].

Phytochemical screening of the leaf endophytic fungus supernatant *U. cordata* using the TLC method. Phytochemical screening is a preliminary stage to provide an overview of the class of compounds contained in the endophytic fungal supernatant. Phytochemical screening of endophytic fungal supernatants was carried out using TLC, sprayed with staining reagent on the TLC plate, and observed the color changes that occurred on the TLC plate. The phytochemical screening found that secondary metabolites were contained in the endophytic fungal supernatant of the leaves of the *U. cordata*; IFED 1 contained alkaloids, flavonoids, phenolics, and terpenoids. The results of phytochemical screening of endophytic mushroom supernatants at IFED 2 were positive for phenolic compounds and flavonoids. In IFED 3, there are alkaloids, phenolic, and flavonoid compounds. Meanwhile, IFED 4 contains alkaloids and terpenoids. The results of the antibacterial activity of endophytic fungi against *P. acnes* and *E. coli*, endophytic fungi isolated from the leaves of the *U. cordata* showed different activities of each isolate in producing its antibacterial metabolites. Antibacterial activity of endophytic fungus leaves of *U. cordata* can be seen in **Table III** dan **Figure 3**.

Table III. Antibacterial activity of endophytic fungus of leaves of *U. cordata*

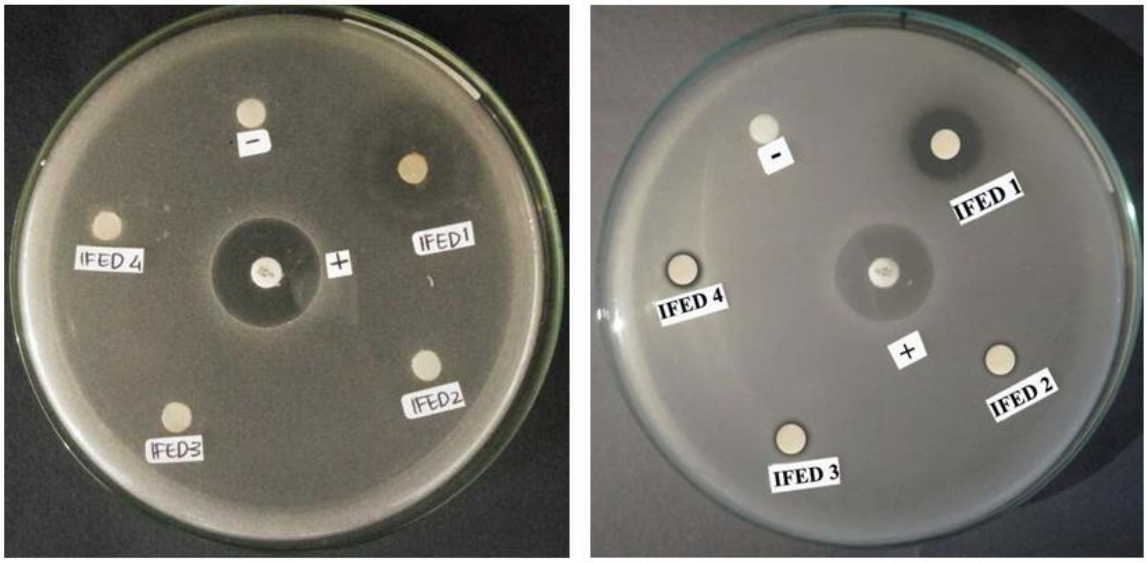
Bacteria	Treatment	Diameter of inhibition zone (mm)			Mean diameter of inhibition zone±SD (mm)
		I	II	III	
Propionibacterium acnes	K(-)	-	-	-	-
	K(+)	23.6	23.2	22.1	22.96±0.77
	IFED 1	17.6	18.7	18.2	18.16±0.55
	IFED 2	6.1	6.4	6.15	6.21±0.16
	IFED 3	6.1	6.25	6.15	6.16±0,07
	IFED 4	6.6	6.7	6.75	6.68±0.07
Escherichia coli	K(-)	-	-	-	-
	K(+)	24.5	25.1	23.3	24.3±0.91
	IFED 1	14.9	14.5	14.3	14.56±0.3
	IFED 2	7.1	6.4	6.1	6.53±0.51
	IFED 3	7.4	6.6	6.15	6.71±0.63
	IFED 4	6.3	7.5	7.9	7.23±0.83

Note: K(-): negative control; K(+): positive control

One-Way ANOVA analysis showed a significant difference ($p < 0.05$) between each endophytic fungal isolate with positive control and negative control on the diameter of the inhibition formed. Tukey's analysis showed that each endophytic fungal isolate differed significantly from *P. acnes* and *E. coli*. The best isolate of endophytic fungi inhibiting *P. acnes* and *E. coli* was IFED 1 (*Nigrospora* sp.).

This study showed that the inhibition zone formed in Gram-positive bacteria was more significant than that of Gram-negative bacteria. This is due to differences in the sensitivity of the bacteria. Gram-positive bacteria have a simpler cell wall than Gram-negative bacteria, which have a more complex cell wall. According to Harti[27], the cell wall structure of Gram-

negative bacteria is relatively more complex; the cell wall structure has two layers, namely the outer layer in the form of lipopolysaccharide and protein and the inner layer in the form of peptidoglycan. While the cell wall structure of Gram-positive bacteria is simple, it has one layer of peptidoglycan[28].



a **b**

Figure 3. Antibacterial activity of endophytic fungus leaves of *U. cordata* against *P. acnes* (**a**) and *E. coli* (**b**).

CONCLUSION

The fungi that were isolated from the leaves of *U. cordata* were 17 isolates. The isolates were continued for antibacterial activity test: IFED 1 (*Nigrospora* sp.); IFED 2 (*Aspergillus* sp.); IFED 3 (*Fusarium* sp.); and IFED 4, whose genus was unknown. The results were that fungal isolates IFED 1 to 4 had activity inhibiting the growth of *P. acnes* and *E. coli*. The results of One-Way ANOVA and Tukey's test showed a significant difference ($p < 0.05$) between the diameter of the inhibition zone with the type of endophytic fungus supernatant isolated from *U. cordata* leaves. The best isolate of endophytic fungi in inhibiting *P. acnes* and *E. coli* was IFED 1 (*Nigrospora* sp.).

ACKNOWLEDGMENT

We thank Sekolah Tinggi Ilmu Farmasi Riau for financing research funds through the Applied Research Grant year 2021 with grant number 12j.15.P3M.STIFAR.VIII.2021. This research was presented at the 2nd International Conference on Pharmacy Science and Practice (ICPSP) 2022 in Pekanbaru, Riau, Indonesia, on January 27th, 2022.

AUTHORS' CONTRIBUTION

Melzi Octaviani: conceptualization, funding acquisition, methodology, visualization, writing-original draft, writing-review & editing. **Winda Yusma Ameliah**: formal analysis, investigation, project administration, resources, writing-original draft. **Neni Frimayanti**: funding acquisition, methodology, supervision, validation. **Meiriza Djohari**: supervision, validation. **Haiyul Fadhli**: supervision, validation, writing-review & editing.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Aremu TO, Oluwole OE, Adeyinka KO. An Understanding of the Drivers of Infectious Diseases in the Modern World Can Aid Early Control of Future Pandemics. Pharmacy. 2021;9(4):181. doi:10.3390/pharmacy9040181

2. Balloux F, van Dorp L. Q&A: What are pathogens, and what have they done to and for us?BMC Biol.2017;15(1):91. doi:10.1186/s12915-017-0433-z

3. McLaughin J, Watterson S, Layton AM, Bjourson AJ, Barnard E, McDowell A. Propionibacterium acnes and Acne Vulgaris: New Insights from the Integration of Population Genetic, Multi-Omic, Biochemical and Host-Microbe Studies. Microorganisms. 2019;7(5):128. doi:10.3390/microorganisms7050128

4. Breijyeh Z, Jubeh B, Karaman R. Resistance of Gram-Negative Bacteria to Current Antibacterial Agents and Approaches to Resolve It. Molecules. 2020;25(6):1340. doi:10.3390/molecules25061340

5. Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. P T. 2015;40(4):277-83.

6. Llor C, Bjerrum L. Antimicrobial resistance: risk associated with antibiotic overuse and initiatives to reduce the problem. Ther Adv Drug Saf. 2014;5(6):229-41. doi:10.1177/2042098614554919

7. Miozi SH. The role of natural products from medicinal plants against COVID-19: traditional medicine practice in Tanzania. Heliyon. 2022;8(6):e09739. doi:10.1016/j.heliyon.2022.e09739

8. Sewani-Rusike CR, Mammen M. Medicinal plants used as home remedies: a family survey by first year medical students. Afr J Tradit Complement Altern Med. 2014;11(5):67-72. doi:10.4314/ajtcam.v11i5.11

9. Ekor M. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. Front Pharmacol. 2014;4:177. doi:10.3389/fphar.2013.00177

10. Sasidharan S, Chen Y, Saravanan D, Sundram KM, Latha LY. Extraction, isolation and characterization of bioactive compounds from plants' extracts. Afr J Tradit Complement Altern Med. 2011;8(1):1-10.

11. Abubakar AR, Haque M. Preparation of Medicinal Plants: Basic Extraction and Fractionation Procedures for Experimental Purposes. J Pharm Bioallied Sci. 2020;12(1):1-10. doi:10.4103/jpbs.jpbs_175_19

12. Gouda S, Das G, Sen SK, Shin HS, Patra JK. Endophytes: A Treasure House of Bioactive Compounds of Medicinal Importance. Front Microbiol. 2016;7:1538. doi:10.3389/fmicb.2016.01538

13. Mengistu AA. Endophytes: Colonization, Behaviour, and Their Role in Defense Mechanism. Int J Microbiol. 2020;2020:6927219. doi:10.1155/2020/6927219

14. Tiwari P, Bae H. Endophytic Fungi: Key Insights, Emerging Prospects, and Challenges in Natural Product Drug Discovery. Microorganisms. 2022;10(2):360. doi:10.3390/microorganisms10020360

15. Wen J, Okyere SK, Wang S, Wang J, Xie L, Ran Y, et al. Endophytic Fungi: An Effective Alternative Source of Plant-Derived Bioactive Compounds for Pharmacological Studies. J Fungi. 2022;8(2):205. doi:10.3390/jof8020205

16. Ingle KP, Deshmukh AG, Padole DA, Dudhare MS. Phytochemicals: Extraction Methods, Identification and Detection of Bioactive Compounds from Plant Extracts. J Pharmacogn Phytochem. 2017;6(1):32–6.

17. Ramachandran G, Rajivgandhi G, Maruthupandy M, Manoharan N. Extraction and Partial Purification of Secondary Metabolites from Endophytic Actinomycetes of Marine Green Algae Caulerpa racemosa Against Multi Drug Resistant Uropathogens. Biocatal Agric Biotechnol. 2019;17:750–7. doi:10.1016/j.bcab.2019.01.016

18. Saxena J, Pant V, Sharma MM, Gupta S, Singh A. Hunt for Cellulase Producing Fungi from Soil Samples. J Pure Appl Microbiol. 2015;9(4):2895-902.

19. Gautam CK, Madhav M, Sinha A, Osborne WJ. VIT-CMJ2: Endophyte of Agaricus bisporus in Production of Bioactive Compounds. Iran J Biotechnol. 2016;14(2):19-24. doi:10.15171/ijb.1287

20. Amelia P, Ayunda R, Bahri S. Screening of Antibacterial Activities of the Endophytic Fungi Isolated from the Leaves of Medinilla speciosa Blume. J Fitofarmaka Indones. 2021;8(3):24-8. doi:10.33096/jffi.v8i3.729

21. Barnett HL, Hunter BB. Illustrated Genera of Imperfect Fungi. 4th ed. St. Paul (US): APS Press; 1998.

22. Watanabe, T. Pictorial Atlas of Soil and Seed Fungi: Morphologies of Cultured Fungi and Key to Species. 3rd ed. Boca Raton (US): CRC Press; 2010.

23. Sciortino Jr CV. Atlas of Clinically Important Fungi. New Jersey (US): John Wiley & Sons; 2017. doi:10.1002/9781119069720

24. Kumala, S. Mikroba Endofit: Pemanfaatan Mikroba Endofit dalam Bidang Farmasi. Jakarta: ISFI Penerbitan; 2014.

25. Diether NE, Willing BP. Microbial Fermentation of Dietary Protein: An Important Factor in Diet–Microbe–Host Interaction. Microorganisms. 2019;7(1):19. doi:10.3390/microorganisms7010019

26. Rosyida VT, Indrianingsih AW, Maryana R, Wahono SK. Effect of Temperature and Fermentation Time of Crude Cellulase Production by Trichoderma reesei on Straw Substrate. Energy Procedia. 2015;65:368–71. doi:10.1016/j.egypro.2015.01.065

27. Harti AS. Mikrobiologi Kesehatan: Peran Mikrobiologi dalam Kesehatan. Yogyakarta: Andi; 2015.

28. Silhavy TJ, Kahne D, Walker S. The bacterial cell envelope. Cold Spring Harb Perspect Biol. 2010;2(5):a000414. doi:10.1101/cshperspect.a000414

Borneo Journal of Pharmacy
Vol 5 Issue 3 August 2022
Page 288 – 298

http://journal.umpalangkaraya.ac.id/index.php/bjop/article/view/3267
DOI: https://doi.org/10.33084/bjop.v5i3.3267
e-ISSN: 2621-4814

Research Article

Bibliometric Analysis of the Utilisation of FINDRISC in Patients with Diabetes: 2005-2021

M. Rifqi Rokhman [1,2]

Bustanul Arifin [3,4*]

Zulkarnain Zulkarnain [5]

Saidah Rauf [6]

Dyah Aryani Perwitasari [7]

[1] Unit of Global Health, Department of Health Sciences, University of Groningen, University Medical Centre Groningen (UMCG), Groningen, The Netherlands

[2] Faculty of Pharmacy, Universitas Gadjah Mada, Sleman, Special Region of Yogyakarta, Indonesia

[3] Faculty of Pharmacy, Universitas Hasanuddin, Makassar, South Sulawesi, Indonesia

[4] Department of Health Behaviour, Environment, and Social Medicine, and Centre of Health Behaviour and Promotion, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Sleman, Special Region of Yogyakarta, Indonesia

[5] Faculty of Medicine, Universitas Syiah Kuala, Banda Aceh, Aceh, Indonesia

[6] Masohi Nursing Study Program, Poltekkes Kemenkes Maluku, Ambon, Maluku, Indonesia

[7] Department of Pharmacology and Clinical Pharmacy, Universitas Ahmad Dahlan, Yogyakarta, Special Region of Yogyakarta, Indonesia

*email: bustanul.arifin.ury@unhas.ac.id

Keywords:
Bibliometric
Diabetes
FINDRISC
Review
Risk factors
Screening

Abstract

Research on risk factors for diabetes (DM) is growing. Identification of these risk factors aims to prevent DM as early as possible. This study intends to identify the utilization of the Finnish diabetes risk score (FINDRISC) and its development using bibliometric analysis. The keywords "FINDRISC AND Diabetes" were used to search for articles published in 2005-2021 in PubMed. A total of 249 articles were analyzed based on the number of publications per year, journals that publish the papers, number of publications by author and year of publication, number of publications by affiliation and year of publication, number of publications by country of origin of authors and year of publication, number of keywords, number of citations, types of articles, specific topics, and theme mapping. The data visualization was obtained from the Scopus database and the VOSviewer and Biblioshiny applications. Despite the increase in publications, the number of publications on FINDRISC in DM patients is still very few per year, with 92.8% being the primary study. Based on clusters of the country of origin, publications are still dominated by researchers from countries in the European region, and the researchers intensely relate to each other through citations. Research themes related to FINDRISC are not limited to DM risk factors. This study is the first study of a bibliometric analysis of the utilization of FINRISC in DM patients. The analysis results can be used to evaluate existing research gaps and identify future research opportunities.

Received: February 12th, 2022
1st Revised: April 17th, 2022
Accepted: June 19th, 2022
Published: August 31th, 2022

How to cite: Rokhman MR, Arifin B, Zulkarnain Z, Rauf S, Perwitasari DA. Bibliometric Analysis of the Utilisation of FINDRISC in Patients with Diabetes: 2005-2021. Borneo J Pharm. 2022;5(3):288-98. doi:10.33084/bjop.v5i3.3267

INTRODUCTION

The International Diabetes Federation estimates that there has been an increase in the number of people with diabetes by 21% between 2013 and 2019[1,2]. The number of people with diabetes is estimated to be 463 million in 2019 and will increase to 700 million by 2045. It is estimated that 50.1% of people with diabetes are still undetected[3]. In addition, people with prediabetes as a high-risk group for developing diabetes usually go undetected clinically[4]. Improper and late intervention in this group will cause prediabetes to develop progressively into type 2 diabetes mellitus within 2-3 years, which in 70% of patients will remain throughout their lives[5]. Diabetes mellitus will burden patients, families, and the health care system[6].

A systematic review and meta-analysis have discovered that lifestyle change interventions such as exercise, weight loss, and dietary management can prevent prediabetes and even slow the progression of type 2 diabetes[7]. Identification of individuals with prediabetes in the population is essential. However, prediabetes is difficult to trace because it is asymptomatic. Therefore, an instrument is needed to identify individuals with a high risk of developing type 2 diabetes so that lifestyle change interventions can be more targeted[8].

The Finnish diabetes risk score (FINDRISC) is an instrument in the form of a questionnaire aimed at identifying individuals with a high risk of developing diabetes[9]. The question items in this instrument are a set of the main risk factors for type 2 diabetes. Initially, this instrument was developed to detect the risk of diabetes in the next ten years in the Caucasian population. However, this instrument has been translated, adapted, and validated over time so it can be applied to other populations, such as Asian populations[10-12]. Several other studies have also compared the performance of the original version of FINDRISC with the simplified version of FINDRISC[13,14].

A study showed that FINDRISC could screen individuals at high risk of developing type 2 diabetes and that lifestyle intervention for these individuals effectively treats their obesity, one of the risk factors for type 2 diabetes[15]. In addition to its ability to identify individuals at high risk of developing diabetes, several studies have also evaluated the ability of FINDRISC to detect individuals with undiagnosed type 2 diabetes, the presence of impaired glucose tolerance, and the risk of developing metabolic syndrome[16-18]. Individuals with higher FINDRISC are also associated with a decreased quality of life[19].

No studies precisely quantify the development of research trends toward the FINDRISC instrument. Bibliometric analysis can be used for this purpose and has been widely used in many disciplines to observe research trends on a particular topic, population, or region. It can also be used to identify the potential for future research development. Therefore, this study aimed to identify the utilization of the FINDRISC using bibliometric analysis.

MATERIALS AND METHODS

Bibliometric analysis was performed by conducting a literature search on PubMed and Scopus with the keywords: "FINDRISC" AND "Diabetes". A total of 249 articles were obtained and analyzed based on the number of publications per year, journals that publish the articles, number of publications by author and year of publication, number of publications based on affiliation and year of publication, number of publications by country of origin and year of publication, number of keywords per year, number of citations per year, types of articles, specific topics, and theme mapping. The data visualization was obtained from the Scopus database with the VOSviewer and Biblioshiny applications.

Data analysis and visualization

The data in the Scopus database are visualized automatically. In contrast, we used VOSviewer and Biblioshiny to show data from the PubMed database. Only four of the 11 figures in this article are obtained from Scopus, notably **Figures 1**, **2**, **8**, and **9**. We used VOSviewer/Biblioshiny to visualize the rest of the data from PubMed.

RESULTS AND DISCUSSION

From the literature search on PubMed and Scopus, the same 249 articles were obtained, with the annual distribution shown in **Figure 1**. Despite the increase in the number of publications, the number of publications on the topic of FINDRISC in patients with diabetes mellitus is still very few per year, where every year, only less than 35 articles are published. The highest number of published articles, 34 articles, was in 2020.

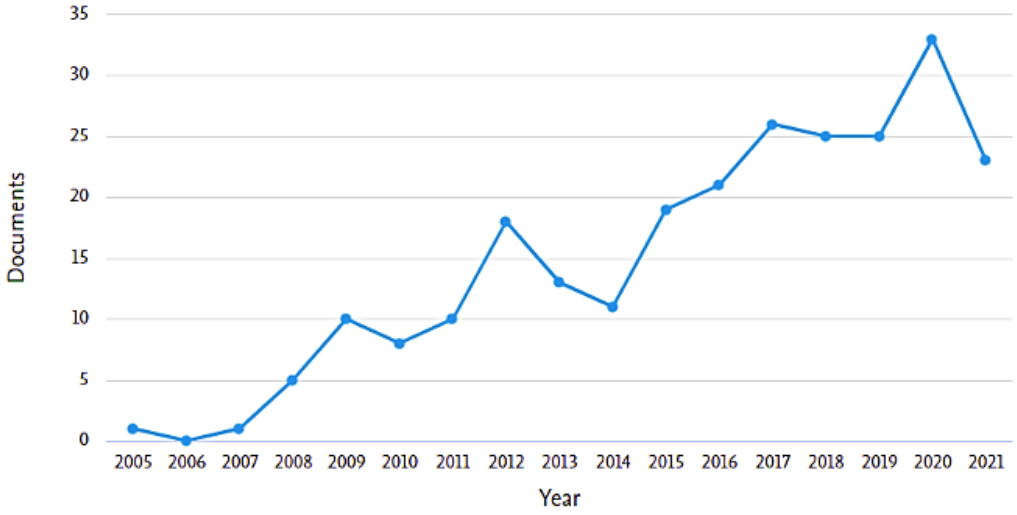


Figure 1. Number of articles on the topics of FINDRISC and diabetes for the period 2005-2021.

A bibliometric analysis of the number of publications related to the utilization of FINDRISC in patients with diabetes revealed an increasing trend since 2005. The overview of publication productivity based on country clusters showed that those researchers from European countries still dominate publications. This situation is likely because the FINDRISC instrument was first developed in a population at high risk of developing diabetes in Finland[20,21]. In 2003, a new Diabetes Risk Screening program was started in Finland, with the development of an instrument called the Diabetes Risk Score instrument. Furthermore, the first publication discussing this topic was in 2005[22]. This finding is in line with the Word Cloud analysis (**Figure 10**), which shows that the word "Finland" is the word with the highest frequency of occurrence.

Figure 2 shows the journals that published articles related to FINDRISC and diabetes from 2005-2021. PLoS One was the journal that published the most articles on this topic in 2016. In 2021, Diabetes Research and Clinical Practice and Primary Care Diabetes published three articles on FINDRISC and diabetes. Research topics regarding FINDRISC and diabetes still have the potential to be explored, as evidenced by PLoS One publishing many articles related to FINDRISC and diabetes.

Figure 3 displays the authors of articles related to FINDRISC and diabetes based on the year of publication and the number of publications. There are eight clusters of authors of articles that are also related to each other. The number of relationships between authors is 344. The authors with yellow circles are included in the latest publication cluster, although the number of published articles is still limited. Tuomilehto was the author with the most publications around 2012-2016, followed by Lindstrom at the same time range.

Figure 4 displays the number of articles by institutional affiliation of the author and year of publication. There are 10 relations between institutions to which the authors are affiliated, and these institutions are divided into three clusters based on the year of publication. Research in 2018 was conducted mainly by the Diabetes Research Group, Dasman Diabetes Institute, and the Department of Chronic Disease. The institution that conducted the most extended years of research was the National Institute for Health.

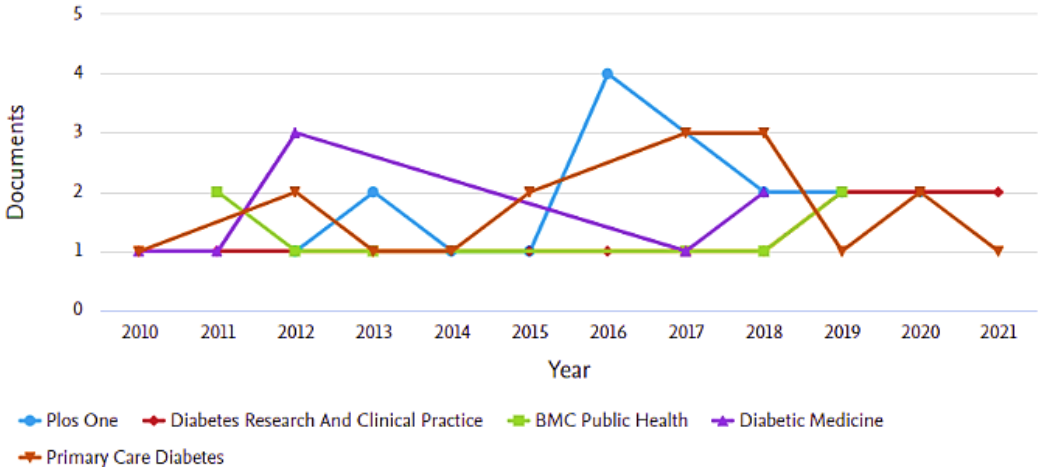


Figure 2. Number of published articles related to FINDRISC and diabetes by journal.

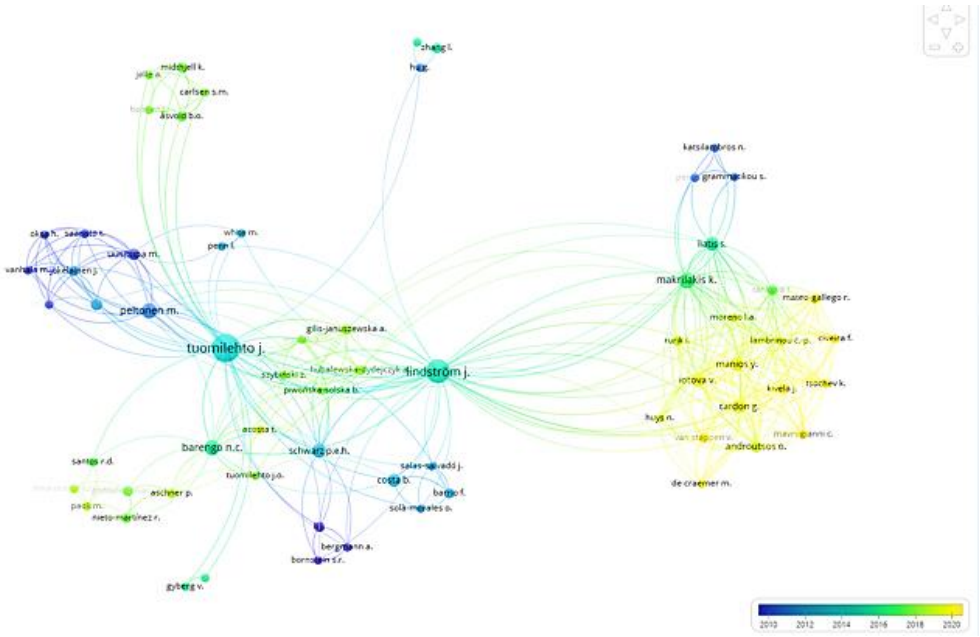


Figure 3. Number of publications on the topics of FINDRISC and diabetes for the period 2005-2021 by author and year of publication.

Figure 4. Number of articles with FINDRISC and diabetes topics in publications for the period 2005-2021 by author affiliation and year of publication.

Figure 5 shows the number of articles by country of origin of the author and year of publication. There are five clusters of countries involved based on the year of publication, with 111 relationships with each other. Recent publications have been by authors from Saudi Arabia, Brazil, Venezuela, Greece, Belgium, and Colombia, although the number of publications is still relatively small. Most publications were by authors from Finland in 2015. This is understandable because FINDRISC originates from Finland.

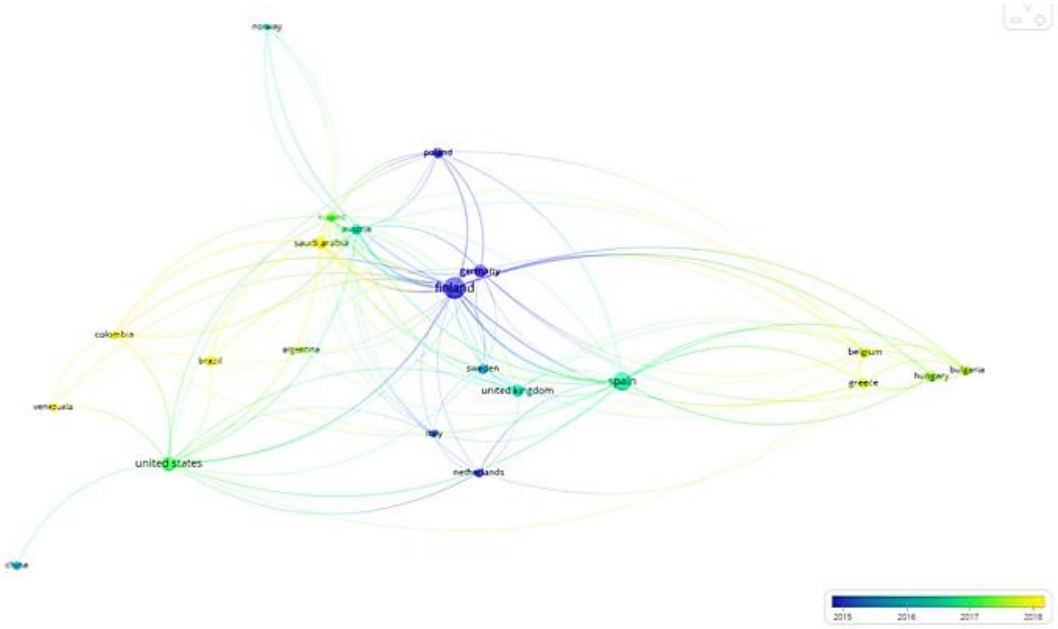


Figure 5. Mapping of publications with FINDRISC and Diabetes topics for the period 2005-2021 by country of origin of author and year of publication.

Figure 6 shows the keywords used by the authors. There are five clusters of these keywords based on the year of publication with 199 relationships with each other. The keyword "diabetes" appeared in 2017 and is almost the same as "type 2 diabetes," which appeared in 2015.

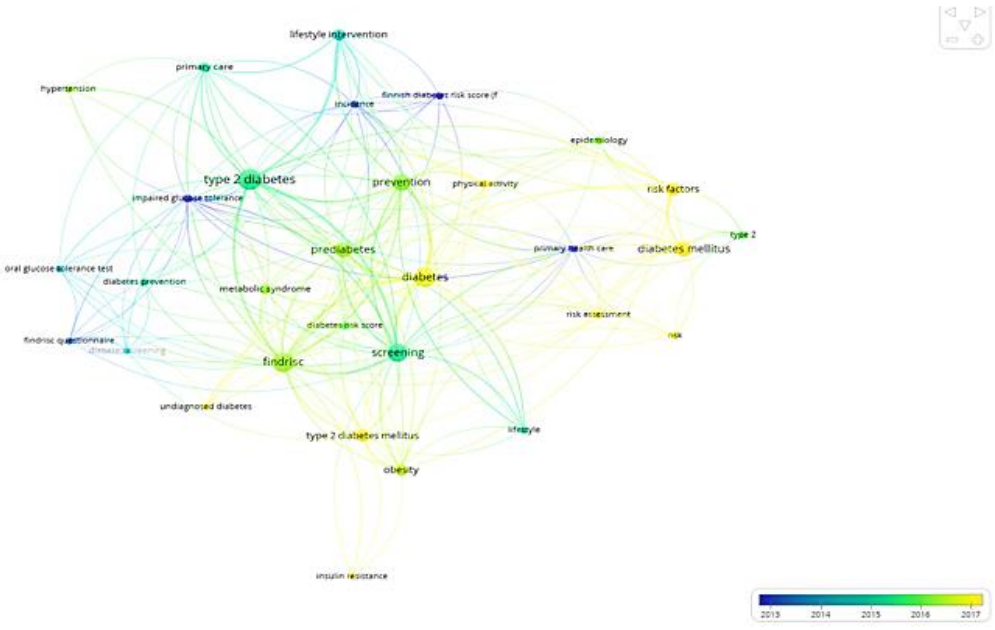


Figure 6. Mapping of keywords related to FINDRISC and Diabetes topics in publications for the period 2005-2021 based on keyword type and year of publication.

Since 2015, articles have been published by researchers from countries in the Americas and Asia. Even though the number of articles is still relatively small, the researchers who research the topic of FINDRISC seem to be intensely related and interact with each other through citations. The lack of publications regarding the utilization of FINDRISC in Asian populations provides an opportunity to conduct a comparative analysis of the validity of the FINDRISC screening instrument in Asian populations. The different characteristics of populations with diabetes mellitus in Europe and Asia are very important to understand by clinicians and policymakers for the prevention of risk factors and management of diabetes. Compared to the European population, the incidence of diabetes in the Asian population is dominated by young people with insulin resistance due to excessive accumulation of body fat and abdominal obesity[23].

Figure 7 exhibits a mapping of citations by author and year of publication. There are six clusters based on the year of publication with 115 relationships between authors. The article by Tuomilehto was most cited in 2014, while the most cited articles published in 2020 were those by Cardon and Iotova. **Figure 8** shows the types of published articles, most of which are research articles (92.8%). While **Figure 9** displays the subject areas of the research, most of which are medicine (58.4%).

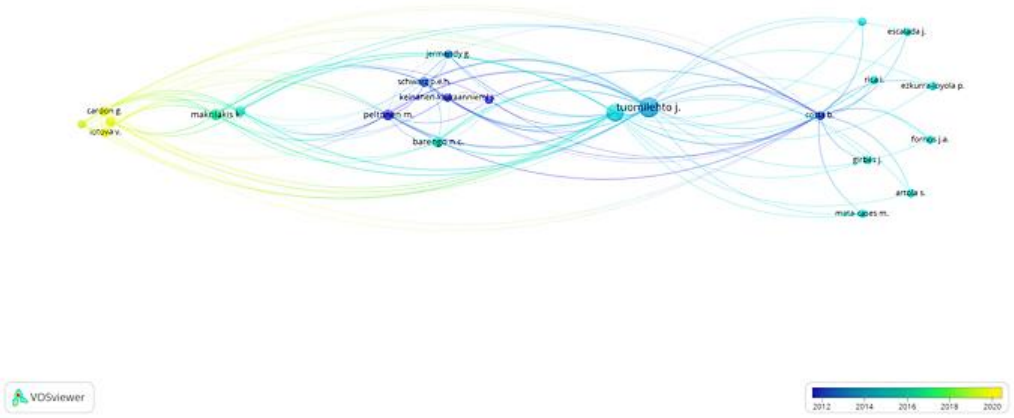


Figure 7. Mapping of citations related to FINDRISC and Diabetes topics in publications for the period 2005-2021 by author and year of publication.

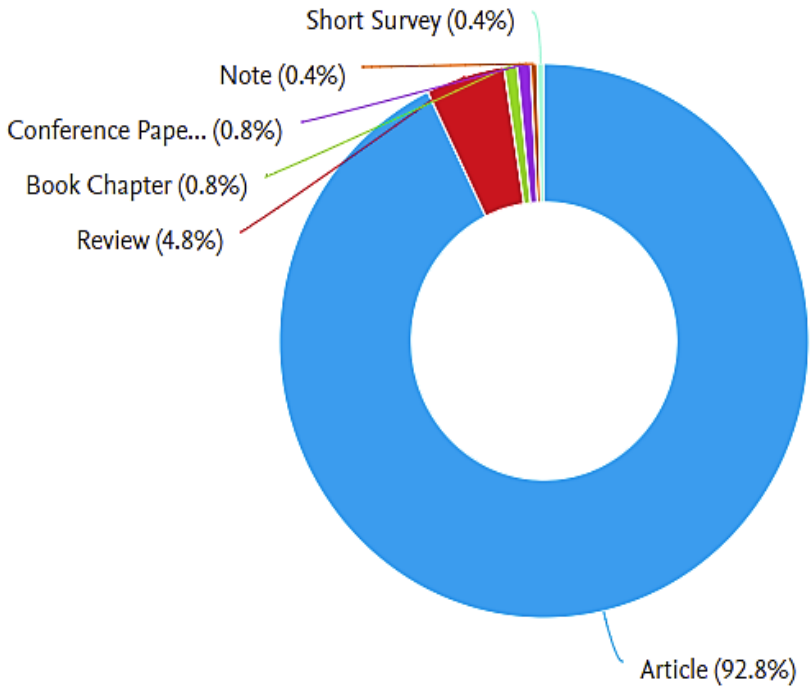


Figure 8. Types of articles on the topics of FINDRISC and Diabetes in publications for the period 2005-2021.

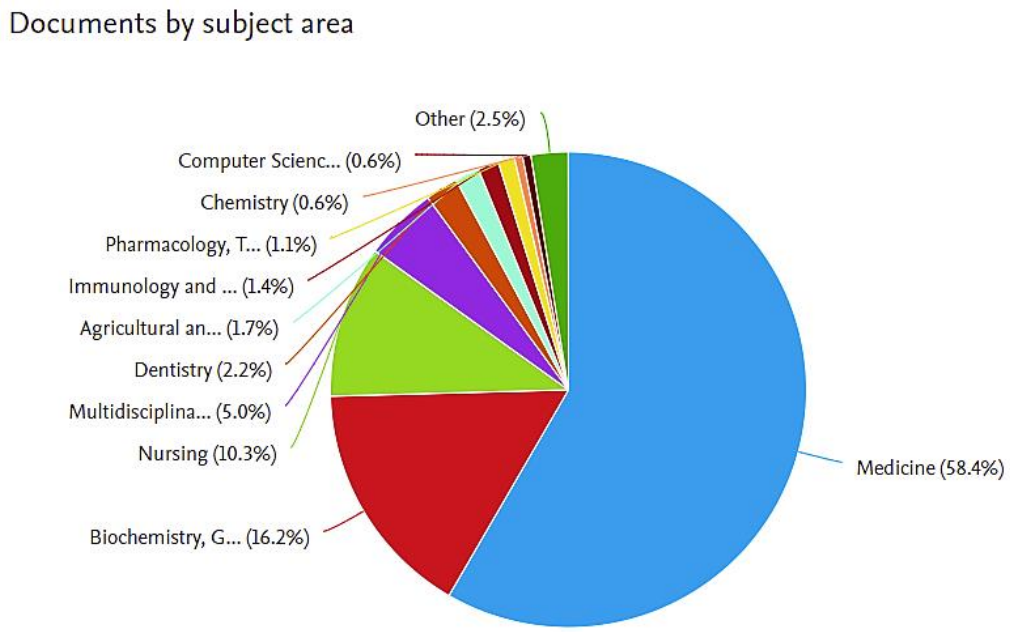


Figure 9. Subject areas of research with the topics of FINDRISC and Diabetes in publications for the period 2005-2021.

The overview of the types of published articles reveals that most of the documents in this bibliometric analysis are original papers or research articles. The number of review articles was found to be less than 5%. It indicates an opportunity to conduct a systematic literature review, scoping review, or meta-analysis method to examine the validity and performance of FINDRISC as an instrument for screening various populations at risk of developing diabetes or having prediabetes. In addition, the existing publications that test the validity of FINDRISC in the new population mainly used a cross-sectional design, and there are still few publications that used prospective data[24].

Figure 10 shows the most frequently occurring words in publications about FINDRISC and diabetes. "Finland" is the most mentioned word in the publications. **Figure 11** is a mapping of themes based on articles found in publications for 2005-2021. The theme clusters can be seen from the color difference. The cluster in gray is the cluster of most discussed themes in research on FINDRISC and diabetes, which include: consensus, diet restriction, clinical effectiveness, Spain, questionnaire, glucose blood level, and others.

Figure 10. Word Cloud in research related to FINDRISC and Diabetes in publications for the period 2005-2021.

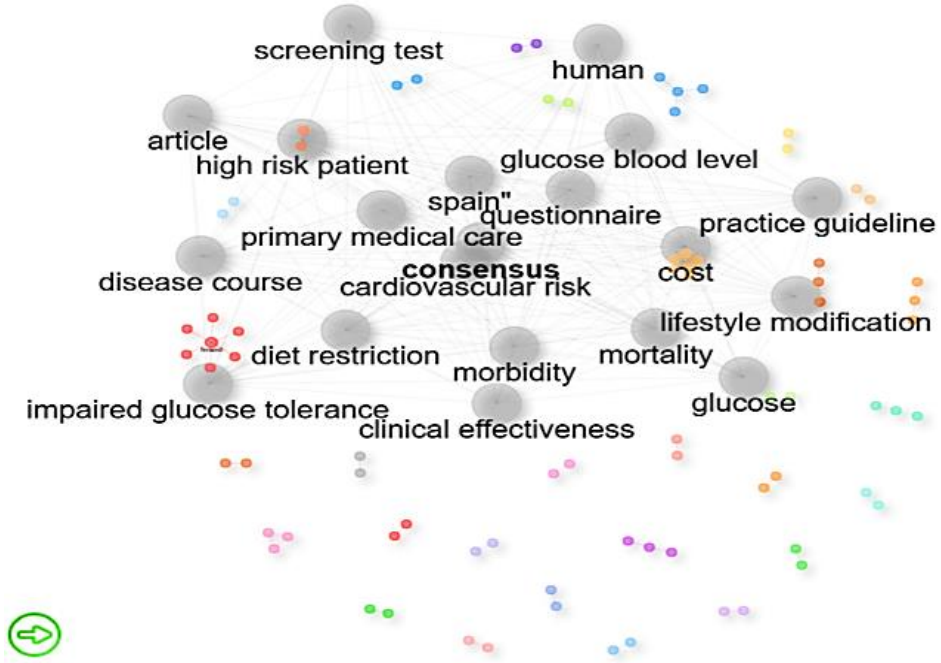


Figure 11. Mapping of themes of research with the topics of FINDRISC and Diabetes in publications for the period 2005-2021.

It seems that the research themes related to FINDRISC are not only limited to immediate diabetes risk factors such as diet restriction, glucose blood level, cardiovascular risk, and lifestyle modification but also intersect with broader themes such as disease course, mortality, morbidity, and costs. Several themes that were also discussed, such as screening tests, high-risk patients, and clinical effectiveness, indicate that the FINDRISC instrument has the potential to be studied further at the clinical level and community level, and it is possible for this instrument to be used to screen for other metabolic disorders such as metabolic syndrome. In addition, the attempt to include FINDRISC in the practice guidelines for primary medical care has become a growing theme[25]. A cost-effectiveness analysis is necessary for the evaluation[26].

On the other hand, although nearly 60% of the subject areas of research on FINDRISC and diabetes are medicine, research from other subject areas such as biochemistry, nursing, dentistry, agriculture, pharmacology, and others provides opportunities for collaborative research on this topic in the future. Various reports on the results of studies related to FINDRISC are still popular and exciting to publish. This is indicated by articles on this topic continuously published by reputable and prestigious journals such as PLoS One, BMC Public Health, and Primary Care Diabetes.

One limitation of this study is that the only databases used were PubMed and Scopus. Scopus will repeat the number of articles based on contributing authors from different countries in one article, so some articles may be counted multiple times if the contributing authors are from several countries[27]. Nevertheless, this is sufficient to serve as a basis for evaluating the utilization of FINDRISC in diabetes screening and identifying research gaps and future research opportunities. Opportunities to conduct research using Indonesia's FINDRISC instrument are still ample. This instrument can be one of the government's considerations, especially in measures for the early detection of diabetes, for example, in the integrated post guidance for non-communicable diseases (*Pos Binaan Terpadu*; *Posbindu*) and the Healthy Campus program initiated by the Ministry of Health of the Republic of Indonesia.

CONCLUSION

Despite the increase in publications, the number of publications on FINDRISC in patients with diabetes mellitus per year is still relatively small, with 92.8% of publications being primary studies. The publications are still dominated by those of researchers from countries in the European region, and these researchers intensely relate to each other and interact with each other through citations. Research themes related to FINDRISC are not limited to diabetes risk factors.

ACKNOWLEDGMENT

We want to thank all those who have helped us in the data collection process and the process of writing this article. Also, thanks to the Office of Research and Publication, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia.

AUTHORS' CONTRIBUTION

DAP was the first to propose this research concept. Then, **DAP** and **MRR** collect and analyze data. In addition to being the corresponding author, **BA** also ensures that the final draft of the manuscript complies with the guidelines of the intended journal. **ZZ** contributes to data visualization. **SR** support in budgeting. All authors provided feedback from the initial draft to the final manuscript and approved the overall process.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. International Diabetes Federation. IDF Diabetes Atlas. 6th ed. Brussels (Belgium): International Diabetes Federation; 2013.
2. International Diabetes Federation. IDF Diabetes Atlas. 9th ed. Brussels (Belgium): International Diabetes Federation; 2019.
3. Saeedi P, Petersohn I, Salpea P, Malanda B, Karuranga S, Unwin N, et al. Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9 th edition. Diabetes Res Clin Pract. 2019;157:107843. doi:10.1016/j.diabres.2019.107843
4. Andes LJ, Cheng YJ, Rolka DB, Gregg E, Imperatore G. Prevalence of prediabetes among adolescents and young adults in the United States, 2005-2016. JAMA Pediatr. 2020;174(2):e194498. doi:10.1001/jamapediatrics.2019.4498
5. Hostalek U. Global epidemiology of prediabetes - present and future perspectives. Clin Diabetes Endocrinol. 2019;5:5. doi:10.1186/s40842-019-0080-0
6. Valensi P, Schwarz EH, Hall M, Felton AM, Maldonato A, Mathieu C. Pre-diabetes essential action: a European perspective. Diabetes Metab. 2005;31(6):606-20. doi:10.1016/S1262-3636(07)70239-2
7. Uusitupa M, Khan T, Viguiliouk E, Kahleova H. Prevention of Type 2 Diabetes by Lifestyle Changes : a systematic review and meta-analysis. Nutrients. 2019;11(11):2611. doi:10.3390/nu11112611
8. Arslanian S, Bacha F, Grey M, Marcus MD, White NH, Zeitler P. Evaluation and Management of Youth-Onset Type 2 Diabetes: A Position Statement by the American Diabetes Association. Diabetes Care. 2018;41(12):2648-68. doi:10.2337/dci18-0052
9. Lindström J, Tuomilehto J. The diabetes risk score: a practical tool to predict type 2 diabetes risk. Diabetes Care. 2003;26(3):725-31. doi:10.2337/diacare.26.3.725

10. Lim HM, Chia YC, Koay ZL. Performance of the Finnish Diabetes Risk Score (FINDRISC) and Modified Asian FINDRISC (ModAsian FINDRISC) for screening of undiagnosed type 2 diabetes mellitus and dysglycaemia in primary care. Prim Care Diabetes. 2020;14(5):494-500. doi:10.1016/j.pcd.2020.02.008

11. Zhang M, Zhang H, Wang C, Ren Y, Wang B, Zhang L, et al. Development and validation of a risk-score model for type 2 diabetes: a cohort study of a rural adult Chinese population. PLoS One. 2016;11(4):e0152054. doi:10.1371/journal.pone.0152054

12. Dugee O, Janchiv O, Jousilahti P, Sakhiya A, Palam E, Nourti JP, et al. Adapting existing diabetes risk scores for an Asian population: A risk score for detecting undiagnosed diabetes in the Mongolian population. BMC Public Health. 2015;15:938. doi:10.1186/s12889-015-2298-9

13. Bernabe-Ortiz A, Perel P, Miranda JJ, Smeeth L. Diagnostic accuracy of the Finnish Diabetes Risk Score (FINDRISC) for undiagnosed T2DM in Peruvian population. Prim Care Diabetes. 2018;12(6):517-25. doi:10.1016/j.pcd.2018.07.015

14. Bergmann A, Li J, Wang L, Schulze J, Bornstein SR, Schwarz PEH. A simplified Finnish Diabetes Risk Score to predict type 2 diabetes risk and disease evolution in a German population. Horm Metab Res. 2007;39(9):677-82. doi:10.1055/s-2007-985353

15. Saaristo T, Moilanen L, Korpi-Hyövälti E, Vanhala M, Saltevo J, Niskanen L, et al. Lifestyle intervention for prevention of type 2 diabetes in primary health care. Diabetes Care. 2010;33(10):2146-51. doi:10.2337/dc10-0410

16. Mavrogianni C, Lambrinou CP, Androutsos O, Lindström J, Kivelä J, Cardon G, et al. Evaluation of the Finnish Diabetes Risk Score as a screening tool for undiagnosed type 2 diabetes and dysglycaemia among early middle-aged adults in a large-scale European cohort: The Feel4Diabetes study. Diabetes Res Clin Pract. 2019;150:99-110. doi:10.1016/j.diabres.2019.02.017

17. Salinero-Fort MA, Burgos-Lunar C, Lahoz C, Mostaza JM, Abánades-Herranz JC, Laguna-Cuesta F, et al. Performance of the Finnish Diabetes Risk Score and a simplified Finnish Diabetes Risk Score in a community-based, cross-sectional programme for screening of undiagnosed type 2 diabetes mellitus and dysglycaemia in madrid, Spain: the SPREDIA-2 study. PLoS One. 2016;11(7):e0158489. doi:10.1371/journal.pone.0158489

18. Janghorbani M, Adineh H, Amini M. Evaluation of the Finnish Diabetes Risk Score (FINDRISC) as a screening tool for the metabolic syndrome. Rev Diabet Stud. 2013;10(4):283-92. doi:10.1900/RDS.2013.10.283

19. Väätäinen S, Cederberg H, Roine R, Keinänen-Kiukaanniemi S, Saramies J, Uusitalo H, et al. Does future diabetes risk impair current quality of life? a cross-sectional study of health-related quality of life in relation to the Finnish diabetes risk score (FINDRISC). PLoS One. 2016;11(2):e0147898. doi:10.1371/journal.pone.0147898

20. Nieto-Martínez R, González-Rivas JP, Aschner P, Barengo NC, Mechanick JI. Transculturalizing diabetes Prevention in Latin America. Ann Glob Heal. 2017;83(3-4):432-43. doi:10.1016/j.aogh.2017.07.001

21. Toumilehto J, Lindström J, Eriksson JG, Valle TT, Uusitupa M. Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. N Engl J Med. 2013;344(18):1343-50. doi:10.1056/nejm200105033441801

22. Rokhman MR, Arifin B, Zulkarnain, Satibi, Perwitasari DA, Boersma C, et al. Translation and performance of the Finnish Diabetes Risk Score for detecting undiagnosed diabetes and dysglycaemia in the Indonesian population. PLoS One. 2022;17(7):e0269853. doi:10.1371/journal.pone.0269853

23. Ma RCW, Chan JCN. Type 2 diabetes in East Asians: similarities and differences with populations in Europe and the United States. Ann N Y Acad Sci. 2013;1281(1):64-91. doi:10.1111/nyas.12098

24. Jølle A, Midthjell K, Holmen J, Carlsen SM, Tuomilehto J, Bjørngaard JH, et al. Validity of the FINDRISC as a prediction tool for diabetes in a contemporary Norwegian population: a 10-year follow-up of the HUNT study. BMJ Open Diabetes Res Care. 2019;7(1):e000769. doi:10.1136/bmjdrc-2019-000769

25. Fauzi NFM, Wafa SW, Ibrahim AM, Raj NB, Nurulhuda MH. Translation and Validation of American Diabetes Association Diabetes Risk Test: The Malay Version. Malays J Med Sci. 2022;29(1):113-25. doi:10.21315/mjms2022.29.1.11

26. Noyes K, Holloway RG. Evidence from cost-effectiveness research. NeuroRx. 2004;1(3):348-55. doi:10.1602/neurorx.1.3.348

27. Putera PB, Suryanto, Ningrum S, Widianingsih I. A bibliometric analysis of articles on innovation systems in Scopus journals written by authors from Indonesia, Singapore, and Malaysia. Sci Ed. 2020;7(2):177-83. doi:10.6087/kcse.214

Borneo Journal of Pharmacy
Vol 5 Issue 3 August 2022
Page 299 – 306

http://journal.umpalangkaraya.ac.id/index.php/bjop/article/view/2158
DOI: https://doi.org/10.33084/bjop.v5i3.2158
e-ISSN: 2621-4814

Research Article

Comparison of Patient's Satisfaction with Pharmaceutical Care Services in Ownership-Based Pharmacies in Semarang, Indonesia

Nisa Febrinasari*

Abdur Rosyid

Fadhila Huswatunnida

Department of Pharmacy, Universitas Islam Sultan Agung, Semarang, Central Java, Indonesia

*email: nisafebrie@unissula.ac.id

Keywords:
Franchise and non-franchise pharmacies
Medicine information
Pharmacy services
Pharmacy staff

Abstract

Implementation of pharmacists services provided in all pharmacies must meet the standard of pharmaceutical services guidelines, including the provision of drug information. The study aims to compare the patient's perception of drug counseling services by pharmacy staff based on the type of pharmacy ownership (franchise/non-franchise) in Semarang. This research is an observational study with a cross-sectional design. Samples were taken of 286 respondents with a quota sampling technique. Data were collected using an online google form questionnaire tested for validity and reliability. The statistical analysis results used the Mann-Whitney test with a p-value of <0.05. There is no significant difference between the patient's perception of the drug information counseling services by pharmacy staff at the franchise or non-franchise pharmacies in Semarang with a p-value of 0.264. This study also found that the standard information given by pharmacy staff is healthy eating and education about antibiotics used in the common cold. In addition, only 55% of respondents were sure that the pharmacy staff who gave them drug information in pharmacies was a pharmacist. Consequently, we humbly recommend that pharmacists consistently wear their pharmacist identification.

Received: February 25th, 2021
1st Revised: May 26th, 2022
2nd Revised: July 27th, 2022
Accepted: July 31th, 2022
Published: August 31th, 2022

INTRODUCTION

Pharmaceutical services are part of a healthcare system oriented toward patient care and quality medication provision[1]. Pharmacy practice in pharmacies has complied with the pharmacy service standard in pharmacies. The medicine information service is one critical aspect of clinical pharmacy service[2]. The process of providing information by pharmaceutical personnel to patients is obligatory. Pharmaceutical personnel must be proactive in providing medication information services provided to patients[3]. Some medication information that needs to be conveyed to patients includes dosage, method and time of use, amount of consumption in a day, storage method, and how to deal with possible side effects[4].

The pharmaceutical services implementation in Semarang has been evaluated by research conducted in hospitals. The results show that there are still differences in implementing pharmaceutical service standards in several public and private hospitals in Semarang that can potentially prevent medication errors. The difference in the implementation of the service standard will undoubtedly affect the quality of pharmaceutical services provided to patients[5]. Likewise, the pharmaceutical services provided in pharmacies, the implementation of pharmaceutical services provided in the network/franchise, and non-network pharmacies must meet the standards of pharmaceutical services in pharmacies, in the provision of medicine, medical devices, and consumable medical materials, or the clinical pharmacy services, including the provision of medical information[6]. A study of the pharmacist community in 2009 showed that pharmacists are not quite ready, according to the

How to cite: Febrinasari N, Rosyid A, Huswatunnida F. Comparison of Patient's Satisfaction with Pharmaceutical Care Services in Ownership-Based Pharmacies in Semarang, Indonesia. Borneo J Pharm. 2022;5(3):299-306. doi:10.33084/bjop.v5i3.2158

Department of Health and the Association of Indonesian Pharmacists (*Ikatan Apoteker Indonesia; IAI*). The pharmacists in non-franchise pharmacies are even more unprepared than the franchise pharmacies. Franchise pharmacies' pharmacists in the metropolitan city are preparing to face the patient-oriented paradigm and fulfilling the pharmacy service standard. In contrast, the pharmacists in the independent non-franchise pharmacies still emphasize fast service and the lower price of medicine[7].

The government has enforced a standard of pharmaceutical services in pharmacies through the Minister of Health Decree Number 73 of the Year 2016 concerning the standard of pharmaceutical services in Pharmacies, which includes two aspects. The first aspect is the provision management of pharmacy, medical devices, and consumable medical materials; the second is the clinical pharmacy services. Implementing these standards is a practice guide for pharmacists in carrying out the profession in pharmacies, protecting the public from unprofessional services, and carrying out pharmaceutical practices[8]. Pharmacists must improve knowledge, skills, and behavior in interacting with patients by providing complete information on medication usage, side effects, and monitoring. Therefore, pharmacists must comply with pharmaceutical service standards in pharmacies to ensure the quality of public pharmaceutical services[9].

Drug information services are an essential part of the clinical pharmacy service[10]. The quality of pharmaceutical services at networked/franchise and non-networked/non-franchise pharmacies will affect the completeness of the information provided to patients, so it is necessary to pay attention to good service and meet the Pharmacy Service Standards at the Pharmacy (*Standar Pelayanan Kefarmasian di Apotek; SPKA*)[11]. Research on the completeness of drug information to patients is a study that can be carried out to assess and measure the quality of service provided by pharmacists and pharmaceutical personnel in pharmacies[12]. The pharmacy service provider acts as the health worker who provides medicine preparation services and information and education services to improve the health and quality of life[13]. Therefore, it is necessary to investigate the completeness of the information by pharmacy personnel in Semarang pharmacies, either networked/franchise or non-network pharmacies. The purpose of this study is to compare patients' perceptions of drug information services by pharmacy staff according to the type of pharmacy ownership (franchise/non-franchise) in Semarang. Through the research, pharmacies can be informed about the medical information services provided by the pharmacy personnel at the respective pharmacies and, at the same time, can be used as evaluation materials to play a more significant role in improving the quality of medication information services to patients.

MATERIALS AND METHODS

A franchise pharmacy is an authorization or official permission or approval granted by a pharmacy company to distributors, groups, or individual owners to establish a pharmacy, for example, Kimia Farma, K24, and Viva Generik. Non-franchise pharmacy is a pharmacy in the general community. Inclusion criteria for respondents in this study were subjects who had redeemed drugs or purchased pharmaceutical products at a pharmacy in Semarang within a maximum of the last three months, had received direct pharmaceutical services from pharmacists and pharmacy technical staff, the minimum age was 17 years, and subjects who were willing to be respondents. The exclusion criteria were respondents who did not complete their questionnaire. The study design and protocol were approved by the Faculty of Medicine Ethics Committee, Universitas Islam Sultan Agung, with the number EC/244/VII/Komisi Bioetik. The tool used in the research was a modified questionnaire Investigating Consumer Attitudes toward Community Pharmacy Services[14]. The questionnaire obtained permission from the previous author and was translated "backward-forward" into the Indonesian version by the Center for International Language Development (CILAD) Universitas Islam Sultan Agung with DOI https://doi.org/10.5281/zenodo.6777035. From calculation from the Slovin formula with a population assumed as 1000 people, the study requires 286 and 30 respondents for the validity and reliability test. The respondents were recruited by quota sampling from May until October 2020. Data collection was carried out with a Google Form questionnaire. The questionnaire distribution was through Google Form link sharing as https://bit.ly/pelayananfarmasi by social media. The Questionnaire item was available in the Supplement file. Data analysis was conducted with the Mann-Whitney test.

RESULTS AND DISCUSSION

The research was carried out on Google Form, in which all questions were valid and reliable with R >0.361 for 30 respondents with a significance level of 5%. The reliability test shows that Cronbach's α value was 0.832. **Table I** shows that from 286 respondents who met inclusion criteria, franchise pharmacies have more patients than non-franchise ones, with 167 respondents at franchise and 119 at non-franchise pharmacies.

Table I. Respondents based on types of pharmacies

Pharmacy type	Total respondents	%
Franchise	167	58.4
Non-franchise	119	41.6
Total	286	100

Table II shows that in each pharmacy, the number of respondents with females was more dominant than males, with a relatively large ratio. The female respondents in franchised and non-franchised pharmacies were 130 people (77.8%) and 89 people (74.8%), respectively; the rest were males. Most respondents are between 17 and 25 years old with an education diploma or bachelor's degree. The same results were also shown in previous research[15], which explains that women were more concerned about the health of each family member, which affected the frequency of female respondents visiting the pharmacy. In addition, another previous study[16] also shows that women have an essential role as decision-makers in health services for themselves and their families.

Table II. Demographic characteristic of respondents

Parameters	Franchise pharmacy		Non-franchise pharmacy	
	Total respondents	%	Total respondents	%
Gender				
Man	37	22.2	30	25.2
Woman	130	77.8	89	74.8
Ages (y.o.)				
17 – 25	135	80.8	87	73.1
26 – 35	19	11.4	11	9.2
36 – 45	5	3	9	7.6
46 – 55	8	4.8	11	9.2
56 – 65	0	0	1	0.8
>65	0	0	0	0
Education				
Elementary school	4	2.4	0	0
High school	32	19.2	23	19.3
Diploma/Bachelor	130	77.8	95	79.8
Magister	1	0.6	1	0.9
Work background				
Health worker	38	22.8	25	21
Other	129	77.2	94	79

Table III describes that of 167 respondents who visited franchise pharmacies, 93 people (55.7%) believed that those who served respondents in pharmacies was pharmacist, followed by 19 people (11.4%) who answered that those who served respondents were not pharmacists or person staff, and 55 people (32.9%) chose to answer that they did not know the person profession who served them, whether it was a pharmacist or person staff. In non-franchise pharmacy have similar results with 45.4%, 20.2%, and 34.4 %, respectively. **Table III** analyzes the response to information about drug services for patients, indicating that only 55.7% of patients recognized pharmacists in franchise pharmacies and only 45.4% in non-franchised pharmacies. About 33.7% of patients, in general, did not know whom pharmacy staff in charge provided the services. This study shows that pharmacist roles are still not well known by the community. Since pharmacists are health workers obliged to provide drug counseling, they should not be replaced by pharmaceutical technical personnel. In addition, identity as a pharmacist in pharmacies is essential, so it is always advised to use/wear a special identity sign that shows a pharmacist's profession when performing service in the pharmacy[17].

Table III. Distribution of respondents answers to the question "Are you served by a pharmacist?"

Answer category	Franchise pharmacy		Non-franchise pharmacy	
	Total respondents	%	Total respondents	%
Yes	93	55.7	54	45.4
No	19	11.4	24	20.2
Unsure	55	32.9	41	34.4
Total	167	100	119	100

Table IV explains that from 167 respondents in franchise pharmacies, 98 people (58.7%) answered agreed with the assessment that the pharmacy staff who served were experienced, trustworthy, and confident. However, 69 people (41.3%) answered neutrally in that regard. In non-franchise pharmacies have similar results with 58% and 41.2%, respectively.

Table IV. Distribution of respondents' answers to the question "How would you rate the pharmacy staff who served you? Are their experienced/trustworthy/confident/useful?"

Answer category	Franchise pharmacy		Non-franchise pharmacy	
	Total respondents	%	Total respondents	%
Agree	98	58.7	69	58
Netral	69	41.3	49	41.2
Disagree	0	0	1	0.8
Total	167	100	119	100

Table V indicates that the majority of the respondent, 140 respondents (83.8%) in franchise pharmacies and 91 (76.5%) in non-franchise pharmacies, admitted that they had been given advice or information by pharmacy staff at the time of service. However, 27 respondents (16.2%) in franchise pharmacies and 28 (23.5%) in non-franchise pharmacies answered that they were not given advice or information during service. **Table VI** shows that 57 respondents (34.1%) in franchise pharmacies and 31 (26.1%) in non-franchise pharmacies answered that they had been given information and advice about healthy eating. They were followed by 35 respondents (21%) in franchise pharmacies and 24 (20.2%) in non-franchise pharmacies receiving education about the use of antibiotics for flu and the common cold. **Table VII** shows that most respondents were satisfied with pharmacy staff service regarding attitude, instruction, drug information about side effects, and asking about previous health history and counseling place. However, the majority of respondents in both pharmacies disagree with a statement about proper drug storage methods information, with 10.8% in franchise pharmacies and 17.6% in non-franchise pharmacies.

Table V. Distribution of respondents' answers to the question " Have you ever been given advice by a pharmacy staff?"

Answer category	Franchise pharmacy		Non-franchise pharmacy	
	Total respondents	%	Total respondents	%
Yes	140	83.8	91	76.5
Never	27	16.2	28	23.5
Total	167	100	119	100

Table VI. Distribution of respondents' answers regarding suggestions/information ever given by pharmacy staff

Answer category	Franchise pharmacy		Non-franchise pharmacy	
	Total respondents	%	Total respondents	%
Not answer	22	13.2	18	15.1
Smoking cessation	8	4.8	7	5.9
Healthy eating	57	34.1	31	26.1
Physical training	5	3	2	1.7
Steroid anabolic	0	0	0	0
Hypertension	3	1.8	1	0.8
Diabetes	0	0	0	0
Oral contraception	1	0.6	1	0.8
Antibiotic used in common cold and influenza	35	21	24	20.2
Answer >1 choices	36	21.6	35	29.4
Total	167	100	119	100

Table VII. Analysis of responses to questions "Pharmacy staff services at franchise and non-franchise pharmacies" based on median and mean values

No	Question	Median		Mean		p-value
		F	Non-F	F	Non-F	
1	The Pharmacy staff in charge of administering your medicines in a polite manner	3	3	2.89	2.8	0.027
2	Label on each drugs are well instructed by pharmacy staff	3	3	2.82	2.75	0.189
3	Pharmacy staff explain all possible side effects clearly	3	2	2.43	2.29	0.203
4	Pharmacy staff provide written/printed information about drug therapy and/or disease	3	3	2.52	2.34	0.065
5	Pharmacists use information about your previous condition/medication when administering your drug therapy	3	3	2.59	2.49	0.361
6	Pharmacists provide information on proper drug storage methods	3	3	2.44	2.35	0.422
7	Pharmacy provided counseling place to respects your privacy	3	3	2.65	2.58	0.293

According to **Table VII**, the response to information about drug services for patients shows that most respondents agreed that pharmacy staff is in charge, providing medicines politely, always giving a clear label, and explaining all possible medication side effects clearly[18]. The study showed that pharmacy staff in Semarang already implemented technical guidelines for pharmaceutical service standards in pharmacies; these activities are included in the dispensing process. In the technical guidelines, it is stated that before handing over the medicine to the patient, a re-examination of the writing of the patient's name on the label, usage instructions, and the type and amount of medicine (the compatibility between the writing of the label and the prescription) must be done[19].

In addition, most respondents agree that pharmacy staff provides written/printed information on drug therapy and patient diseases. The pharmacy staff also used information about the patient's previous condition/medication when administering medicine therapy. When administering medicine to patients, pharmacist or pharmacy personnel must be attentive to the patient's history of medication or disease, especially related to the medications consumed, to reduce the side effects. The process described in the statement is part of the clinical pharmacy service activity: medication information provision[20]. Based on technical guidelines for the implementation of pharmaceutical service standards in Pharmacies, it is stated that in the drug counseling process, Drug Information Services include activities such as answering questions orally or in writing, making brochures/leaflets containing medication information, as well as providing education and information to patients. In detail, it is necessary to ask the patient questions and data/information in implementing service standards[21]. The counseling process standard also explains that pharmacists need to explore further information by exploring medicine use problems and explaining medicine use problem-solving. The pharmacy services standard's technical guidelines also show that pharmacies must have adequate space, including rooms/places for counseling. At a minimum, there must be a set of tables and chairs for counseling in the counseling space, a book cupboard, reference books, leaflets, posters, counseling supporting tools, and counseling book records and forms to record the patients' treatment[22].

The Mann-Whitney test result shows Asymp Sig. (2-tailed) value of 0.264, as shown in **Table VIII**. The significance of the acquisition result value was more than 0.05, which indicates that the completeness of the medication information by the pharmacy personnel between the franchise and nonfranchise pharmacies has no significant difference. The median value acquisition of franchise pharmacies' respondents is 19.00, while the nonfranchise pharmacies' respondents is 18.00. The median value obtained in the two ownership types of pharmacies shows the category of "very complete" in the pharmacy service, which is in the value range of 18-21. Therefore, it can be interpreted that the completeness of medicine information by pharmacy personnel between the franchise and non-franchise pharmacies has no significant difference.

Table VIII. Mann-Whitney test results, Median value of the franchise and non-franchise pharmacies

Test	Sig.	Interpretation
Non-parametric test: Mann-Whitney test	0.264	Not significantly different
Median of franchise pharmacies	19.00	Very complete
Median of non-franchise pharmacies	18.00	Very complete

In general, not all patients are informed and aware of what to do with the medicines that have been obtained, so medication services are needed to prevent medicine abuse and unwanted medicine interactions. In this case, medicine information

services are still lacking compared to the need for speed in service and information about patients' medicines. Pharmacy personnel must provide patient information. Besides, pharmacy personnel must proactively provide medication information services to patients. Some information that should be conveyed to patients includes the dosage of drugs, methods, the timing of use, the amount of medicine consumed in a day, how to store medicines, and how to deal with possible side effects are possible[23].

In many countries, the sustainability of pharmacy practice has been carried out; pharmacists have integrated regulations to support patients in selecting medications and providing appropriate information advice[24,25]. For results, pharmaceutical practices' sustainability needs to be maintained for patient quality of service and life. Furthermore, patient's perceptions of the pharmacy profession as a product rather than a service certainly influence satisfaction with community pharmacy services. Interestingly, in some study[26], patient satisfaction was high despite the low counseling level rating. According to other studies[27,28], the higher the frequency of counseling and monitoring, as well as the more targeted the guidance, the higher the satisfaction rating[29]. It has also been stated that patient counseling may not be as frequent or as comprehensive due to a lack of demand for these services. These findings emphasize the importance of educating the public about pharmacists' services.

CONCLUSION

Patients' perceptions about pharmaceutical services based on the type of pharmacy ownership (franchise/non-franchise) in Semarang do not significantly differ. Unfortunately, many respondents are still unaware of the pharmacist profession. Therefore, We humbly recommend that pharmacists wear identification as pharmacists as they do pharmacy services to the community.

ACKNOWLEDGMENT

We would like to thank all the participants who contributed to this study for their time and efforts.

AUTHORS' CONTRIBUTION

Nisa Febrinasari: conceptualization, supervision, writing- review and editing. **Abdur Rosyid**: supervision. **Fadhilla Huswatunnida**: investigator, writing original draft.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Bobbins AC, Burton S, Fogarty TL. Different models of pharmaceutical services and care in primary healthcare clinics in the Eastern Cape, South Africa: Challenges and opportunities for pharmacy practice. Afr J Prim Health Care Fam Med. 2020;12(1):e1-11. doi:10.4102/phcfm.v12i1.2323

2. Lopes H, Lopes AR, Farinha H, Martins AP. Defining clinical pharmacy and support activities indicators for hospital practice using a combined nominal and focus group technique. Int J Clin Pharm. 2021;43(6):1660-82. doi:10.1007/s11096-021-01298-z

3. Eiland LS, Benner K, Gumpper KF, Heigham MK, Meyers R, Pham K, et al. ASHP-PPAG Guidelines for Providing Pediatric Pharmacy Services in Hospitals and Health Systems. J Pediatr Pharmacol Ther. 2018;23(3):177-91. doi:10.5863/1551-6776-23.3.177

4. Jimmy B, Jose J. Patient medication adherence: measures in daily practice. Oman Med J. 2011;26(3):155-9. doi:10.5001/omj.2011.38

5. Satibi, Marin VYW, Suwarni S, Kuswardhani. Analisis Perbedaan Implemantasi Standar Pelayanan Kefarmasian dengan Potensi Medication Error di Beberapa Rumah Sakit Kota Semarang. J Manajemen Pelayanan Farmasi J Manag Pharm Pract. 2017;7(3):125-31. doi:10.22146/jmpf.33251

6. Costa FA, Scullin C, Al-Taani G, Hawwa AF, Anderson C, Bezverhni Z, et al. Provision of pharmaceutical care by community pharmacists across Europe: Is it developing and spreading? J Eval Clin Pract. 2017;23(6):1336-47. doi:10.1111/jep.12783

7. Mulyagustina, Wiedyaningsih C, Kristina SA. Implementation of Pharmaceutical Care Standard in Jambi City's Pharmacies. J Manajemen Pelayanan Farmasi J Manag Pharm Pract. 2017;7(2):83-96. doi:10.22146/jmpf.30284

8. Wiryanto, Tanjung H, Rumonda R. Implementation of Standards for Managing Pharmaceutical, Medical Devices and Disposable Medical Materials in Community Pharmacy in Medan City. Open Access Maced J Med Sci. 2019;7(22):3769-73. doi:10.3889/oamjms.2019.532

9. Ilardo ML, Speciale A. The Community Pharmacist: Perceived Barriers and Patient-Centered Care Communication. Int J Environ Res Public Health. 2020;17(2):536. doi:10.3390/ijerph17020536

10. Alamri SA, Al Jaizani RA, Naqvi AA, Al Ghamdi MS. Assessment of Drug Information Service in Public and Private Sector Tertiary Care Hospitals in the Eastern Province of Saudi Arabia. Pharmacy. 2017;5(3):37. doi:10.3390/pharmacy5030037

11. Gobel N, Tuloli TS, Madania. Studi Penjaminan Mutu (Quality Assurance) Dalam Pelayanan Kefarmasian Di Apotek. J Syifa Sci Clin Res. 2022;4(2):237-46. doi:10.37311/jsscr.v4i2.13956

12. Athiyah U, Setiawan CD, Nugraheni G, Zairina E, Utami W, Hermansyah A. Assessment of pharmacists' knowledge, attitude and practice in chain community pharmacies towards their current function and performance in Indonesia. Pharm Pract. 2019;17(3):1518. doi:10.18549/PharmPract.2019.3.1518

13. Hermansyah A, Wulandari L, Kristina SA, Meilianti S. Primary health care policy and vision for community pharmacy and pharmacists in Indonesia. Pharm Pract. 2020;18(3):2085. doi:10.18549/pharmpract.2020.3.2085

14. El-Sharif SI, Alrahman NA, Khaled N, Sayah N, Gamal E, Mohammed A. Assessment of Patient's Satisfaction with Pharmaceutical Care Service in Community Pharmacies in the United Arab Emirates. Arch Pharma Pract. 2017;8:22-30.

15. Aljuffali LA, Alshabanah MO, Almalag HM. Cross-sectional study to evaluate burnout among pharmacy staff in Saudi Arabia during COVID-19 pandemic. Saudi Pharm J. 2022;30(4):440-53. doi:10.1016/j.jsps.2022.01.017

16. Osamor PE, Grady C. Women's autonomy in health care decision-making in developing countries: a synthesis of the literature. Int J Womens Health. 2016;8:191-202. doi:10.2147/ijwh.s105483

17. Tommasello AC. Substance abuse and pharmacy practice: what the community pharmacist needs to know about drug abuse and dependence. Harm Reduct J. 2004;1(1):3. doi:10.1186/1477-7517-1-3

18. Brown MT, Bussell JK. Medication Adherence: WHO Cares? Mayo Clin Proc. 2011;86(4):304-14. doi:10.4065/mcp.2010.0575

19. Manchanayake MGCA, Bandara GRWSK, Samaranayake NR. Patients' ability to read and understand dosing instructions of their own medicines - a cross sectional study in a hospital and community pharmacy setting. BMC Health Serv Res. 2018;18(1):425. doi:10.1186/s12913-018-3252-1

20. Fitzgerald RJ. Medication errors: the importance of an accurate drug history. Br J Clin Pharmacol. 2009;67(6):671-5. doi:10.1111/j.1365-2125.2009.03424.x

21. Toklu HZ, Hussain A. The changing face of pharmacy practice and the need for a new model of pharmacy education. J Young Pharm. 2013;5(2):38-40. doi:10.1016/j.jyp.2012.09.001

22. Ali S, Shimels T, Bilal AI. Assessment of Patient Counseling on Dispensing of Medicines in Outpatient Pharmacy of Tikur-Anbessa Specialized Hospital, Ethiopia. Ethiop J Health Sci. 2019;29(6):727-36. doi:10.4314/ejhs.v29i6.9

23. Saqib A, Atif M, Ikram R, Riaz F, Abubakar M, Scahill S. Factors affecting patients' knowledge about dispensed medicines: A Qualitative study of healthcare professionals and patients in Pakistan. PLoS One. 2018;13(6):e0197482. doi:10.1371/journal.pone.0197482

24. Bou-Saba AW, Kassak KM, Salameh PR. The current trends and challenges towards good community pharmacy practice and the way forward. Explor Res Clin Soc Pharm. 2022;6:100152. doi:10.1016/j.rcsop.2022.100152

25. McConnell KJ, Delate T, Newlon CL. The sustainability of improvements from continuing professional development in pharmacy practice and learning behaviors. Am J Pharm Educ. 2015;79(3):36. doi:10.5688/ajpe79336

26. Ali HS, Alhadab AS, Mohamed EB, Prajapati SK, Badulla WFS, Alshakka M, et al. Patients' Perspectives on Services Provided by Community Pharmacies in Terms of Patients' Perception and Satisfaction. J Young Pharm. 2019;11(3):279-84. doi:10.5530/jyp.2019.11.56

27. Al-Arifi MN. Patients' perception, views and satisfaction with pharmacists' role as health care provider in community pharmacy setting at Riyadh, Saudi Arabia. Saudi Pharm J. 2012;20(4):323–30. doi:10.1016/j.jsps.2012.05.007

28. Hasan S, Sulieman H, Stewart K, Chapman CB, Hasan MY, Kong DCM. Assessing patient satisfaction with community pharmacy in the UAE using a newly-validated tool. Res Social Adm Pharm. 2013;9(6):841–50. doi:10.1016/j.sapharm.2012.10.002

29. Larasanty LPF, Cahyadi MF, Sudarni NMR, Wirasuta IMAG. Patient satisfaction with pharmaceutical care services provided at primary-level and secondary-level health facilities in Indonesia's health coverage system. J Health Res. 2019;33(1):80–8. doi:10.1108/JHR-06-2018-0033