Vol. 11 No. 2 August 2024

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia

E-ISSN: 2580-8303
P-ISSN: 2406-9388

PUBLISHED BY:
FACULTY OF PHARMACY UNIVERSITAS AIRLANGGA in collaboration with
INDONESIAN PHARMACISTS ASSOCIATION (IAI) OF EAST JAVA

Accredited SINTA 2
No: B/1796/E5.2/KI.02.00/2020

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia

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Jurnal Farmasi dan Ilmu Kefarmasian Indonesia (Pharmacy and Pharmaceutical Sciences Journal) P-ISSN: 2406-9388; E-ISSN: 2580-8303 is an official journal published by the Faculty of Pharmacy, Universitas Airlangga in collaboration with Indonesian Pharmacists Association (IAI) of East Java which the articles can be accessed and downloaded online by the public (open-access journal).

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Jurnal Farmasi dan Ilmu Kefarmasian Indonesia
Pharmacy and Pharmaceutical Sciences Journal
Published by Faculty of Pharmacy Universitas Airlangga
OPEN ACCESS
E-ISSN 2580-8303
P-ISSN 2406-9388

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia Vol. 11 No. 2 August 2024, 128-136
DOI: 10.20473/jfiki.v11i22024.128-136
Available online at https://e-journal.unair.ac.id/JFIKI/

Antioxidant Properties of Various Yacon Leaf Water Extracts and Physicochemical Profile of Decoction During Refrigerated Storage

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Submitted: 27 December 2023
Revised: 3 July 2024
Accepted: 30 July 2024

Abstract

Background*: Yacon (Smallanthus sonchifolius (Poepp.) H.Rob.) leaves show promising antioxidant properties, and have traditionally been used for diabetes management in Baturraden, Banyumas, Central Java, Indonesia. This study evaluated the effects of traditional extraction methods and crude drug-to-solvent ratios on the content and activity of antioxidants and physicochemical properties of yacon leaf water extracts during storage.* ***Methods****: Crude drugs were extracted by infusion, short-time decoction, and longer-time decoction at ratios of 1:10, 1:20, and 1:100 (w/v). Antioxidant content was analyzed using standard total flavonoid content (TFC) and total phenolic content (TPC). The antioxidant activity was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity and ferric-reducing antioxidant power (FRAP) assays. Yacon leaf water extracts at a ratio of 1:20 (w/v) were stored in tightly closed bottles at 4±2°C for 26 days. The organoleptic characteristics, color, pH, TFC, TPC, and DPPH scavenging activity were evaluated on days 0,1, 3, 6, 12, 18, and 26.* ***Results****: The different methods and crude drug-to-water ratios generated different antioxidant activities and contents of the yacon extracts. Yacon leaf decoction for 15 min in a ratio of 1:20 (w/v) produced extract with the best scavenging activity (450.27±5.48 mM Trolox equivalent (TE)/100 g dry weight (DW)), TFC (6.43±0.18 mg Quercetin equivalent (QE)/g DW), and TPC (3.91±0.04 mg gallic acid equivalent (GAE)/g DW). The yacon leaf decoction started to undergo aroma and PH changes on days 3 and 6, respectively. On day 12, the TFC, TPC, and DPPH SA of yacon leaf decoction remained 93.93±3.70, 96.52±1.81, and 89.99±0.91% of the freshly prepared extract, respectively.* ***Conclusion****: Our results suggest that extraction using the decoction method for 15 min at a water-to-crude drug ratio of 1:20 (w/v) generated an extract with the best antioxidant profile, which chemically started to change on day 12 during refrigerated storage.*

Keywords*: antioxidant, extraction, refrigerated storage, Smallanthus sonchifolius, stability*

How to cite this article:
Wicaksana, F., Hartanti, D. & Hamad, A. (2024). Antioxidant Properties of Various Yacon Leaf Water Extracts and Physicochemical Profile of Decoction During Refrigerated Storage. *Jurnal Farmasi dan Ilmu Kefarmasian Indonesia*, 11(2), 128-136. http://doi.org/10.20473/jfiki.v11i22024.128-136

P-ISSN: 2406-9388
E-ISSN: 2580-8303

INTRODUCTION

Yacon (*Smallanthus sonchifolius* (Poepp.) H.Rob.) leaves are traditionally used for diabetes treatment by people in Baturraden, Banyumas, Central Java, Indonesia (Utaminingrum et al., 2020). The antidiabetic activities of yacon leaves have been evaluated in in vitro and in vivo models, with promising results (Aligita et al., 2018; Simamora et al., 2020). Yacon leaves may contribute to the antidiabetic activity via indirect antioxidant mechanisms. The ethanolic extracts of yacon leaves showed considerably high 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity and ferric-reducing antioxidant power (FRAP), which was strongly correlated with total phenolic content (TPC) (Hartanti et al., (2022).).

The extraction method greatly affects the quantity and quality of extracted antioxidant compounds. Optimal extraction conditions enable high yields of bioactive components while maintaining their antioxidant activity. Infusion and decoction are commonly used to prepare traditional herbal formulations. Infusion is a dilute extract prepared by pouring boiling water into crude drugs and straining it to obtain a lukewarm preparation. On the other hand, a decoction is obtained by boiling crude drugs in water on a decoct apparatus for specific times (Sujarwo et al., 2015). Hence, the temperature and time of extraction are different for both the traditional methods. The extraction temperature and time significantly affected the TPC and DPPH scavenging activity of *Clinacanthus nutans* (Sulaiman et al., 2017). Furthermore, different extraction methods result in different bioactivity profiles. The extracts obtained from the infusion of a polyherbal formulation showed a better reduction in blood pressure in hypertensive patients than the decoction (Triyono et al., 2018). Similarly, the ratio of plant material to solvent also affected the extraction efficiency. Generally, a small ratio is associated with the saturation of the solvent by the target compounds, which limits the mass transfer during extraction (Abubakar & Haque, 2020). The Indonesian Herbal Pharmacopeia (IHP) suggests a crude drug-to-solvent ratio of 1:10 (w/v). In contrast, *jamu godhog*, a traditional herbal drink, is prepared by decoction at a ratio of 1:20 (w/v) (Hartanti et al., 2023; Indonesian MoH, 2017).

Both decoction and infusion are traditionally prepared in small quantities for one-day use only, because they are often susceptible to degradation mechanisms influenced by temperature, light, oxygen exposure, and microbial spoilage if kept longer. Storage water extracts at low temperatures might preserve them longer because such conditions slow down the degradation processes (Jovanović et al., (2022)). . Hence, it is essential to understand the changes in pH, color, TPC, TFC, and DPPH scavenging activity during storage, which are crucial for maintaining their stability and shelf life. This study evaluated the effects of traditional extraction methods and crude drug-to-water ratios on DPPH scavenging activity, FRAP, TPC, and total flavonoid content (TFC) of yacon leaves. The physicochemical profile of the extract with the best antioxidant properties was also evaluated during refrigerated storage for 26 days.

MATERIALS AND METHODS

Materials

Reagents such as DPPH, 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ), Folin-Ciocalteu reagent, gallic acid, quercetin, Trolox, acetic acid, aluminum chloride, hydrochloric acid, sodium acetate, sodium carbonate, sodium hydroxide, and solvents, that is, chloroform, deionized water, ethanol, and methanol, were of analytical grade (Sigma, United States). Crude drugs were prepared from mature yacon leaves collected from Sumbang, Banyumas, Central Java, Indonesia. The identity of the plants was confirmed to be *Smallanthus sonchifolius* (Poepp.) H.Rob. (Asteraceae) by Tusrianto, the botanist at the Laboratory of Pharmaceutical Biology, Universitas Muhammadiyah Purwokerto, Banyumas, Central Java, Indonesia (Ref. 272-RDS/(2022).

Method

Extraction

The yacon leaves were dried using the rack-drying method. The crude drugs were pulverized into a fine powder. Powdered crude drugs were extracted with water by infusion and decoction (Abubakar & Haque, 2020). The crude drugs were poured into freshly boiled water (95±2°C) and incubated for 5 min to obtain an infusion. They were boiled in a water bath (set at 100°C) for 15 minutes (Decoction-15) and 30 minutes (Decoction-30) using the decoction method. Three crude drug-to-solvent ratios were used for each extraction method: 1:10, 1:20, and 1:100 (w/v), respectively. The water extract was filtered and used for further antioxidant content and activity analyses.

Antioxidant content determination

TFC and TPC of the extracts were determined according to the compendial methods of the Indonesian Herbal Pharmacopeia (Indonesian MoH 2017). A 0.5 ml of properly diluted extract sample was

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E-ISSN: 2580-8303

homogenously mixed with 1.5 ml of ethanol, 0.1 ml of 10% $AlCl_3$, 0.1 ml of 1M CH_3COONa, and 2.8 ml of water. After standing at room temperature for 30 min, the absorbance of the reaction mixture was read at 426 nm. The absorbance was plotted on a calibration curve (y=0.0055x-0.1342), and the TFC was presented as mg quercetin equivalent (QE)/g DW crude drugs. For TPC evaluation, appropriately diluted extract samples (1.0 mL) were homogenously mixed with 7.5% Folin-Ciocalteu reagent (5.0 mL). The reaction mixture was allowed to stand for 8 min and was subsequently added to 1% NaOH (4.0 mL). After 40 min, the absorbance was recorded at 741 nm and plotted on a calibration curve (y=0.0401x+0.0437). TPC is presented as mg gallic acid equivalent (GAE)/g DW crude drug.

Antioxidant activity evaluation

The DPPH scavenging activity and FRAP of the extracts were analyzed using a previously reported method (Hartanti et al., (2022)). . A 0.5 ml properly diluted extract was homogenously mixed with 25 µg/ml DPPH solution (5.0 mL) in ethanol. After incubating at room temperature, the reaction mixture was read at 517 nm and protected from light for 30 min. The absorbance of each sample was calculated as the inhibitory percentage of the blank. The percentage inhibition was plotted on a calibration curve (y=0.0654x+9.1889), and the DPPH scavenging activity was expressed as mM Trolox equivalent (TE)/100 g DW crude drugs. For FRAP analysis, 0.21 ml of properly diluted extract sample was homogenously mixed with freshly prepared FRAP reagent (4.0 mL). The reaction mixture was allowed to stand for 40 min and the absorbance was recorded at 596 nm. The absorbance of the samples was plotted on a calibration curve (y=0.0401x+0.0437). TPC is presented as mM TE/g DW of crude drugs.

Storage condition

The yacon leaf water extracts in a crude drug-to-solvent ratio of 1:20 *(w/v)* were stored for evaluation of physicochemical properties following a previously reported method (Vongsak et al., 2013). A total of 15 ml of the extract was stored in tightly closed containers at 4±2°C for 26 days.

Physicochemical properties evaluation

Physicochemical properties were evaluated on days 0,1, 3, 6, 12, 18, and 26. Three untrained panelists organoleptically evaluated the taste, aroma, and color of the extracts. The color of the extract was read using a Chroma Meter (Konica Minolta, Japan) and reported as the color distance (Prommachart et al., 2020). The TFC, TPC, and DPPH scavenging activities of the extracts were determined using the same methods used to determine antioxidant content and activity.

Data analysis

The effect of the extraction method and crude drug-to-solvent ratio on the TFC, TPC, DPPH scavenging activity, and FRAP of the extracts was evaluated by two-way ANOVA. The effect of storage time on pH, TFC, TPC, and DPPH SA was analyzed using one-way ANOVA. The mean separation of the variants was evaluated using Duncan's post-hoc test. The correlation between TFC-TPC and DPPH scavenging activity (FRAP) was analyzed using Pearson's correlation test. Significant effects, differences, and correlations were considered at $p < 0.05$. All analyses were conducted utilizing SPSS ver. 26 (IBM, US).

RESULTS AND DISCUSSION

Infusion and decoction differed according to the heating intensity of the plant materials and target compounds. In this study, contact with the high temperature at the highest intensity occurred in the decoction for 30 min, followed by the decoction for 15 min and infusion. The temperature of the decoction was assumed to be approximately 90°C. Temperature plays a significant role in the extraction process. Heat enhances the extraction efficiency as it improves the solubility of the target compounds in the solvent and modifies the transfer of the compound outside the plant materials. However, heat may cause reactions that eventually lead to the degradation of thermolabile compounds (Abubakar & Haque, 2020). The crude drugs used in this study were characterized, and the TFC and TPC were chosen as the chemical contents of the crude drugs for the standardization process (Hartanti et al., (2022).).

Both the extraction method and crude drug-to-solvent ratio affected the extracted flavonoids in yacon leaf crude drugs, with decoction for 30 min of 100 parts of crude drugs in a part of water generated the extract with the highest TFC (9.08±0.08 mg QE/g DW). Similarly, the extraction method and ratio significantly affected the TPC of crude drugs. Infusion and short-time decoction in a ratio of 1:20 *(w/v)* generated the highest extracted phenolic compounds from yacon leaf crude drugs, with values of 6.06±0.37 and 3.91±0.04 mg GAE/g DW, respectively (Figure 1).

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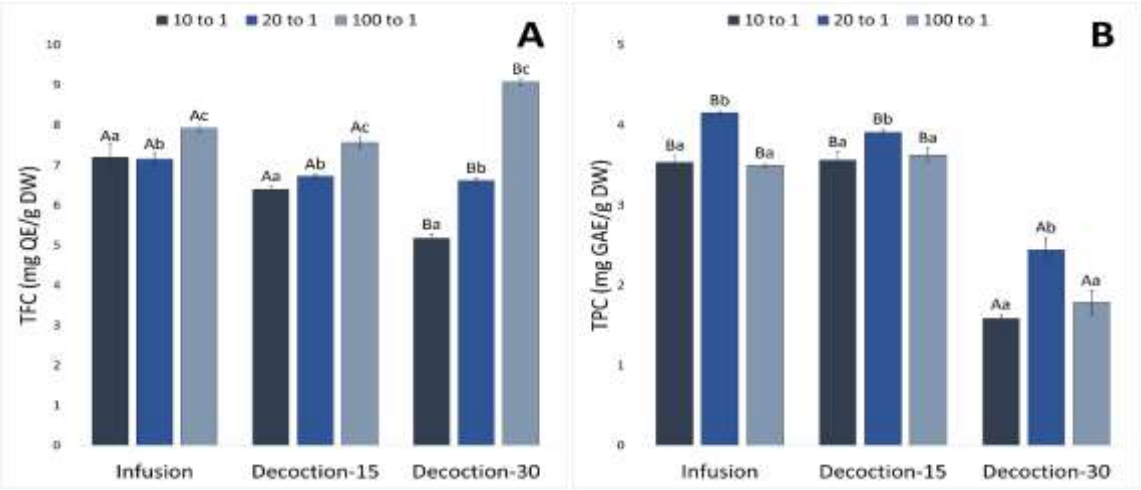

Figure 1. TFC (A) and TPC (B) of crude yacon leaf drugs. Ten to 1, 20 to 1, and 100 to 1 represent the crude drug-to-solvent ratios of 1:10, 1:20, and 1:100 (w/v), respectively. The different uppercase and lowercase alphabets on each bar represented significantly different values by extraction method and crude drug-to-solvent ratio, respectively (n = 3)

Considerably large quantities of apigenin, luteolin, myricetin, and rutin, both in free and bound forms, have been identified in yacon leaves (Khajehei et al., 2017; Padilla-González et al., 2020; Russo et al., 2015 Russo et al., 2015). The higher TFC of extracts from the decoction for 30 min resulted from the extended contact between the plant materials and heat. This result was similar to that of a study that reported that a longer extraction time resulted in mangosteen leaf extract with a higher flavonoid content (Rusli et al., 2024). Our results also indicate that the extracted flavonoids are likely to be stable under these conditions. Hence, our results are similar to those of *Actinidia arguta* and *Actinidia deliciosa* fruits, in which extracts obtained from infusion contained lower TFC than the decoction (Silva et al., 2019). However, longer contact with heat during decoction resulted in more flavonoid degradation. After heating at 90°C for 15 and 30 min in a food heat-treatment model, rutin was degraded by approximately 15% and 25%, respectively (Ioannou et al., 2020). Compared to ethanol extracts from an ultrasonic-assisted process of the same crude drugs in the same crude drug-to-solvent ratio, yacon water extract contained a lower TFC (Hartanti et al., (2022)). . Our results suggested that extraction using a crude drug-to-solvent ratio of 1:100 (w/v) resulted in the highest flavonoid-containing extracts, regardless of the method used. Higher flavonoid content in extracts obtained from a higher ratio of crude drug-to-solvent has also been reported in maceration-processed *Moringa oleifera* seeds, *Rosa canina*, *Hippophae rhamnoides*, and *Crataegus monogyna* fruit extracts (Ghafar et al., 2017; Predescu et al., 2016).

Several phenolic compounds such as caffeic acid, caffeoylquinic acid, chlorogenic acid, p-coumaric acid, and ferulic acid have been identified in considerable quantities in yacon leaves (Khajehei et al., 2017; Padilla-González et al., 2020; Russo et al., 2015; Russo et al., 2015). Similar to flavonoids, the extraction of phenolic compounds from plant matrices is also a temperature-sensitive process. The optimum temperature for the extraction of these compounds from *the aerial parts of O. basilicum* and *Robinia pseudoacacia* flowers was 90.7 and 60°C, respectively (Do et al., 2020; Gajic et al., 2019). However, our yacon leaf extract results showed that contact with heat at approximately 90°C for 30 min resulted in the highest TPC-containing extracts, comparable to its ethanol counterpart (Hartanti et al., (2022)). . A higher TPC in extracts from decoctions over infusions has also been reported in *Actinidia arguta* and *Actinidia deliciosa* fruits and *Centella asiatica* aerial parts (Silva et al., 2019; Zainal et al., 2019). Extraction using a crude drug-to-solvent ratio of 1:20 (w/v) is commonly used to prepare *jamu gendong*, which showed the highest TPC. Hence, our TFC results support the traditional preparation methods.

Both the extraction methods and ratios affected the scavenging activity and FRAP of yacon leaf crude drugs. 30-min decoctions in crude drug-to-solvent ratio of 1:20 (w/v) produced extract with the best DPPH scavenging activity, with the value of 450.27±5.48 mM TE/100g DW. On the other hand, decoction of the crude drugs for 15 min produced FRAP of 101.73±1.04 mM TE/100g DW (Figure 2).

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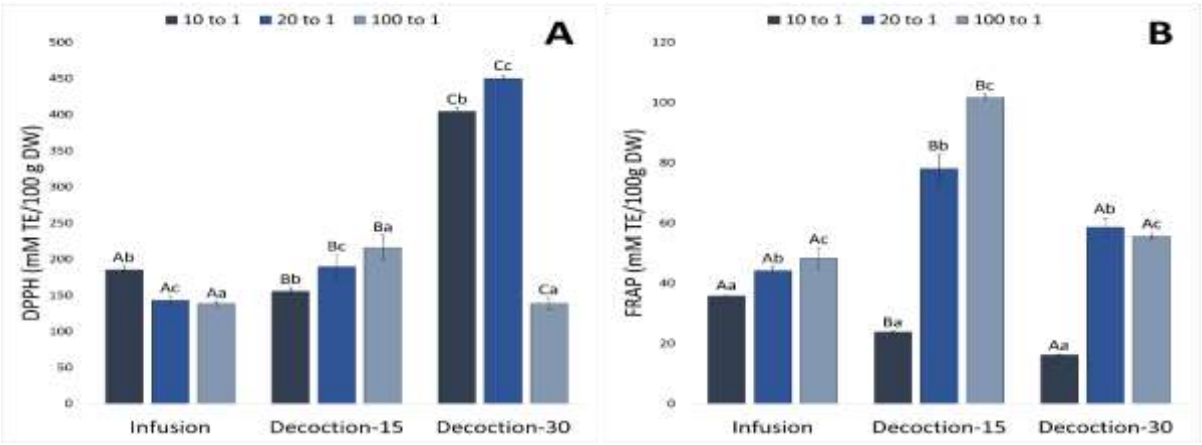

Figure 2. DPPH scavenging activity (A) and FRAP (B) of crude yacon leaf drugs. Ten to 1, 20 to 1, and 100 to 1 represent the crude drug-to-solvent ratios of 1:10, 1:20, and 1:100 (w/v), respectively. The different uppercase and lowercase alphabets on each bar represented significantly different scavenging activity by extraction method and crude drug-to-solvent ratio, respectively (n = 3)

Longer extraction times and higher extraction temperatures generally increased the extraction of antioxidant compounds. However, antioxidant activity might decrease with increasing temperature. For example, the optimum temperature for preserving the antioxidant flavonoids in *Dryopteris erythrosora* leaves was 75°C (Zhang et al., 2019). The antioxidant activity of water extracts obtained from infusions and decoctions and the solvent-to-solid ratio can vary depending on several factors. Some plant infusions showed higher activity, whereas others showed higher free scavenging activity. For example, the *Thymus sipyleus* aerial part infusion was much higher than that of its decoction counterpart (Ustuner et al., 2019). The same trend was observed in *Ayapana triplinervis, Dodonaea viscosa, Hubertia ambavilla,* and *Pelargonium graveolens*. However, comparable DPPH scavenging activity was shown by infusion and decoction of *Aphloia theiformis, Hypericum lanceolatum, Psiloxylon mauritianum,* and *Syzygium cumini* (Checkouri et al., (2022).). FRAP of decoction of *Lavandula angustifolia* and *Lavandula x intermedia* was also significantly higher than that of their infusion (Dobros et al., 2022).). Similar results were also demonstrated in Morrocan-originated *Haloxylon scoparium* aerial parts (Lachkar et al. 2021).

Correlations between antioxidant content and activity varied from none to strong, both positive and negative. Strong positive correlations were observed between TPC and DPPH scavenging activity in extracts obtained from decoctions of all evaluated crude drug-to-solvent ratios. In contrast, TPC and FRAP in yacon leaves were observed in the short-term decoction at a ratio of 1:100 *(w/v).* On the other hand, TFC and DPPH scavenging activities were strongly correlated in the 15-min decoction, while those of TFC and FRAP were observed in extracts from the 15-min decoction at ratios of 1:10 and 1:100 *(w/v)* (Table 1).

Table 1. Correlation between antioxidant content and antioxidant activity of yacon leaf extracts

Method or ratio	Content	Pearson's correlation coefficient	
		DPPH	FRAP
Infusion	TPC	-0.437	0.214
	TFC	-0.516	0.623
Decoction-15	TPC	0.807*	0.809*
	TFC	0.890*	0.878*
Decoction-30	TPC	0.957*	-0.034
	TFC	-0.872*	0.736*
1:10 (w/v)	TPC	0.950*	-0.901*
	TFC	-0.856*	0.945*
1:20 (w/v)	TPC	0.973*	-0.165
	TFC	-0.721*	-0.630
1:100 (w/v)	TPC	0.966*	0.997*
	TFC	-0.622	-0.578

The asterisk indicates a significant correlation between antioxidant content and activity

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Table 2. Organoleptic characters of yacon leaf decoctions during storage

Aspect`	Day						
	0	**1**	**3**	**6**	**12**	**18**	**26**
Aroma	Grassy, unpleasant, aromatic	Grassy, unpleasant, aromatic	Grassy, unpleasant, aromatic	Grassy, unpleasant, aromatic	Grassy, unpleasant, aromatic	Grassy, unpleasant, aromatic	Grassy, unpleasant, aromatic
Taste	Bitter +, astringent +	Bitter +, astringent +	Bitter ++, astringent ++	Bitter ++, astringent ++	Bitter +++, astringent ++	Bitter +++, astringent ++	Bitter +++, astringent ++
Color	Dark green	Dark green	Dark green	Dark green	Dark green	Dark green	Dark green

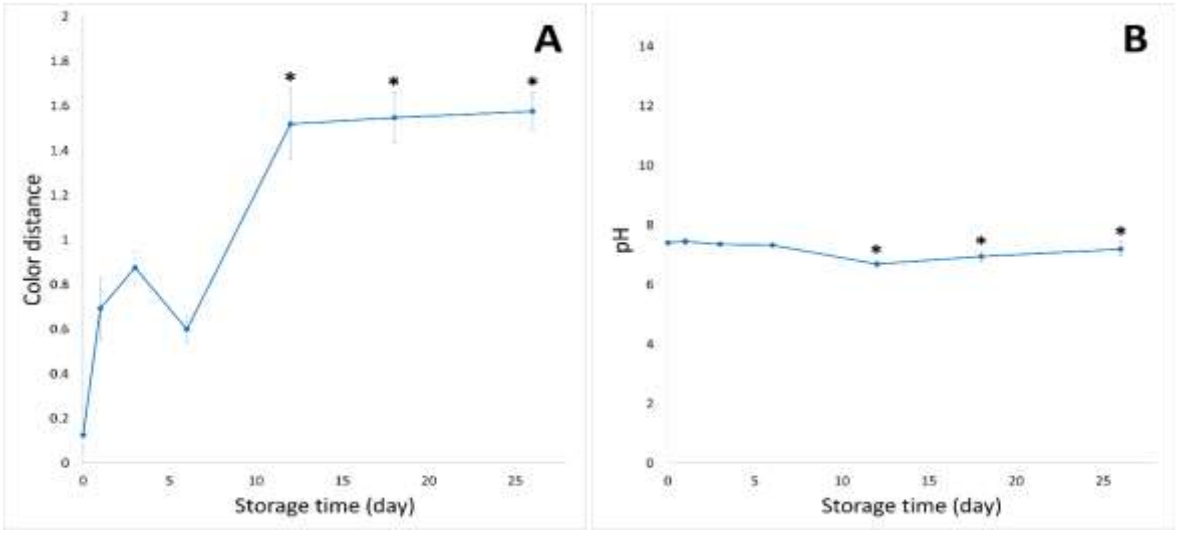

Figure 3. Color distance (A) and pH (B) of the yacon leaf decoction during refrigerated storage. The asterisk indicated a significantly different value from that of the freshly prepared extract (n = 3)

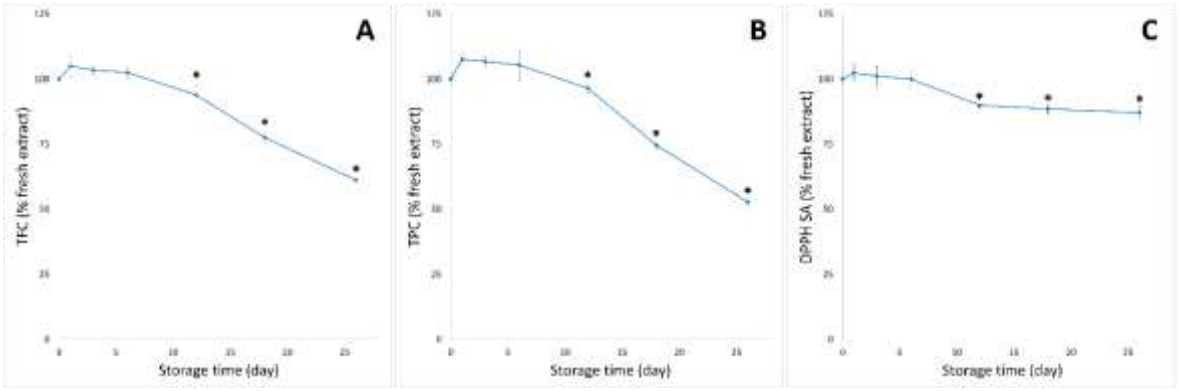

Figure 4. TFC (A), TPC (B), and DPPH scavenging activity (C) of the yacon leaf decoction during refrigerated storage. The asterisk indicated a significantly different value from that of the freshly prepared extract (n=3)

Flavonoids and phenolic compounds in the yacon leaf and Malayan cherry fruit contributed significantly to their antioxidant activities. The phenolic groups of these compounds enable the transfer of hydrogen atoms, while their single electrons scavenge free radicals. Both mechanisms were evaluated using the DPPH scavenging activity assay. The double bonds of these compounds facilitated single-electron transfer, which was exclusively determined in FRAP assays (Santos-Sánchez et al., 2019). The same correlation between these parameters has been previously reported in germany-grown yacon tubers (Khajehei et al., 2018).

The yacon leaf decoction occurred as a dark green liquid with a grassy, unpleasant, aromatic odor, and bitter and astringent taste. The bitterness and astringency of the extracts increased with the storage time (Table 2). The yacon leaf decoction undergoes a slight color change during storage. In contrast, the pH began to decrease on day 12 (Figure 3).

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E-ISSN: 2580-8303

The profiles of TFC, TPC, and DPPH scavenging activity of the yacon leaf decoction during storage were similar. The TFC of the yacon leaf decoction started to decrease on day 12, remaining at 93.93±3.70% of the fresh extracts. Similarly, the TPC and DPPH scavenging activity also began to decrease at day 12, with 96.52±1.81 and 89.99±0.91% of the new counterpart, respectively.

Changes in the color of the decoction represented changes due to chemical reactions or degradation processes of the compounds. For instance, phenolic compounds in the extract may be oxidized or condensed, leading to changes in color. In addition, exposure to light and oxygen might degrade certain phenolic compounds, resulting in pigments altering the color of the solutions. However, a decrease in pH might occur due to the degradation of compounds. This phenomenon has been observed in apple and chokeberry liqueurs (Petrović et al. 2021). The degradation of phenolic compounds and flavonoids may be responsible for the changes in the color and pH of the decoction. During storage, these compounds may undergo various reactions under exposure to light, oxygen, enzymatic activity, and interactions with other components present in the water extract. Degradation of flavonoids and phenolic compounds and decreasing antioxidant activities during refrigerated storage were observed in watermelon and carrot juices (Hwang et al., 2023; Salin et al., (2022).

CONCLUSION

The traditional extraction method and crude drug-to-water ratio significantly affected the TFC, TPC, DPPH scavenging activity, and FRAP of the yacon leaf water extracts. Extraction by decoction for 15 min at a crude drug-to-solvent ratio of 1:20 (w/v), as in the traditional preparation of *jamu*, generated yacon leaf extracts with the best antioxidant properties. The storage time affected the physicochemical properties of the yacon leaf decoction, in which changes in physicochemical parameters started to be noticeable on day 12. It is recommended to store the yacon leaf water extract under refrigerated storage and consume it within a week.

ACKNOWLEDGMENT

The authors acknowledge Majelis Pendidikan Tinggi, Penelitian, dan Pengembangan Pimpinan Pusat Muhammadiyah for funding this study through Hibah Fundamental RisetMu batch VI.

AUTHOR CONTRIBUTIONS

Conceptualization, D.H., A.H.; Methodology, D.H., A.H.; Validation, D.H., A.H.; Formal Analysis, A.H., D.H..; Investigation, F.W.; Resources, D.H., A.H.; Data Curration; D.H., A.H., F.W.; Writing - Original Draft, D.H.; Writing - Review & Editing, DH, A.H., F.W.; Visualization, D.H.; Supervision, D.H., A.H.; Project Administration, D.H.; Funding Acquisition, D.H., A.H.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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P-ISSN: 2406-9388
E-ISSN: 2580-8303

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P-ISSN: 2406-9388
E-ISSN: 2580-8303

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P-ISSN: 2406-9388
E-ISSN: 2580-8303

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia
Pharmacy and Pharmaceutical Sciences Journal
Published by Faculty of Pharmacy Universitas Airlangga
OPEN ACCESS
E-ISSN 2580-8303
P-ISSN 2406-9388

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia Vol. 11 No. 2 August 2024, 137-146
DOI: 10.20473/jfiki.v11i22024.137-146
Available online at https://e-journal.unair.ac.id/JFIKI/

Application of the Simplex Lattice Design Method to Determine the Optimal Formula of Diclofenac Sodium Nanoemulsion

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Submitted: 18 May 2024
Revised: 27 June 2024
Accepted: 8 July 2024

Abstract

Background*: The success of nanoemulsion preparation, with the aim of producing good characteristic values, is determined by the ratio of each component. The design of experiments (DoE) approach using the Simplex Lattice Design (SLD) method can be used to determine the optimal formula for nanoemulsions, with variable factors consisting of oleic acid, Tween 20:ethanol (4:1), and water. The observed response variables included droplet size, PDI, and pH.* ***Objective****: DoE can help reduce the energy, cost, and time needed to make the optimal formula for diclofenac sodium nanoemulsions.* ***Methods****: Nanoemulsions were prepared using low-energy emulsification. Their characteristics were evaluated and analyzed using Design Expert software.* ***Results****: The optimal nanoemulsion formulation consisted of 4.17% oleic acid, 37.5% emulsifier (Tween 20: ethanol, 4:1), and 58.33% water. The nanoemulsion characteristics were good, with 20.37 a droplet size, 0.42 PDI, of 4.75 pH. The observed values were not significantly different from the predicted values, and the formula could effectively trap 1% diclofenac sodium.* ***Conclusion****: The simplex lattice design method is very useful for pharmaceutical development, such as nanoemulsion optimization.*

Keywords*: diclofenac sodium, nanoemulsion, design of experimental, simplex lattice design, optimizing formula*

How to cite this article:
Nahdhia, N., Rijal, M. A. S., Hendradi, E. & Widodo, R. T. (2024). Application of the Simplex Lattice Design Method to Determine the Optimal Formula of Diclofenac Sodium Nanoemulsion. *Jurnal Farmasi dan Ilmu Kefarmasian Indonesia*, 11(2), 137-146. http://doi.org/10.20473/jfiki.v11i22024.137-146

P-ISSN: 2406-9388
E-ISSN: 2580-8303

INTRODUCTION

Nanoemulsion is a novel drug delivery system consisting of water and oil phases stabilized by an emulsifier, which is a combination of a surfactant and cosurfactant. The aim is to reduce the surface tension to obtain nanoscale droplet sizes (10-100 nm). Because of their small size, nanoemulsions can be thermodynamically stable with transparent, monophasic, and low viscosity characteristics (Donthi et al., 2023; Nastiti et al., 2017). Nanoemulsions have recently become a research topic of great interest owing to their high stability, ease of manufacture, and ability to increase the bioavailability of hydrophobic drugs (Jadhav et al., 2020; Shaker et al., 2019).

The manufacturing method and the ratio of each component greatly influence the success with good characteristic values of nanoemulsions, such as transparency, small droplet size, high droplet homogeneity, and appropriate pH. An experimental design-based approach can help obtain the optimal dosage formula from the nanoemulsion to reduce the costs and time required.

The simplex lattice design (SLD) method can be used to obtain an optimal formula with a proportion of the total number of ingredients of one (100%). The maximum and minimum limits for each factor (Hidayat et al., 2020) in the nanoemulsions were determined from the pseudo-ternary diagram (Duangjit et al., 2014). SLD has succeeded in designing optimal formulas for ketoconazole microemulsions (Duangjit et al., 2014), p-coumaric acid microemulsions (Nasser et al., 2024), andrographolide SNEEDS (Indrati et al., 2020), and furosemide SNEEDS (Fithri et al., 2017). Using this method, we can analyze the influence of each component as a causal factor on the response variable.

In this study, the active ingredient used is diclofenac sodium (DS), which has low solubility (partition coefficient 13.4). Diclofenac sodium is an NSAID that inhibits prostaglandin synthesis as an inflammatory agent by inhibiting COX-1 and COX-2 enzymes (Hendradi et al., 2021). Diclofenac sodium has disadvantages, such as first-pass metabolism, and long-term use causes ulcers and stomach bleeding (Hendradi et al., 2017; Latifah et al., 2023; Md et al., 2020; Sacha et al., 2019). In this study, an O/W nanoemulsion of diclofenac sodium was formulated to increase its solubility and bioavailability and reduce its side effects using the simplex lattice design method to determine the most optimal formula.

MATERIALS AND METHODS

Materials

Diclofenac sodium was provided by PT Dexa Medica (Indonesia); oleic acid and Tween 20 were purchased from PT Brataco (Indonesia); absolute ethanol was purchased from Merck (Germany); and distilled water. All excipients were of pharmaceutical grade.

Tools

Design-Expert software version 13, a UV-Vis spectrophotometer (Hitachi UH5300, Japan), a particle size analyzer (Delsa^{TM} Nano C, US), and a pH meter (Eutech pH 700, US).

Methods

Preparation of pseudo-ternary phase diagram

An aqueous titration method was adopted to develop a pseudo-ternary phase diagram to draw the nanoemulsion region and define the concentration ratios of the individual components. Tween 20 as a surfactant and ethanol as a co-surfactant, at a ratio of 4:1, were added to oleic acid at different weight ratios. The mixture was then stirred gently for 5 min. The aqueous phase was added dropwise with vigorous stirring. The preparations obtained were observed visually; preparations with a clear appearance and easy-to-flow were categorized as nanoemulsions (Gul et al., 2022).

Determining the optimal formula of nanoemulsion with SLD

The largest area that formed an equilateral triangle was determined from the nanoemulsion area in the pseudoternary diagram. The upper and lower limits of each component were input into Design-Expert software using the SLD method. SLD forms 14 formulas with different component ratios, which are then incorporated into nanoemulsion systems. Each formula was then tested for its characteristics, including droplet size (Y_1), PDI (Y_2), and pH (Y_3). The optimal formula was selected based on the specified acceptance criteria, namely, maximum oil, minimum Smix, minimum droplet size, PDI <0.5, and pH 2-8.

Preparation of diclofenac sodium-loaded nanoemulsion

The optimal blank nanoemulsion formula based on the SLD results was used to trap 1% diclofenac sodium. Diclofenac sodium was added to the mixture of oil and the co-surfactant until it dissolved. The surfactant was then added and the mixture was stirred. Water was then added dropwise (1.000 rpm, 30 min).

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Characterization of nanoemulsion

a) Droplet size and PDI

A nanoemulsion sample diluted with aquadest was placed into a cuvette using a particle analyzer (Delsa^{TM} Nano C, US). The data (output) are the droplet size values calculated from the average fluctuation of the light scattering intensity and PDI, which describes the particle size distribution.

b) pH value

The electrode of the pH meter was submerged in the sample by dipping. The pH result was denoted by the value displayed on the instrument.

c) Percent transmittance

The percent transmittance (%T) of the nanoemulsions was measured using a UV-Vis spectrophotometer at 650 nm with distilled water as a blank.

d) Viscosity

Using an Ostwald viscometer, a 5 mL sample was inserted into the viscometer. Using a filler pipette, the sample fluid was sucked until it was slightly above the top mark on the capillary tube. The time required for the liquid to flow from the top to the bottom of the viscometer was recorded. The viscosity value was calculated using the following formula (Poggio et al., 2015):

$$\eta_{sample} = \frac{\eta_{water} \times \rho_{sample} \times t_{sample}}{\rho_{water} \times t_{water}}$$

η = viscosity (mPa.s)
ρ = density (g/mL)
t = The time for the liquid to flow from the top to the bottom mark (s)

RESULTS AND DISCUSSION

Preparation of pseudo-ternary diagram

In the oil:Smix ratio from 1:1 to 1:7 until the addition of 100% distilled water, a cloudy and thickened preparation was formed (Table 1). This is due to the lack of Smix, which reduces the surface tension between oil and water. In oil:Smix 1:8, a visual change occurred after the continuous addition of distilled water (up to 100%) from cloudy to translucent with a blue glint (Figure 1 F8). However, we did not categorize this preparation as a nanoemulsion because according to Jintapattanakit (2018), colloidal dispersions with this appearance have a droplet size of >100 nm and a transmittance of <90%. The ratio of oil to mix used was 1:9 (Table 2).

Table 1. The appearance of preparation with ratio of oil:Smix 1:1 until 1:9

Formula	Oil (g)	Smix (g)	Smix (surf : co-surf)	Appearance	Transmittance
F1	1	1		Cloudy	
F2	1	2		Cloudy	
F3	1	3		Cloudy	
F4	1	4		Cloudy	<5 %
F5	1	5	4 : 1	Cloudy	
F6	1	6		Cloudy	
F7	1	7		Cloudy	
F8	1	8		Translucent with blue glint	73.40 ± 0.26 %
F9	1	9		Transparent	99.23 ± 0.21 %

Figure 1. The appearance of preparation with ratio of oil:Smix 1:1 until 1:9

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Table 2. The formula of nanoemulsion which can produce a nanoemulsion region

Oil (g)	Smix (g)	Water (g)
1	9	14 - 41
1	10	14 - 56
1	11	16 - 71
1	12	16 - 101
1	13	16
1	14	18

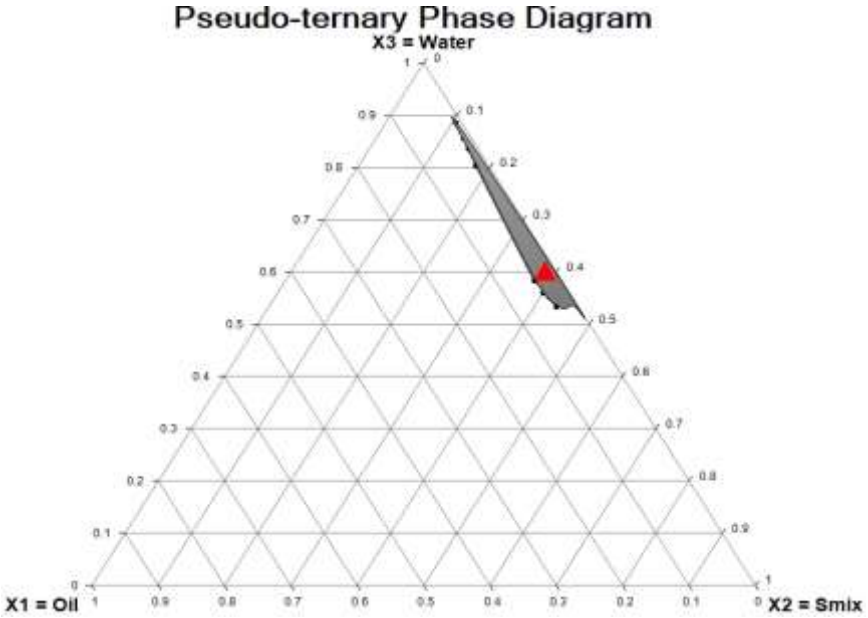

Figure 2. The pseudo-ternary phase diagram of oil (oleic acid), Smix (Tween 20-Ethanol 4:1), and water

Table 3. The characterization of nanoemulsions in determined formulas by SLD

Formula	X_1 Oil (%)	X_2 Smix (%)	X_3 Water (%)	Y_1 Droplet Size (nm)	Y_2 PDI	Y_3 pH
1	1.00	37.50	61.50	19.90	0.40	5.12
2	2.59	39.09	58.33	11.20	0.05	4.81
3	3.11	38.03	58.86	20.10	0.34	4.87
4	1.00	40.67	58.33	11.40	0.09	5.19
5	4.17	37.50	58.33	17.60	0.46	4.78
6	1.00	39.09	59.92	10.40	0.08	5.05
7	1.53	39.61	58.86	16.50	0.17	5.02
8	2.06	38.56	59.39	14.80	0.13	4.90
9	1.00	37.50	61.50	19.60	0.22	5.06
10	4.17	37.50	58.33	18.80	0.43	4.88
11	1.53	38.03	60.44	16.70	0.37	4.92
12	1.00	40.67	58.33	8.90	0.04	5.20
13	2.59	37.50	59.92	18.30	0.49	4.84
14	2.59	39.09	58.33	18.30	0.30	4.82

P-ISSN: 2406-9388
E-ISSN: 2580-8303

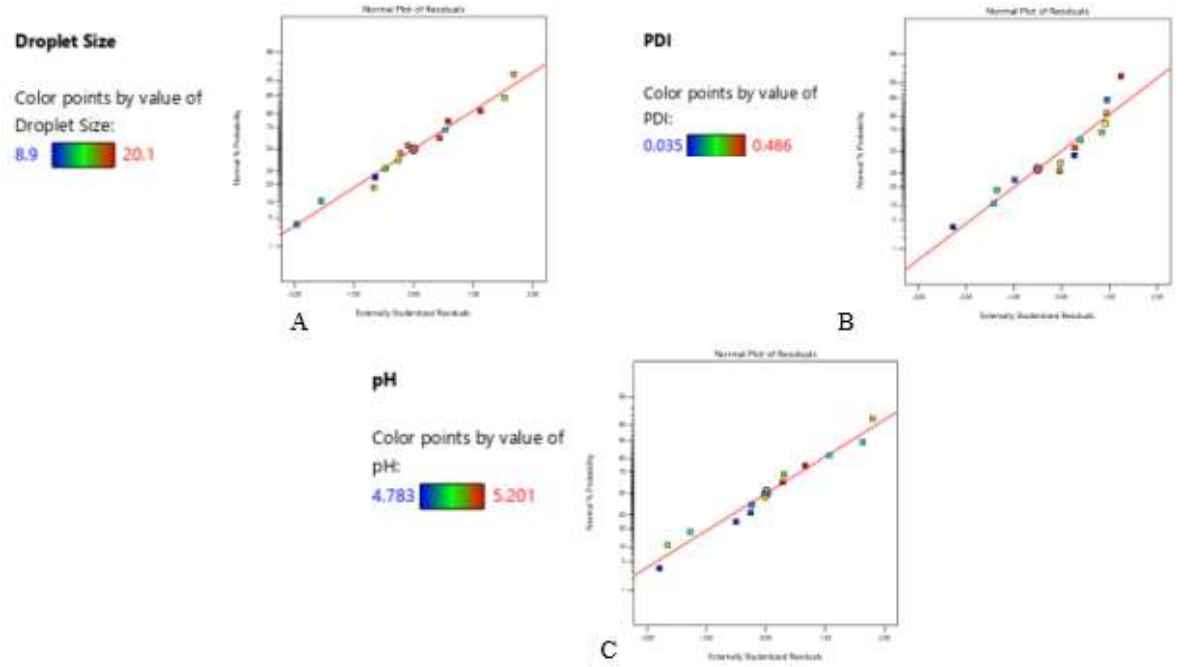

Figure 3. Normal plot of residuals of responses: (A) droplet size, (B) PDI, and (C) pH

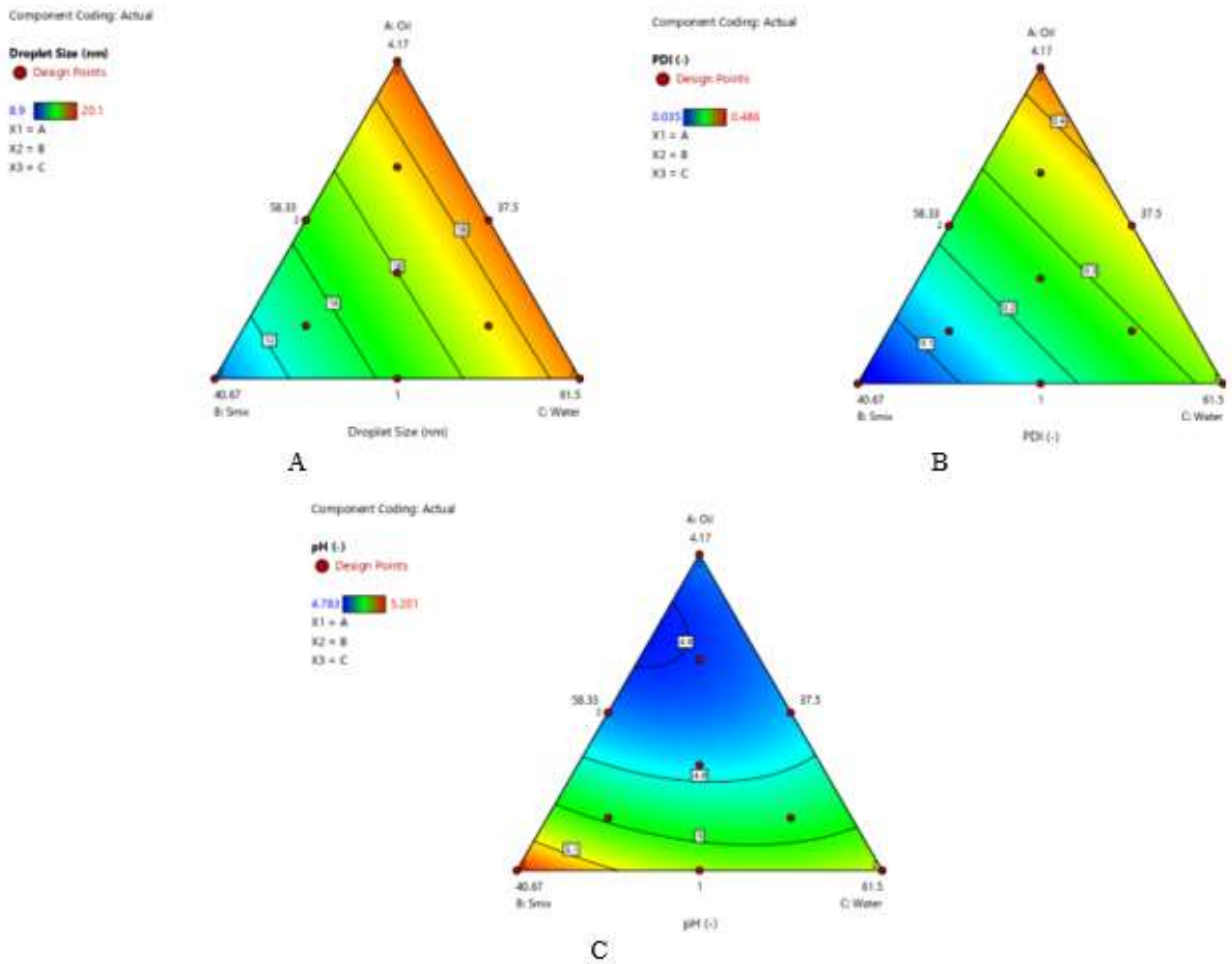

Figure 4. Model graph of nanoemulsion characteristics: (A) droplet size, (B) PDI, and (C) pH

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Table 4. Analysis of variance and lack of fit tests of the model for the responses

Responses	Range	Model	Regression equation	*p*-value		Lack of fit (*p*-value)	
Y_1 (droplet size)	8.9 – 20.1 nm	Linear	Y = 19.01A + 10.27B + 18.68C	0.00	Significant	0.63	Not significant
Y_2 (PDI)	0.035 – 0.486	Linear	Y = 0.4346A + 0.0132B + 0.3232C	0.00	Significant	0.72	Not significant
Y_3 (pH)	4.78 – 5.20	Quadratic	Y = 4.84A + 5.19B + 5.08C – 0.72AB – 0.40AC – 0.31BC	0.00	Significant	0.36	Not significant

Table 5. Summary of the regression analysis of the responses

Responses	R^2	Adjusted R^2	Predicted R^2	The different between adjusted R^2 and predicted R^2 (must be <0.2)
Y1 (droplet size)	0.6412	0.5759	0.4894	0.0865
Y2 (PDI)	0.7043	0.6505	0.5534	0.0971
Y3 (pH)	0.9362	0.8963	0.8058	0.0865

The ratios of each component were plotted in a pseudo-ternary phase diagram using ProSim Ternary software. The gray areas represent the nanoemulsion regions. From this area, an equilateral triangular area (red area) (Fig.2) was selected as the upper and lower boundaries of each component, which was used as the ratio of the independent variable in the SLD method, and the following equation was obtained:

$1 \leq X_1 \leq 4.17$

$37.5 \leq X_2 \leq 40.67$

$58.33 \leq X_3 \leq 61.33$

$X_1 + X_2 + X_3 = 100\%$

Characterizations of nanoemulsion

The normal curve plot of the residual analysis (Fig.3) showed that the data for the three response variables were normally distributed because the data were spread around the diagonal line and followed the direction of the diagonal line; therefore, it was continued with ANOVA analysis (Annisa, 2021). The characterization data showed a good relationship between factors and response variables, marked by the *p*-value of the model, which was significant ($p<0.05$), and the lack of fit was not significant ($p>0.05$) for all responses (Table.4), especially for the pH response, which has a value of R^2 approaching 1 (Table.5).

(a) Droplet size

The droplet size of nanoemulsions ranges from 10-100 nm (Nastiti et al., 2017). In the nanoemulsion formula, the droplet size was 8.9 – 20.1 nm (Table.4). Based on the regression equation (Table.4), oleic acid was the most dominant factor affecting the droplet size. Oleic acid induced an increase in particle size, whereas Smix induced a decrease in particle size (Fig.4 A)**.** When the proportion of oil increases, the droplet size also increases owing to the expansion of nanoemulsion droplets; therefore, the proportion of Smix decreases. With increasing Smix, the nanoemulsion droplet size decreases because Smix can reduce the surface tension between oil and water and produce smaller droplet sizes (Ahmed et al., 2022; Bashir et al., 2021).

(b) PDI

The PDI value of a good preparation was <0.5 (Bashir et al., 2021). The nanoemulsions produced PDI values between 0.035 and 0.486. Based on the regression equation (Table.4), the oil factor influences the PDI value more than the Smix and water factors. Large amounts of oil reduce the proportion of Smix so that its ability to reduce surface tension is reduced, and fewer homogeneous droplets are produced (Bashir et al., 2021). A low PDI occurred when the number of Smix increased (Fig.4 B).

(c) pH

The nanoemulsion formula produced a pH between 4.78 and 5.20 with normally distributed data (Fig.3). The regression equation for the pH response model is quadratic (Table.4). This implies that the response is not only influenced by each factor, but also by the presence of a mixed interaction between the two factors. The dominant factor affecting the pH value was Smix, as indicated by the regression equation. In addition, pH is influenced by the interaction between two factors: oleic acid – Smix (A–B) (sig. 0.0014). The pH response is not influenced by the interaction between oleic acid – water (A–C) (sig. 0,0589), or Smix – water (B–C) (sig. 0.1251). The pH value of the nanoemulsion can be increased by Smix, whereas it can be decreased by oleic acid (Fig.4 C).This is based on the materials' pH, where oleic

P-ISSN: 2406-9388
E-ISSN: 2580-8303

acid has a pH of 4.32 ± 0.16, Tween 20 7.39 ± 0.03 (Rowe et al., 2012), and ethanol 7.32 ± 0.12. A low oleic acid pH can reduce the pH value when oleic acid levels are high.

Determination of the optimal formula

SLD determined the optimal formula of nanoemulsion based on pre-arranged acceptance criteria, namely the maximum amount of oil (to increase the ability to dissolve diclofenac sodium), minimum Smix (reduces irritation), water within range, minimum droplet size, PDI <0.5, and the pH in the range (because it corresponds to the stable pH of diclofenac sodium, namely 2-8 (Manjunatha et al., 2007)). The predicted optimal formula was 4.17% oil, 37.5% Smix (4:1), and 58.33% water with a high desirability value of 0.965 (Table.6). A high desirability value (close to 1) indicates that the formula can satisfy the desired criteria for all responses with a high level of compliance.

Verify the optimal formula

The verification results of the optimal formula showed no significant difference (P > 0.05) between the predicted and observed values (Table.7). These results indicate the validity of the proposed model (Annisa, 2021).

The characterizations of diclofenac sodium-loaded nanoemulsion

(a) Organoleptic

The nanoemulsion formed in the blank and DS-loaded nanoemulsion produced a transparent, non-separation, liquid (easily flowing), with a slightly bright yellow color (Tab.8). This characteristic indicates that the nanoemulsion has small droplets with high stability because of the working mechanism of surfactants and co-surfactants, which prevents destabilization mechanisms, including flocculation, coalescence, Ostwald ripening, and creaming (Donthi et al., 2023; Nastiti et al., 2017).

(b) Droplet size

The small droplet size (nanoscale) increases the surface area to release a higher drug content at the target location. The manufacturing process and components of each nanoemulsion can influence the droplet size. In this study, the method for preparing nanoemulsions was low-energy. According to Santana et al. (2013), the low-energy emulsification method has advantages over high-energy emulsification, because it can produce smaller droplet sizes and higher stability. High levels of surfactant and co-surfactant also influence the droplet size with a working mechanism, namely, providing a mechanical barrier and reducing the surface tension between the adsorbing oil-water interface, thus preventing coalescence. The selection of nanoemulsion components is also an essential factor. Tween 20 (HLB 16.7) was chosen because it has an HLB close to that of oleic acid (HLB 17) (Rao et al., 2015). When the HLB in the emulsification system approaches the value of the HLB of oil, the surfactant molecules are arranged more tightly in the oil-water interfacial film, resulting in greater interfacial film strength and increased electrical repulsion between droplets (Rao et al., 2015). Moreover, the droplet size can be smaller when the surfactant HLB is high (Fadhel & Rajab, 2022).

(c) PDI

The PDI value provides information on the physical stability of the dispersed system. The particle size distribution became more uniform at low PDI values (<0.5). This indicates a more stable system in the long term, as it can prevent flocculation, coalescence, and creaming by reducing the Ostwald ripening rate (Bashir et al., 2021; Nastiti et al., 2017).

(d) pH

The pH of the nanoemulsion formed was at a pH where diclofenac sodium was stable (2-8). The independent t-test showed a significant difference between the blank nanoemulsion and DS-loaded nanoemulsion (Table.8). The pH of the DS-loaded nanoemulsions increased. Diclofenac sodium dissociates in the solution to produce diclofenac and sodium ions (Na^+). Diclofenac ions can bind H^+ ions from the solution, reducing acidity and increasing the pH.

(e) Transmittance

Both the blank and loaded nanoemulsions produced high transmittance, that is, >95% (Table.8), which also aligns with the nanoscale droplet size. The transparency of the system is caused by the dispersed phase droplets being no greater than ¼ of the wavelength of visible light (Sintov & Shapiro, 2004); therefore, the nanoemulsion reflects little light and appears transparent.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Table 6. The optimal formula of nanoemulsion and predicted value selected by SLD

Number	Component (%)			Predicted Value			Desirability	
	Oil	Smix	Water	Droplet Size	PDI	pH		
1	4.17	37.5	58.33	19.01	0.43	4.84	0.965	Selected

Table 7. Comparison of predicted and observed values of optimal formula of nanoemulsion

Responses	Predicted value	Observed value*	*p*-value	
Droplet size (nm)	19.01	20.37 ± 3.80	0.600	Not significant
PDI	0.43	0.42 ± 0.02	0.137	Not significant
pH	4.84	4.75 ± 0.16	0.412	Not significant

*Mean ± SD (n=3)

Table 8. The characterization of blank nanoemulsion and DS-loaded nanoemulsion from optimal formula

Characterization	Blank nanoemulsion	DS-loaded nanoemulsion	*p*-value	
Organoleptic	Transparent, liquid (easy to flow), no separation, light yellow	Transparent, liquid (easy to flow), no separation, light yellow		
Droplet size (nm)	20.37 ± 3.80	16.77 ± 0.84	0.18	Not significant
PDI	0.42 ± 0.02	0.25 ± 0.09	0.04	Significant
pH	4.75 ± 0.16	6.03 ± 0.02	<0.00	Significant
Transmittance (%)	99.20 ± 0.44	98.60 ± 0.69	0.273	Not significant
Viscosity (mPa.s)	76.98 ± 1.60	145.84 ± 3.55	<0.00	Significant

(f) Viscosity

Nanoemulsions are characterized by their low viscosity and easy flow. Low viscosity can accelerate the drug release process at the target site (Bashir et al., 2021), and viscosity is greatly influenced by the components that make up the nanoemulsion. The viscosity test results showed a significant increase in the viscosity of the loaded nanoemulsion compared to the blank because of the presence of diclofenac sodium adsorbed in the core (Tabke.8). However, the DS-loaded nanoemulsion was still a liquid preparation with a viscosity of 145.84 mPa.s and a good droplet size, PDI, and transmittance.

CONCLUSION

This study showed that the blank nanoemulsion was successfully optimized using the simplex lattice design method. The optimal nanoemulsion formula comprised 4.17% oleic acid, 37.50% Smix (Tween 20:ethanol 4:1), and 58.33% water. There was no significant difference between the predicted and observed values, resulting in good characteristic results. This formula has also been successful in loading 1% diclofenac sodium, with good results. This indicates that the simplex lattice design method is advantageous for optimizing nanoemulsion formulations.

ACKNOWLEDGMENT

The authors express their gratitude to the Project Management Unit (PMU) of Maulana Malik Ibrahim State Islamic University for funding this research, which allowed it to be successfully completed.

AUTHOR CONTRIBUTIONS

Conceptualization, N.N., M.A.S.R., E.H.; Methodology, N.N., M.A.S.R., E.H.; Software, N.N.; Validation, N.N., M.A.S.R., E.H.; Formal Analysis, N.N.; Investigation, N.N., E.H.; Resources, N.N., M.A.S.R., E.H; Data Curration; N.N.; Writing - Original Draft, N.N.; Writing - Review & Editing, M.A.S.R., E.H.; Visualization, N.N., M.A.S.R., E.H.; Supervision, M.A.S.R., E.H.; Project Administration, E.H.; Funding Acquisition, N.N., E.H.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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P-ISSN: 2406-9388
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P-ISSN: 2406-9388
E-ISSN: 2580-8303

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia
Pharmacy and Pharmaceutical Sciences Journal
Published by Faculty of Pharmacy Universitas Airlangga
OPEN ACCESS
E-ISSN 2580-8303
P-ISSN 2406-9388

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia Vol. 11 No. 2 August 2024, 147-155
DOI: 10.20473/jfiki.v11i22024.147-155
Available online at https://e-journal.unair.ac.id/JFIKI/

A Comparative Study of Randu Honey Antimicrobial Activity from Several Regions in Java

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Submitted: 21 May 2024
Revised: 11 July 2024
Accepted: 24 July 2024

Abstract

Background*: Randu honey is monofloral honey sourced from a type of plant nectar. The geographical location of randu (Ceiba pentandra) as the source of nectar is one factor that influences the antimicrobial activity of random honey. This research used randu honey from several regions in Java such as Sidoarjo (RSH), Pusat Perlebahan Nasional Bogor (RBH), Kediri (RKH), and Malang (RMH).* ***Objective****: To compare the antimicrobial activity of several random honeys (RSH,RBH,RKH, and RMH) against Gram-negative Escherichia coli ATCC 25922, Gram-positive Staphylococcus aureus ATCC 6538, methicillin-resistant Staphylococcus aureus (MRSA) ATCC 33592, and Candida albicans ATCC 10231.* ***Methods****: This study used well diffusion and dilution antimicrobial test methods. The diameter of the inhibition zone formed by the well diffusion method was measured using a Vernier caliper. The diffusion method was used as a screening test before determining the quantitative minimum inhibitory concentration (MIC) using serial dilution at a ratio1 of 2 (v/v). Streptomycin and Ketoconazole were used as positive controls. Nutrient broth and Sabouraud broth were incubated at 37°C for 24 h (antibacterial tests) and 25°C for 48 h (antifungal test), respectively.* ***Results****: The well diffusion test revealed that all random honey samples could inhibit the test bacteria and fungi with the appearance of an inhibition zone. Diameter inhibition zone ranged from 14.66±0.52 mm to 27.86±0.43 mm. The MICs of RSH,RBH,RKH, and RMH ranged from 3.12% to 25% against all test bacteria and fungi.* ***Conclusion****: The results of this study showed randu honey from Bogor (RBH) has the highest antimicrobial activity based on diffusion and dilution tests.*

Keywords*: antimicrobial activity, dilution, diffusion, MIC, randu honey*

How to cite this article:
Wardani, N. P., Primaharinastiti, R., Poernomo, A. T. & Khatib, A. (2024). A Comparative Study of Randu Honey Antimicrobial Activity from Several Regions in Java. *Jurnal Farmasi dan Ilmu Kefarmasian Indonesia,* 11(2), 147-155. http://doi.org/10.20473/jfiki.v11i22024.147-155

INTRODUCTION

Randu honey is a monofloral type of honey sourced from the dominance of nectar from a random plant (*Ceiba pentandra*). Randu plants grow widely in Asia, especially in Indonesia, Philippines, and Malaysia. The total area of the plantation was 250,500 hectares, and the honey yield ranges from 52,358.74 to 540,227.27 kg/year. Randu honey is a type of honey that is widely produced in Indonesia, especially in the Java region, where around 75% of the total honey is produced by beekeepers in Java (Badan Pusat Statistik, 2018). Randu honey is harvested from farms located around random forests during the flowering season. The best season for harvesting randu honey is from May to October during the flowering period because random nectar content will be abundant. The existence of beekeeping in random forests can help pollination and increase the productivity of random honey by approximately 20-40% (Basuki, 2018).

Randu honey has the physical characteristics of a clear yellowish-brown color, sticky sweet taste with a slightly sour taste, and distinctive aroma. The main substances in randu honey are sugar and other compounds such as water, protein, vitamins, free amino acids, and volatile organic compounds as minor components (Burgut, 2020). These compounds are known to be active compounds that exert antimicrobial activity through different mechanisms. The antimicrobial mechanism of hydrogen peroxide in honey is reactive and can break bonds in the outer membrane of bacteria until lysis. Phenolic compounds found in high amounts in honey contribute to antimicrobial activity via membrane dysfunction and binding to bacterial DNA (Almasaudi, 2021).

The chemical composition determines the quality of randu honey and its antibacterial activity. The antibacterial activity of randu honey is influenced by several factors, including the biochemical profile of the randu plant nectar used as a food source for honey-producing bees. The biochemical profile of nectar is qualitatively and quantitatively influenced by plant genetics and physiology, environmental factors (climatic conditions), soil characteristics, and pollinator bee typology (Kocsis et al., 2022). Dezmirean et al. (2017) and Tomczyk et al. (2019) conducted research on the influence of geographic origin, plant source, and polyphenolic substances on the antimicrobial properties of honey.

Currently, much research on randu honey is limited to its antibacterial activity, such as research regarding the benefits of randu honey as an antimicrobial was carried out by Djakaria et al. (2020) who successfully reported the antimicrobial activity of randu honey from *Apis dorsata* bees from Sumbawa, Riau, Belitung and *Apis cerana* from Sukabumi, Bogor, Banyuwangi against *Propionibacterium acnes*. Research by Hasan et al. (2020) showed that randu honey from Riau has potential as an antimicrobial against *Staphylococcus aureus* and *Eschericia coli*. The growth of *Staphylococcus aureus* and *Eschericia coli* can also be inhibited by administering honey from Bandung (Dewi et al., 2017).

In this research, a comparative study will be carried out on the antimicrobial activity of randu honey from several regions in Java with different geographical conditions such as Sidoarjo (RSH), Bogor National Beekeeping Center (RBH), Kediri (RKH), and Malang (RMH). This location was chosen according to geographical conditions for the growth of randu plants, such as the altitude of the area (Bogor at an altitude of 1600 m above sea level, Malang 760 m above sea level, Sidoarjo 20 m above sea level, and Kediri 350 m above sea level), rainfall, temperature, and air humidity (Widodo et al., 2017). The selection of sampling locations was based on the location of the honey bee farm. Honey bee farms in Bogor and Kediri are managed by the government; therefore, there is guidance regarding the quality of the honey produced. The Malang honey bee farm is owned by a company that has national standards, whereas the Sidoarjo honey bee farm is owned by an individual. This difference can be observed in its influence on the antibacterial activity of randu honey against Gram-negative *E. coli* ATCC 25922, Gram-positive *S. aureus* ATCC 6538, methicillin-resistant *S. aureus* (MRSA) ATCC 33592, and *C. albicans* ATCC 10231.

MATERIALS AND METHODS

Materials

Randu honey samples were obtained from beekeepers at Pusat Perlebahan Nasional Bogor (RBH), Karang Ploso Malang (RMH), Sumber Podang Kediri (RKH), and Sidoarjo (RSH) in May 2022, during the harvest season, as shown in Figure 1. All the samples were stored in amber glass at 4°C until further processing. Nutrient agar (NA) (E.Merck) was used as culture and antibacterial activity test media, Sabouraud Dextrose agar (SDA) (E.Merck) was used as culture and antifungal activity test media, ketoconazole 2%(*w/v*) (Genero) and streptomycin injection 200 mg/mL (Meiji) as positive control, NaCl 0.9% p.a (E.Merck), test microbes *E. coli* ATCC 25922, *S. aureus* ATCC 6538,

P-ISSN: 2406-9388
E-ISSN: 2580-8303

methicillin-resistant *S. aureus* (MRSA) ATCC 33592, *C. albicans* ATCC 10231 were obtained from the Faculty of Agriculture Muhammadiyah University Jember.

Tools

Autoclave vertical type stream sterilizer (HL-340 series), micropipette (Eppendorf® research plus), vortex (IKA® maximix II), incubator (Memmet IN110®), analytical balance (Sartorius Type BP22IS®), UV-Vis spectrophotometer (Lambda EZ201 Perkin Elmer), vernier caliper (Jason).

Method

In this study, antimicrobial activity was tested using well diffusion and dilution methods to determine the ability of randu honey to inhibit (static) pathogenic microorganisms. The diffusion method was used to determine the sensitivity of test microorganisms to the randu honey, while the dilution method was used to determine the minimum inhibitory concentration (MIC) of randu honey. The test microorganisms were selected to determine the inhibitory power of randu honey against the growth of gram-negative bacteria (*E. coli* ATCC 25922), gram-positive bacteria (*S. aureus* ATCC 6538), resistant bacteria that often cause nosocomial infections (methicillin-resistant *S. aureus* (MRSA) ATCC 33592), and yeast that causes opportunistic infections (*C. albicans* ATCC 10231).

Preparation of antimicrobial test media

The antimicrobial test media used were divided into those for the antibacterial and antifungal tests. Antibacterial test media were prepared by dissolving 28 g NA powder in 1 L distilled water. Meanwhile, 65 g of SDA was weighed and dissolved in 1 L of distilled water in a different container for the antifungal test media. Each medium was magnetically stirred until the solution became clear. Each medium was then filled separately in a 12 mL reaction tube as a base layer and 8 mL as a seed layer, covered with cotton, and sterilized at 121°C for 15 min.

Preparation of test microbes

The preparation was initiated by regenerating the test microbes. First, one Ã–se of each test microbe (*E. coli* ATCC 25922, *S. aureus* ATCC 6538, MRSA ATCC 33592) was streaked onto NA slant agar medium and incubated at 37 Â °C for 24 h. *C. albicans* ATCC 10231 was streaked onto SDA agar slant medium and incubated at 25°C for 48 h. The culture results in the form of colonies on slanted agar were used to prepare the inoculum. A total of 10 mL of 0.9% saline solution was added to slanted agar medium containing colonies of *E. coli* ATCC 25922, *S. aureus* ATCC 6538, MRSA ATCC 33592, and *C. albicans* ATCC 10231. These were vortexed until the test microbes were separated from the medium (marked by the presence of turbidity). The turbidity was measured at a wavelength of 580 nm until the transmittance reached 25%. A test microbial inoculum was obtained with a bacterial count range of 10^7-10^9 cfu/mL (Kemenkes RI, 2020).

Figure 1. Sampling location of randu honey (RBH, RMH, RKH, RSH)

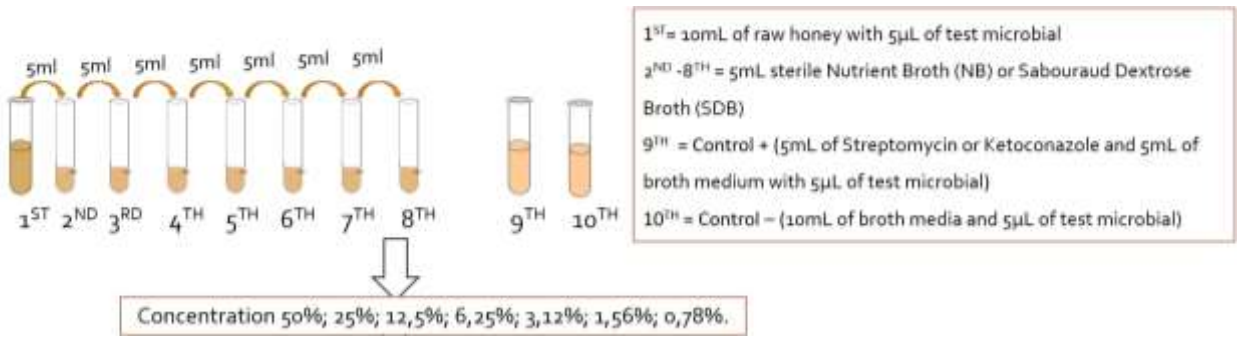


Figure 2. Serial dilution method with ratio 1:2 (v/v)

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Antimicrobial activity test

Well diffusion test

The antimicrobial activity test was performed using the diffusion method described by Irfanah (2018), with modifications. A well-diffusion technique was used in this study. This technique was carried out by filling a hole measuring 7.50 mm in diameter with a random honey sample solution. According to Anand et al. (2019), the diffusion method can provide better results than the other methods. This is because, in the well diffusion method, the test substance has more contact with the medium, so more of it diffuses and interacts with the test microbes. The working principle is the diffusion of active antimicrobial compounds in honey into media containing the test microorganisms.

First, 12 mL of NA as the base layer medium was poured into a sterile Petri dish. The test bacterial inoculum was pipetted 5µL and put into an 8mL seed layer. The mixture of the seed layer medium and bacterial inoculum was vortexed. The seed layer was then poured on top of the solidified base layer. Once the agar was solid, holes were created using a ring (diameter: 7.50 mm). The medium was perforated in five holes consisting of 100µl of randu honey with three replications, one positive control, and one negative control. Finally, the media was incubated at 37°C for 24 h to test antibacterial activity and at 25°C for 48 h to test antifungal activity. The diameter of the inhibition zone formed around the well was measured using a caliper with an accuracy of 0.05 mm. The diameter of the inhibition zone was considered a measure of antibacterial activity. The diameter of the inhibition zone exhibited a linear relationship with the antimicrobial activity of the samples. An inhibitory zone diameter of less than 7 mm is defined as a no-obstacle zone (Banerjee *et al*., 2022).

Dilution test

The minimum inhibitory concentration (MIC) was determined using the serial dilution method at a ratio of 1:2 (v/v) in 10 sterile reaction tubes. Liquid medium (broth) was used for dilution. The first tube contained 10 mL of randu honey to which 5µL of the test microbial inoculum was added, and the second to eighth tubes were filled with 5 mL of sterile Nutrient Broth (NB) medium for bacterial MIC and Sabouraud Dextrose Broth (SDB) for fungal MIC. Approximately 5 mL of randu honey in the first tube was pipetted into the second tube using a micropipette. The solution was centrifuged until homogeneous and a 50% solution was formed. The same procedure was performed up to the eighth tube, and all extract concentrations were obtained in a ratio of 1:2 to obtain concentrations of 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, and 0.78%, respectively (Figure 2). The ninth tube contained 5mL Streptomycin or Ketoconazole as a positive control and 5mL mL broth media with 5µL inoculum. The 10th negative control tube contained 10 mL broth medium and 5 µL test microbial inoculum. All tubes were incubated at 37°C for 24 h for bacteria and at 25°C for 48 h for fungi (Kemenkes RI, 2020). After incubation, each tube was vortexed and the transmittance was immediately measured using a spectrophotometer. Transmittance measures the amount of light passing through a sample (Akinduti et al., 2019). Transmission through a sample solution can be easily measured by measuring the intensity of the incident and transmitted light.

The dilution method aims to determine the smallest concentration of randu honey that can inhibit the growth of the test microorganism, or, , plays a role in determining the minimum inhibitory concentration (MIC). The presence or absence of growth of test microorganisms was observed by measuring turbidity using a spectrophotometer at a wavelength of 580 nm. Incubation results that show turbidity indicates that there is growth of the test microorganisms, whereas a clear sample means that the active antimicrobial compounds in randu honey from Bogor (RBH), Malang (RMH), Kediri (RKH), and Sidoarjo (RSH) can inhibit the growth of the test microorganisms.

Statistical analysis

This research used two-way Analysis of Variance (ANOVA) statistical analysis via the IBM SPSS (Statistical Package for Social Sciences) version 26 application to determine whether there were significant differences in the antimicrobial activity produced by randu honey samples.

RESULTS AND DISCUSSION

Antimicrobial activity test results using the diffusion method

The test result is said to be positive if a clear area is formed around the sample (Figure 3). This clear area shows that the growth of microorganisms is inhibited; therefore, it is called the inhibition zone. Observations of the inhibition zone were adjusted according to the growth temperature of the test microbes. Incubation was carried out at 37 °C according to the growth temperature of the mesophyll bacteria and 32 °C according to the growth temperature of the fungi. An inhibition zone appeared after 24 h of incubation in the antibacterial activity test and after 48 h in the antifungal test.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

The results of the antimicrobial activity test by diffusion showed that each sample of randu honey inhibited the growth of the test microbes. The formation of the inhibition zone varied in size. Overall, the inhibition zone formed was between 11.57 ± 0.67 and 27.86 ± 0.43 mm, indicating that all randu honey samples had potent antimicrobial activity against the test microbes.

In Table 1, it can be seen that randu honey from Bogor (RBH) produces an inhibitory zone diameter between 17.59 ± 0.13 mm and 27.86 ± 0.43 mm. The inhibition zone for MRSA ATCC 33592 and *C. albicans* ATCC 10231 was more than 20 mm; therefore, it was included in the very strong inhibitory category based on the classification by Abu-Zaid et al. (2022).Randu honey from Malang (RMH) forms an inhibitory zone diameter of 15.90 ± 0.57 to 21.58 ± 0.56 mm, which is included in the strong inhibitory category. The RMH sample exhibited the highest antimicrobial activity against *S. aureus* (ATCC 6538). Randu honey from Sidoarjo (RSH) and Kediri (RKH) also showed strong inhibitory power, with a range of inhibitory zone diameters of 14.66 ± 0.52 – 20.91 ± 0.29 mm, respectively, and 11.57 ± 0.67 - 17.51 ± 0.57 mm. These two randu honey samples had the highest inhibitory power against *C. albicans* ATCC 10231 compared with the other tested microbes.

Compared to other other randu honey samples, the randu honey sample from Bogor (RBH) had the highest inhibitory activity against *C. albicans* ATCC 10231. In contrast, the antimicrobial activity produced by the RKH sample was the lowest among the four tested microbes. This is in accordance with the two-way ANOVA statistical test, which gives a value of F = 83.386 > F table (2.25) and a significance value of 0.000 < α = 0.05, indicating that there is a significant difference in the diameter of the inhibition zone between the randu honey groups and test microbes. Randu honey with the highest antimicrobial activity was tested through post-hoc multiple comparisons, obtaining the largest mean difference in samples RBH (21.1642) and *C. albicans* ATCC 10231 (21.2350). It can be concluded that there is a match between the observation results and the statistical analysis.

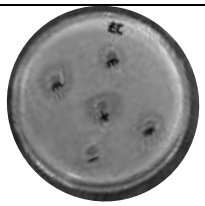
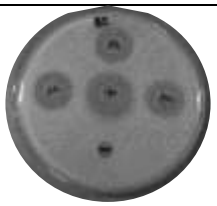
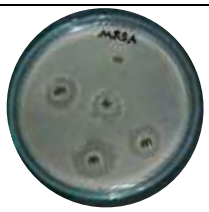
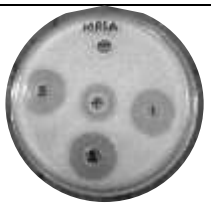
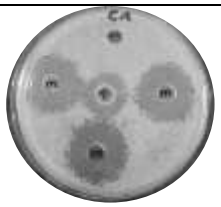
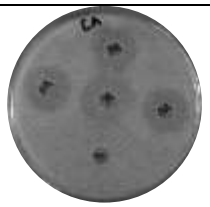
Test microbes	RBH	RMH	RKH	RSH
Eschericia coli ATCC 25922 (EC)				
Staphylococcus aureus ATCC 6538 (SA)				
MRSA ATCC 33592				
Candida albicans ATCC 10231 (CA)				

Figure 3. Results of well diffusion test (M= sample replication, + = positive control, - = negative control)

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Table 1. Diameter of inhibition zone randu honey samples

Test Microbes	Diameter of Inhibition Zone (mm)					
	RBH	**RMH**	**RSH**	**RKH**	**Positive Control**	**Negative Control**
Escherichia coli ATCC 25922	17.59±0.13	17.90±0.57	16.05±0.29	13.73±0.71	25.80	0.00
Staphylococcus aureus ATCC 6538	18.24±0.36	21.58±0.56	17.94±0.24	15.13±1.35	27.35	0.00
MRSA ATCC 33592	20.97±1.03	20.58±0.73	14.66±0.52	11.57±0.67	18.30	0.00
Candida albicans ATCC 10231	27.86±0.43	18.66±0.74	20.91±0.29	17.51±0.57	25.19	0.00

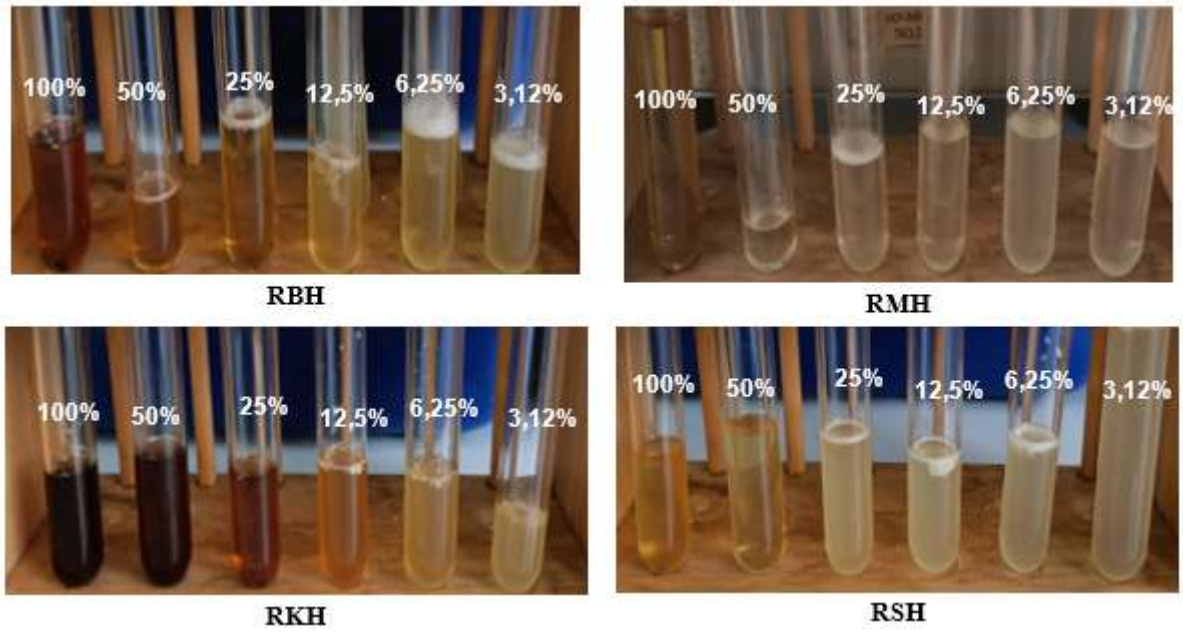


Figure 4. Results of dilution test against *Escherichia coli* ATCC 25922 with 100%; 50%; 25%; 12.5%; 6.25%; 3.12% (v/v) concentration of randu honey

Antimicrobial activity test results using the dilution method

MIC was visually observed as the smallest concentration that did not cause turbidity. The results of the dilution tests are shown in Figure 4.

The sample with the highest transmittance value in Table 2 is the positive control, which contains media and antibiotics to suppress the growth of the test microorganisms. As a result, there is no turbidity and more light can pass through the solution, resulting in a high transmittance percentage. The opposite is true for negative controls. Meanwhile, if we observe the transmittance at each concentration of randu honey, the higher the concentration of the randu honey sample, the higher the transmittance produced because the number of test microorganisms that grow decreases. A high concentration of randu honey indicates that it contains more antimicrobial compounds, therefore the inhibitory power for the growth of microorganisms is higher. The MIC value is the lowest concentration of randu honey that can inhibit microbial growth, at concentrations less than the MIC (bold numbers in Table 2) there is no inhibitory effect (Vaou et al., 2021). This is because the transmittance produced at this concentration is already lower than that produced by the negative control, which only contains the medium and test microbes without randu honey.

From the data in Table 2, it can be seen that the minimum inhibitory concentration produced by each sample of randu honey using the turbidimetric method was in the concentration range of 25% to 3.12% (v/v). The average transmittance of the MIC of the four samples was 31.70 ± 0.63%. u The MIC of RKH sample against *S. aureus* ATCC 6538 was 25% (v/v), and the other samples were 12.5% (v/v). The MICs of RKH, RMH, RSH, and RBH samples against MRSA ATCC 33592 were 6.25%, 12.5%, 25%, and 3.12% (v/v). Meanwhile, the inhibitory ability of RKH, RMH, RSH, and RBH randu honey samples against *C. albicans* ATCC 10231 was at a concentration of 25%, 12.5%, and 3.12% (v/v). The MIC value shows RKH, RMH and RSH honey had moderate antibacterial power according to the Kuete (2010) classification which differentiates antibacterial power into 3 levels: strong (<100µg/mL), moderate (100- 625µg/mL) and weak (>625µg/mL). Based on the MIC data, it can be said that the most

P-ISSN: 2406-9388
E-ISSN: 2580-8303

potent inhibitory power is exhibited by the RBH sample with a concentration of 3.12%, which is considered to have strong inhibitory ability against pathogens.

Overall, the randu honey sample from Bogor (RBH) showed the highest ability to inhibit the growth of *C. albicans* ATCC 10231, both using diffusion and dilution methods. Based on research by Irish et al. (2021), honey with antimicrobial activity that depends on hydrogen peroxide is more effective in inhibiting dermatophyte fungi and Candida species. This suggests that these randu honey samples may have a broader spectrum and may be valuable antifungal agents.

Table 2. %Transmittance of randu honey sample against test microbes

	Concentration %(v/v)	**Average of % transmittance**			
		Escherichia coli ATCC 25922	*Staphylococcus aureus* ATCC 6538	MRSA ATCC 33592	*Candida albicans* ATCC 10231
RKH	100%	78.40 ± 0.55	88.16± 0.29	80.09± 0.15	73.51± 0.47
	50%	57.57 ± 1.01	64.23 ± 0.59	71.21 ± 0.21	48.69 ± 0.50
	25%	37.70 ± 1.10	**37.99 ± 0.73**	58.09 ± 0.43	**27.09 ± 0.41**
	12.50%	**28.64 ± 0.93**	25.10± 0.24	40.67± 0.83	19.53± 0.35
	6.25%	23.45 ± 0.58	19.36 ± 0.54	**32.07 ± 0.24**	17.24 ± 0.36
	3.12%	19.34± 0.99	15.13 ± 0.22	23.99 ± 0.17	11.93 ± 0.41
	1.56%	14.36 ± 0.98	13.15 ± 0.74	17.14 ± 0.28	9.97 ± 0.12
	0.78%	11.61 ± 1.23	10.66 ± 0.27	12.12± 0.45	7.01± 0.23
	Control -	25.71 ± 0.06	25.83 ± 0.10	25.84 ± 0.09	20.74 ± 0.13
	Control +	95.47 ± 0.06	95.37 ± 0.38	90.41± 0.06	97.46± 0.20
RMH	100%	67.96 ± 1.07	80.16± 0.18	72.88± 0.46	89.04± 0.65
	50%	51.86 ± 0.67	66.09± 0.27	57.25± 0.35	72.47± 0.63
	25%	46.17 ± 1.00	46.71 ± 0.97	44.89 ± 0.12	53.87 ± 0.35
	12.50%	34.86± 0.52	**31.48 ± 0.56**	**28.98± 0.31**	**36.57± 0,51**
	6.25%	**27.21 ± 1.04**	20.07 ± 0.91	21.32 ± 0.34	23.11 ± 0.27
	3.12%	19.03 ± 0.86	14.34± 0.59	16.03± 0.19	18.03± 0.06
	1.56%	9.98 ± 0.84	9.25± 0.59	14.00± 0.32	14.90± 0.29
	0.78%	8.56 ± 0.47	7.31 ± 0.60	10.02± 0.30	11.09± 0.21
	Control -	25.67 ± 0.13	25.52 ± 0.27	25.81 ± 0.16	20.84 ± 0.05
	Control +	95.57 ± 0.09	95.02 ± 0.19	90.47 ± 0.07	97.24 ± 0.11
RSH	100%	64.65 ± 1.42	70.03± 0.80	65.29± 0.46	80.64± 0.44
	50%	47.23 ± 1.25	59.73 ± 0.73	52.93 ± 0.19	62.23 ± 0.28
	25%	**32.53 ± 0.82**	40.17± 0.48	**33.75± 0.82**	49.96± 0.68
	12.50%	24.99 ± 0.16	**30.35 ± 1.28**	25.06 ± 0.34	**37.02 ± 0.13**
	6.25%	18.37 ± 0.49	23.18± 0.26	18.92± 0.11	19.93± 0.32
	3.12%	13.87 ± 0.38	17.34 ± 0.42	14.93 ± 0.28	17.36± 0.60
	1.56%	10.01 ± 0.63	12.90 ± 0.57	12.02 ± 0.10	11.98 ± 0.19
	0.78%	6.66 ±1.15	9.99 ±0.23	10.70 ±0.16	10.14 ±0.44
	Control -	25.64 ± 0.10	25.70 ± 0.12	25.81 ± 0.06	20.83 ± 0.11
	Control +	95.57 ± 0.07	95.63 ± 0.13	90.58 ± 0.11	97.17 ± 0.15
RBH	100%	71.37 ± 0.57	85.31 ± 0.18	84.86 ± 0.38	83.08 ± 0.59
	50%	61.96 ± 0.17	70.30 ± 0.45	65.68 ± 0.37	72.79 ± 0.69
	25%	48.41± 0.15	46.11± 0.15	57.77 ± 0.53	56.94± 0.61
	12.50%	**37.04± 0.23**	**32.43± 0.40**	48.34 ± 0.39	50.28 ± 0.39
	6.25%	23.16 ± 0.24	20.20 ± 0.38	31.33 ± 0.43	41.03 ± 0.67
	3.12%	17.40 ± 0.45	15.30 ± 0.51	**26.05 ± 0.69**	**28.03 ± 0.68**
	1.56%	13.13 ± 0.15	13.17 ± 0.33	16.05 ± 0.29	19.01 ± 0.44
	0.78%	9.80± 0.24	9.79± 0.23	7.02 ± 0.75	13.28± 0.51
	Control -	25.77 ± 0.06	20.80 ± 0.08	25.65 ± 0.07	25.09 ± 0.14
	Control +	90.61± 0.04	97.19 ± 0.12	95.63 ± 0.05	94.91 ± 0.59

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Brudzynski (2020) said that hydrogen peroxide is the main antimicrobial agent in honey because it is capable of producing an inhibitory power (MIC) in the range of 10–10000µg/ml. The reactive hydrogen peroxide in randu honey can break the bonds of the microbes' outer membrane, resulting in lysis of the microbes. Therefore, factors that influence the production and breakdown pathways of hydrogen peroxide also influence the antimicrobial activity.

Clearwater et al. (2018) stated that water content is one of the factors that influences the formation of hydrogen peroxide. Their research found that hydrogen peroxide levels in honey harvested between May and August 2006 (rainy season) in the Czech Republic were higher than those in honey harvested in July (summer). This is because the water content in honey increases, and water is needed as a reactant for the formation of hydrogen peroxide by the enzyme glucose oxidase. This is one of the factors that causes the RBH sample to have a higher inhibitory power than the other samples. The geographical conditions of the area of origin of the RBH sample, the Pusat Perlebahan Bogor, are located in an area with rainfall of approximately 3500–4000 mm per year. This rainfall is higher than that in Sidoarjo (1300–1700 mm per year), Malang (1596 mm per year), and Kediri (1652 mm per year).

It was found that even though the samples came from the same type of honey (monoflora honey) from randu plants and were harvested at the same time because the harvest location had different geographical conditions, the antimicrobial activity produced could also be different. In addition to being influenced by geographical conditions, climate, and water availability, it can also be influenced by the nutrition of plant nectar sources and bee entomological factors (Abu-Zaid et al., 2022). In the case of honey that relies on hydrogen peroxide, such as randu honey, antimicrobial activity is related to the stability of the enzyme glucose oxidase, the enzyme responsible for the production of hydrogen peroxide (Almasaudi, 2021).

CONCLUSION

In conclusion, all samples of randu honey from several regions in Java, in this case Sidoarjo (RSH), Bogor National Beekeeping Center (RBH), Kediri (RKH), and Malang (RMH), had active antimicrobial activity against *E. coli* ATCC 25922, *S. aureus* ATCC 6538, methicillin-resistant *S. aureus* (MRSA) ATCC 33592, *C. albicans* ATCC 10231. The RBH sample showed strong inhibitory activity, with a minimum inhibitory concentration (MIC) of 3.12%. The other three honey samples, namely RMH, RKH, and RSH honey, had moderate inhibitory power. Further research is needed to identify the active antimicrobial ingredients in randu honey.

AUTHOR CONTRIBUTIONS

Conceptualization, N.P.W., R.P., A.T.P.; Methodology, N.P.W., R.P., A.T.P.; Software, N.P.W.; Validation, N.P.W., R.P.; Formal Analysis, N.P.W.; Investigation, N.P.W.; Resources, N.P.W.; Data Curration; N.P.W., R.P., A.T.P.; Writing - Original Draft, N.P.W., R.P., A.T.P.; Writing - Review & Editing, N.P.W., R.P., A.T.P.; Visualization, N.P.W.; Supervision, R.P., A.T.P.; Project Administration, N.P.W., R.P., A.T.P.; Funding Acquisition, N.P.W.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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P-ISSN: 2406-9388
E-ISSN: 2580-8303

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P-ISSN: 2406-9388
E-ISSN: 2580-8303

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia
Pharmacy and Pharmaceutical Sciences Journal
Published by Faculty of Pharmacy Universitas Airlangga
OPEN ACCESS
E-ISSN 2580-8303
P-ISSN 2406-9388

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia Vol. 11 No. 2 August 2024, 156-173
DOI: 10.20473/jfiki.v11i22024.156-173
Available online at https://e-journal.unair.ac.id/JFIKI/

Systematic Review of Green Seaweed *Caulerpa racemosa* as an Anti-Inflammatory Agent: Current Insights and Future Perspectives

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Submitted: 30 May 2024
Revised: 11 August 2024
Accepted: 31 August 2024

Abstract

Background*: Seaweed is a marine biota with many benefits, one of which is C. racemosa. It is one type of seaweed that is quite widely found in Indonesia.* ***Objective*** *This study investigated the anti-inflammatory activity of C. racemosa using various in vitro and in vivo approaches.* ***Methods****: A literature review was conducted by searching for research data on C. racemosa. The literature was obtained from PUBMED, ScienceDirect, Scopus, SpringerLink, and Google Scholar using the keywords C. racemosa, sea grapes, in vivo, in vitro, and anti-inflammatory. The search identified 1313 articles with 100 articles in Scopus, 100 articles in ScienceDirect, 0 articles in PubMed, 3 articles in SpringerLink, and 1,110 articles in Google Scholar.* ***Results****: The study showed 12 articles found C. racemosa has the ability as an anti-inflammatory both with in vitro and in vivo study approaches and supported by data on proximate composition which is quite high and substance consisting of various bioactive constituents including flavonoids, phenolics, phytosterols, terpenoids, saponins and alkaloids where the anti-inflammatory active isolate caulerpin was successfully isolated. C. racemosa is able to reduce the inflammatory response by inhibiting NO production and the release of cytokines and inflammatory mediators such as AMPK, mTOR, TNF-α and IL4.* ***Conclusion****: C. racemosa indicated that this species is a rich source of phytochemicals with many pharmacological activities, one of which is anti-inflammatory. Further research is required to explore the relationship between secondary metabolites and their activities.*

Keywords*: Caulerpa racemosa, marine natural products, anti-inflammatory, in-vitro, in-vivo*

How to cite this article:
Prayogo, E. W., Sholikhah, I., Suciati, Dej-adisai, S. & Widyowati, R. (2024). Systematic Review of Green Seaweed *Caulerpa racemosa* as an Anti-Inflammatory Agent: Current Insights and Future Perspectives. *Jurnal Farmasi dan Ilmu Kefarmasian Indonesia*, 11(2), 156-173. http://doi.org/10.20473/jfiki.v11i22024.156-173

P-ISSN: 2406-9388
E-ISSN: 2580-8303

INTRODUCTION

Seaweed is one of Indonesia's marine and fishery commodities and has a high economic value because of its many benefits. The value of seaweed production in Indonesia in 2022 reached 231,829.70 tonnes (Central Bureau of Statistics, 2023). This shows that there is a lot of market demand for seaweed because every year the Indonesian government always tries to increase seaweed cultivation. One type of seaweed that has many benefits is sea grape (Stuthmann et al., 2023).

Sea grapes (*Caulerpa racemosa*) are a common type of seaweed in Indonesia. It possesses a thallus that exhibits characteristics similar to those of grass and displays green coloration. This thallus is composed of numerous erect branches measuring approximately 2.5-6.0 cm in height. The length of the primary stem ranged from 16 to 22 cm. At the apex of each branch, there are spherical structures similar to grapes, with a length ranging from approximately 2.5 to 10.0 cm. The characteristics of sea grapes include a thallus with stolons measuring approximately 5 cm each. The roots are relatively large and tapered like nails, with ramuli reaching 8 cm in length. Ramuli is a branch organ or branch of the stolon as the main organ, and its substance is rather soft and seems empty. These ramuli are between 2-4 mm in diameter. The ramuli arise on stolons that are branched, have rounded, flattened ends and stalks, and are arranged around and along the ramuli (Yudasmara, 2015).

Sea grapes have an economic function in that they can be used as food ingredients, where the processing process is quite easy. Sea grapes that came from the sea were taken and washed thoroughly using boiled water. It is then boiled to kill pathogenic bacteria in seaweed (Ersalina et al., 2020). Currently, sea grapes are widely used in the food sector as ingredients in jelly candy (Estrada et al., 2020), ice cream, cream soup, and seaweed flour (Stuthmann et al., 2023). In addition to the food sector, seagrapes can be used as medicines in the pharmaceutical sector. In general, the chemical composition of sea grapes has a protein content of 10.41%, ash content of 38.94%, total fat of 1.58%, moisture content of 92.37%, carbohydrates of 35.69%, dietary fiber of 34.08%, energy from the fat of 14.22 kCal/100 g and total energy of 198.58 kCal/100 g (Sedjati, 1999). Furthermore, sea grapes contain minerals such as Na, Ca, and K and amino acids in the form of L-threonine and L-glycine (Sinurat et al., 2021). The secondary metabolite content in sea grapes can be used as an antioxidant (Sinurat et al., 2021), antibacterial (Belkacemi et al., 2020), anticancer (Permatasari, Wewengkang et al., 2022), antidiabetic (Mandlik et al., 2022), antinociceptive (De Souza et al., 2009), antiobesity (Kurniawan et al., 2023), and anti-inflammatory (Worms & Adrian, 2023).

Sea grapes can be used as anti-inflammatory agents because they contain sulfated polysaccharides. This polysaccharide is a negatively charged polysaccharide present in the cell walls of seaweeds and is currently widely used in the food and pharmaceutical industries (Ribeiro et al., 2020). In addition, the most abundant carotenoids in sea grapes are β-carotene and canthaxanthin. The current investigation showed the capability of *C. racemosa* carotenoids as innate suppressors of inflammation through modulation of the AMPK-mTOR-TNF-α signaling cascade. Furthermore, it has been demonstrated that clinical AMPK activation reduces inflammation-related pain by blocking NF-κB, mTOR, and IL-1β activation. (Kurniawan et al., 2023) .

Numerous investigations have been conducted on *C. racemosa* to validate and affirm its biological characteristics. Several investigations have been conducted using both in vivo and in vitro models. Therefore, to efficiently conduct future research while minimizing resource waste and maximizing time optimization, retrospective and systematic research methods were employed to outline the technique and present the collected results.

MATERIALS AND METHODS

Focus question

The feasibility of the C. racemosa anti-inflammatory activity test was evaluated through activity tests using in vivo and in vitro model approaches and the mechanisms that occur.

Search strategy

Searching and collecting article data were conducted online from September 2023 to February 2024 using the keywords "*C. racemosa* AND anti-inflammatory AND in-vitro AND in-vivo" in several online databases, such as PubMed, Google Scholar, ScienceDirect, SpringerLink, and Scopus. Furthermore, the collected articles were filtered using EndNote X9.3.3. The initial round of screening involved thorough examination of the article search results to identify any instances of duplication. Subsequently, the duplicate articles that were identified were segregated and separated from the others. After the article separation process, the sorting process continued, including the appropriateness of the title and abstract regarding the research content. The anti-inflammatory activity of C. racemosa was investigated using in vitro and in vivo models. In

P-ISSN: 2406-9388
E-ISSN: 2580-8303

addition, eligibility assessment was conducted by thoroughly reviewing the entirety of the article's content to ascertain its compatibility with the pre-established inclusion criteria. A team of five individuals conducted the method of gathering and categorizing publications, with two additional individuals performing a secondary review. Subsequently, the risk of bias for each article was evaluated using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses checklist. A comprehensive literature review was conducted to identify relevant studies on the potential anti-inflammatory properties of *C. racemosa*. The search identified 1313 articles with details of 100 articles in Scopus, 100 articles in ScienceDirect, three journals in SpringerLink, and 1110 articles in Google Scholar (Figure 1).

Eligibility criteria

The eligibility criteria employed in this systematic review were established by considering the research questions formulated according to the PICO (population, intervention, comparator, outcome) framework.

•Population: Species *C. racemosa*

•Intervention: In vivo and In vitro study of anti-inflammatory properties

•Comparison: Positive control and negative control

•Outcome: *C. racemosa* species' anti-inflammatory effects both in vitro and in vivo

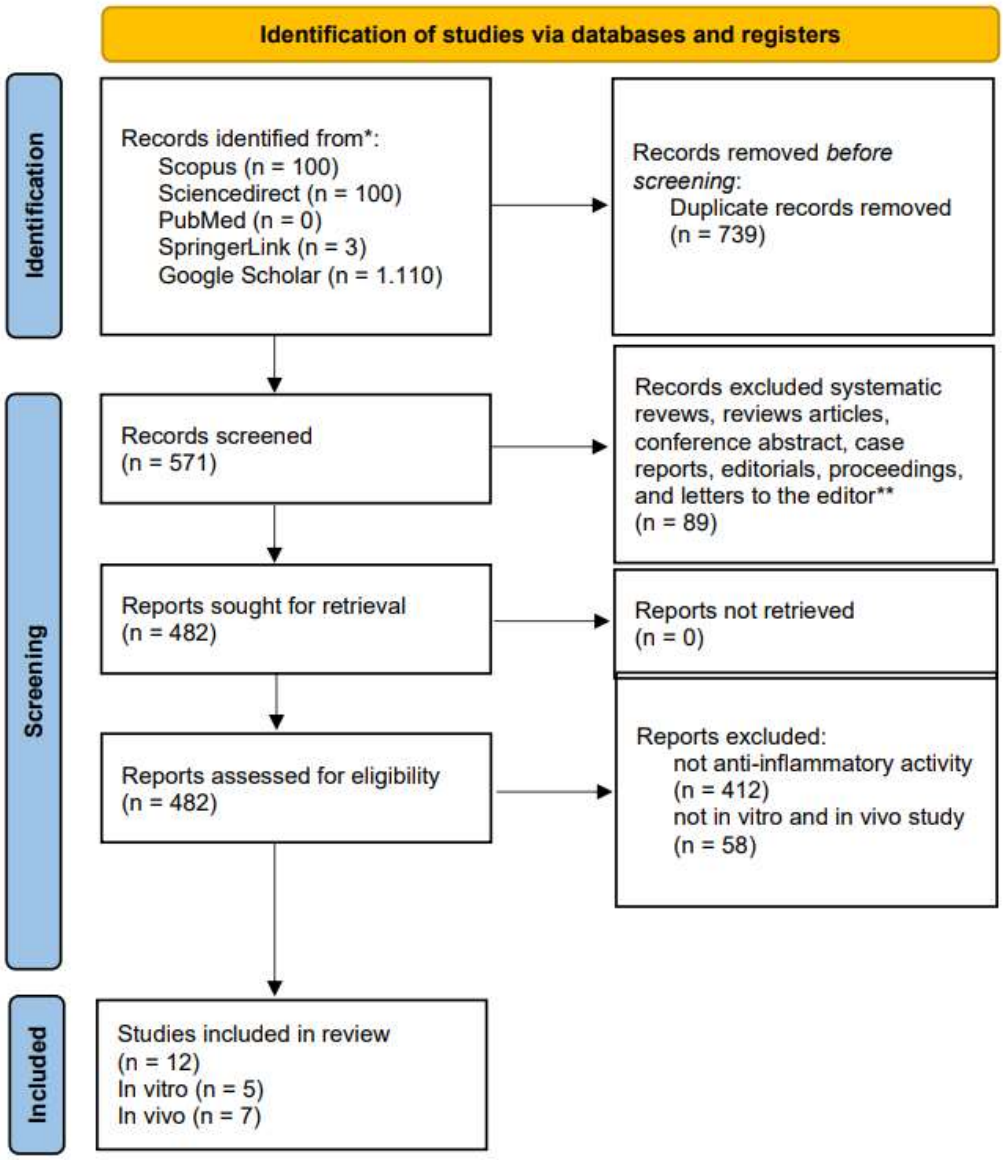

Figure 1. The research PRISMA 2020 flow diagram

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E-ISSN: 2580-8303

The PICO framework was employed in systematic reviews to construct literature search algorithms that guarantee thorough and unbiased searches. It is commonly employed in evidence-based practice, particularly in the field of evidence-based medicine, to generate clinical or healthcare-related questions and propose answers to the research problem at hand (Methley et al., 2014). In this study, the PICO format was used to specify articles collecting data from several databases.

The inclusion criteria for this study were studies related to *C. racemosa* that have anti-inflammatory activities using in vitro and in vivo models, as well as articles containing comparative analysis and results from both methods from 2000 to 2024. The exclusion criteria were articles in languages other than English and systematic reviews, review articles, conference abstracts, case reports, editorials, proceedings, and letters to the editor.

RESULTS AND DISCUSSION

C. racemosa can be found in shallow waters up to 100 m deep in the warm tropics. It is a type of senositic Chlorophyta with chloroplasts that can migrate inside the cells through a network of protein fibers. This type has a branch morphology or a short, erect rachis originating from the stolon horizontally attached to the sediment or substrate using rhizomes. A branch or rachis appears every few centimeters along the stolon, and the rachis height can reach 30 cm. In murky waters, the rachis erect grows tall, while in waters with sufficient currents, strong, shorter erect rachises. In every branch upright (rachis), the ramuli or small branches are oval to round (Gopi et al., 2019). *C. racemosa* type commonly found in various waters in Indonesia. It is known locally as sea grapes and is widely used by coastal communities as vegetables or lalap. Caulerpa is known to have a high nutritional content, making it a food ingredient (Ridhowati et al., 2016). *C. racemosa* contains fatty acids with higher unsaturation than saturated fatty acids, with the highest acid content fat being oleic acid. The lipid content in *C. racemosa* also has an index of low atherogenicity and thrombogenicity. The amino acid content is relatively more balanced between essential and nonessential amino acids (Magdugo et al., 2020).

Characteristics of study design

A complete list of the studies is presented in Table 1. Twelve studies that satisfied the inclusion criteria were identified, and these studies were published within the time frame of 2009 to 2024. The studies encompassed five in vitro investigations and seven in vivo studies. These 12 studies examined the anti-inflammatory properties of *C. racemosa*, focusing on various activities. The animal species used in these experiments were *Wistar albino* rats (Magdugo et al., 2020), adult female *Mus musculus* Swiss mice, and male *Rattus norvegicus* Wistar mice (Mandlik et al., 2022). The initial utilization of the in vitro anti-inflammatory investigation, as delineated in this review, involved the stimulation of RAW 264.7, through the administration of lipopolysaccharide (LPS). Bacterial LPS is widely recognized as a highly effective stimulus for inducing substantial production of nitric oxide (NO), thereby facilitating the upregulation of pro-inflammatory proteins, including cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). This experimental approach involving the use of RAW 264.7 cells was employed to evaluate the anti-inflammatory effects of C. racemosa (Vairappan et al., 2013). In a separate study conducted on in vitro cell culture experiments for anti-inflammatory effects, *C. racemosa* increased TNF-α and mTOR expression, as well as decreased AMPK expression after 6 and 24 h of incubation in RAW 2647 cells (Kurniawan et al., 2023). In an alternative in vitro model, the researchers employed CRC HT-29 cells were used to suppress the expression of TGF-β1, a pro-inflammatory protein that stimulates the proliferation of colon cancer cells (Permatasari, Bulain, et al., 2022). The anti-inflammatory activity of *C. racemosa* was assessed using a carrageenan-induced paw edema test as an in vivo test paradigm (Radhika et al., 2012). In additional investigations utilizing acetic acid induction, inflammation was evaluated using the rat abdominal writhing test and the hot plate test to measure the heat-resistant latency time reactivity of rat paws before and after *C. racemosa* treatment. Anti-inflammatory assays using commercial kits for the liver and pancreas after and without *C. racemosa* administration were conducted in streptozotocin-induced diabetic animal models to evaluate the levels of inflammatory biomarkers, including cytokines, TNF-α and IL-4, serum markers (AST and ALT), and ALP (Mandlik et al., 2022). The majority of the research examined the anti-inflammatory activity of C. racemosa in diverse experimental settings, encompassing a range of extract types, fractions, isolates, and doses.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Table 1. Characterize a study including 12 papers, including 5 articles on in-vitro investigations and 7 articles on in-vivo research regarding the anti-inflammatory capabilities of *C. racemosa*

Types of Extract	Plant sources	Study Type	Conclusion	Ref
Methanol	Sepanggar sea, Kota Kinabalu, Sabah, Malaysia	In Vitro	Extraction: Dried algal thallus was extracted by maceration with methanol for 5 days. The MeOH solution was concentrated in a vacuum and partitioned between diethyl ether and water. The Et2O solution was washed with water, dried over anhydrous sodium sulfate, and evaporated to leave a dark green oil. Method: Samples were tested for anti-inflammatory activity in lipopolysaccharide (LPS) stimulated RAW 264.7 cells. Parameter: Inhibitory effects of nitric oxide (NO) Control: (+) RAW 264.7 cell induced LPS (-) RAW 264.7 cell Results: The green seaweeds, *C. racemosa* var. laete-virens, suppressed 30–40% of NO production.	(Vairappan et al., 2013)
Ethanol 96%	Cultivation pond in Jepara Regency, Central Java Province, Indonesia	In Vitro	Extraction: Simplica powder from each green algae was mixed with 2 L of 96% ethanol solvent in a 1:2 ratio and put into a dark bottle. Simplica was soaked for three days. and the condensed extract was sequentially partitioned into equal volumes using EtOAc and n-hexane solvents. Method: In Vitro Anti-Inflammatory Assays via Mammalian Target of rapamycin (mTOR) Kinase, AMP-Activated Protein Kinase (AMPK), and Tumor Necrosis Factor-Alpha (TNF-α) Assay Parameter: AMPK, mTOR, and TNF-α expression Control: (+) RAW 264.7 cell induced LPS (-) RAW 264.7 cell Results: AMPK expression was generally enhanced while TNF-α and mTOR expression was suppressed by the carotenoid extract of *C. racemosa*. After six or twenty-four hours of	(Kurniawan et al., 2023)

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E-ISSN: 2580-8303

			incubation, the CrE led to a greater elevation of AMPK expression when compared to the other groups' treatment.	
Ethanol 70%, and Fraction of Hexane, Chloroform, ethyl acetate. Isolated squalane from *C. racemosa*	Southwestern coastal area Sri Lanka	In Vitro	Extraction: Algae powder was extracted four times using 70% ethanol and filtered under vacuum. The filtrate was concentrated by a rotary evaporator to obtain the crude extract. The crude extract (CRE) was suspended in deionized water and fractionated between hexane (CREH), chloroform (CREC), and ethyl acetate (CREE). Method: Samples were tested for anti-inflammatory activity in lipopolysaccharide (LPS) stimulated RAW 264.7 cells. Parameter: Inhibitory effects of nitric oxide (NO) Control: (+) RAW 264.7 cell induced LPS (-) RAW 264.7 cell Results: Solvent fractions, CREE and CREH showed higher potency to dose-dependently inhibit LPS-induced NO production in RAW cells compared to the inhibition by CREC and CREW.	(Fernando et al., 2018)
ethanolic extract	Mantehage, North Sulawesi, Indonesia	In Vitro	Extraction: The extract was macerated in 96% ethanol for 72 hours. The filtrate was concentrated and evaporated at 40°C to obtain a thick extract. Method: Quantitative measurement of apoptosis was measured using flowcytometry and the expression of Bcl-2, BAX, and cleaved-caspase 3 as pro and anti-apoptotic proteins were measured using immunofluorescence Parameter: Pro-apoptosis through expression of Bcl-2 and BAX Control: HeLa cells Results: The TGF-β pathway may be disrupted by the *C. racemosa* extract, which could have several negative knock-on consequences that prevent CRC from progressing. Through the inhibition of TGF-β1 expression and the disturbance of its receptor activation, it modifies growth factors, apoptotic processes, and the cancer microenvironment, offering a possible treatment pathway for colorectal cancer. Extracts that exhibit pro-apoptotic effects correlate	(Permatasari, Bulain, et al., 2022)

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E-ISSN: 2580-8303

			with anti-inflammatory activity by modulating key signaling pathways and reducing the production of cytokines and inflammatory mediators.	
Methanol (soxhlet apparatus)	Tuticorin coast, Tamilnadu, India.	In Vitro	Extraction: Extracts of the freeze-dried and powdered biomass were prepared using methanol as solvent using a soxhlet Method: Antibacterial activity of seaweed extracts using Disc Diffusion Parameter: Highest inhibition bacterial zone *Vibrio cholera, Salmonella typhoid, Escherichia coli* and *Klebsiella pneumonia* Control: Bacterial agar plates *Vibrio cholera, Salmonella typhoid, Escherichia coli* and *Klebsiella pneumonia* without extract Results: A peak value of 9 mm zone of inhibition was observed against *Vibrio cholera* with *C. racemosa* extract. Cholera toxin (CT) is the main virulence factor of *Vibrio cholera* that causes the signs and symptoms of cholera. CT production can be inhibited through various mechanisms, some of which also exhibit anti-inflammatory properties	(Radhika et al., 2012)
Methanol and Fraction of hexane, chloroform, ethyl acetate, n-butanol.	João Pessoa, State of Paraiba, Brazil	In Vivo	Extraction: The fresh sample of *C. racemosa* was exhaustively extracted with MeOH at room temperature. The solvent was removed under reduced pressure at <40 °C and a dark green residue was obtained. Part of the crude methanol extract was submitted to solid-liquid partition successively with hexane, chloroform, ethyl acetate, and n-butanol Method: Formalin-induced nociception test. Parameter: Measured the time the animal spent licking the right hind paw from 15 to 30 minutes from the time after injection. Control: (+) Indomethacine (-) control received vehicle-only Results: In assessing the anti-inflammatory activity in formalin-induced experimental animals. In the inflammatory phase, only ethyl acetate (75.43%) and indomethacin (47.83%) induced significant response inhibition in this model.	(Souza et al., 2009)

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N-hexane, chloroform, ethyl acetate, methanol, and water in a Soxhlet device.	João Pessoa, Paraíba State, Brazil	In Vivo	Extraction: Fresh algae were lyophilized and extracted thoroughly with hexane, chloroform, ethyl acetate, methanol, and water in a Soxhlet apparatus, to obtain extracts. Method: The formalin test was performed according to the method of Hunskaar and Hole. Parameter: Measured the time the animal spent licking the right hind paw from 15 to 30 minutes from the time after injection and evaluate the activity of these species in a cell migration model Control: (+) Indomethacine (-) control received vehicle-only Results: In assessing the anti-inflammatory activity in formalin-induced experimental animals, only the ethyl acetate fraction (68,7 % inhibition) and treatment with indomethacin (48.7% inhibition) inhibited the inflammatory phase. In the leukocyte migration inhibition test into the peritoneal cavity, the AE fraction inhibited 71.7%, compared to Indomethacin treatment, which inhibited 65.4% of leukocyte migration.	(Da Matta et al., 2011)
Ethanol (95%)	The coastal area of Okha Port in the Gujarat state of India	In Vivo	Extraction: Fresh algae were lyophilized and extracted thoroughly with hexane, chloroform, ethyl acetate, methanol, and water in a Soxhlet apparatus, to obtain extracts. Method: Measurement of NO, IL4, and TNF-α levels in the serum blood of streptozotocin-induced diabetic rat model Parameter: NO, IL4, and TNF-α levels Control: (+) Glipizide (-) Blank citrate buffer Results: The inhibitory effect of *C. racemosa* (200 mg/kg/day) on serum TNF-α and IL-4 levels was greater than that observed after glipizide (5 mg/kg/day) treatment.	(Mandlik et al., 2022)
Total sulfated polysaccharides (TSP) extraction and Total	From beach at Pedra Rachada in Sa˜o Gonc¸alo-Ce, Brazil	In Vivo	Extraction: The dried algae were hydrated in 250 mL of sodium acetate buffer with papain, cysteine, and EDTA. This mixture was kept at 60°C for 6 hours, filtered, and the residue washed with water. TSP was precipitated using cetylpyridinium chloride (CPC) and centrifuged. The precipitate was washed with CPC solution, dissolved in NaCl-ethanol	(Ribeiro et al., 2020)

P-ISSN: 2406-9388
E-ISSN: 2580-8303

sulfated polysaccharides (TSP) fraction			solution, and re-precipitated with ethanol. The final product was washed, dialyzed, lyophilized. Method: Evaluation the levels of TNF-α, and IL-1β in Wistar rats modeled on TMJ hypernosis that were pretreated (iv) 30 minutes prior to the administration of formalin. Parameter: TNF-α, and IL-1β Control: (-) Formalin Control not treatment Results: *C. racemosa* had anti-inflammatory properties in a TMJ hypernociception experimental model. These effects were associated with a reduction in plasma extravasation, a peripheral stimulation of HO-1, and a decrease in TNF-α, and IL-1β	
Ethanol 50%	From the St. Martin's Island shore in Bangladesh	In Vivo	Extraction: The algae extract was obtained by 50% ethanol maceration with a ratio of 1 gram/10 mL (1:10) for 7 days. Method: To evaluate the reduction of paw swelling/edema, the volume displacement of the left hind paw was re-measured for each rat with a plethysmometer after ½ hours, 1 hour, 2 hours, 3 hours, 4 hours, and 5 hours, 6 hours, and 8 hours after carrageenan induction. Parameter: Reduction of paw swelling/edema Control: (-) Without any treatment (+) Diclofenac Results: A 50% ethanol extract of *C. racemosa* 50mg/kg body weight showed better anti-inflammatory activity at six hours of investigation and inhibited 155.60% of edema.	(Chowdhury et al., 2023)
Aqueous extract	Collected from habitat in Southern, Northern, and North-western coastal areas in Sri	In Vitro In Vivo	Extraction: Each dry powder sample was suspended/dissolved separately in distilled water. Then, the samples were dissolved via sonication for 1 hour using an ultrasonic sonicator. The temperature was maintained at 40°C throughout the process. Then, the samples were shaken in a roller overnight at room temperature, and the extraction results were centrifuged at 15,000 rpm for 10 min at 4°C. Method: Cell migration induction of seaweed extracts was assessed by scratch wound	(Premarathna et al., 2020)

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	Lanka		healing test using the L929 cell line. For in vivo studies to evaluate the whole skin cut to create wounds in mice. as well as the expression levels of Tumor Necrosis Factor (TNF-α) and Transforming Growth Factor-β (TGF-β) through RT-PCR were measured once every three days until the end of the test. Parameter: Cell migration activity, enhanced wound healing activity in mice, and expression levels of TNF-α and TGF-β Control: In Vitro - In vivo (-) Without forming wounds and without providing any treatment. Results: The aqueous extract of C. racemosa has properties that make it able to increase the healing activity of scratch wounds in vitro and in vivo and the evaluation results of cytokine of TNF-α and TGF-β expression	
Isolated caulerpin from *C. racemosa*	Collected in the Northeast of Brazil	In Vivo	Extraction: The methanol extract of *C. Racemosa* was partitioned between H2O and hexane, chloroform, ethyl acetate, and n-butanol. The separation of chloroform fraction resulted in the isolation of orange-red pigment. Based on UV, IR, and NMR spectra data as well as chemical properties, the structure of caulerpine is shown. Method: Formalin-induced nociception Parameter: Measured the time the animal spent licking the right hind paw from 15 to 30 minutes from the time after injection. Control: (+) Indomethacine (-) control received vehicle-only Results: The possible anti-inflammatory activity observed in the second phase in the formalin test of caulerpin (100 μmol/kg, p.o.) was confirmed on the capsaicin-induced ear edema model, where inhibition of 55.8% was presented.	(De Souza et al., 2009)

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E-ISSN: 2580-8303

Figure 2. *C. racemosa* post-harvesting

The secondary metabolites such as alkaloids, flavonoids, phenolic, phytosterol, tannins, terpenoids, and saponins of various *C. racemosa* extracts are screened in Table 2. These secondary metabolites have many therapeutic applications and are widely used in the pharmaceutical industry. The benefits of alkaloids in the medical field include stimulating and combating microbiological infections, altering blood pressure, and stimulating the neurological system (Vairappan et al., 2013). Flavonoids help the body absorb vitamin C, preventing and treating allergies, viral infections, arthritis, and inflammatory conditions (Pires et al., 2013). Phenolic compounds have antioxidant, antidiabetic, anti-filaria, anticancer, cardioprotective, anti-inflammatory, and antiviral effects against the SARS-CoV-2 virus, which causes severe acute respiratory syndrome (Palaniyappan et al., 2023). Phytosterols have cholesterol-lowering effects by inhibiting the absorption of cholesterol from the intestine, avoiding cholesterol in bile salt micelles, and increasing the excretion of bile salts. Phytosterols also improve blood cholesterol regulation at normal levels (He et al., 2023). Tannin binds and precipitates proteins, treats diarrhea and hemorrhoids, stops inflammation, and is a natural alternative for cleaning dentures (Wu et al., 2022). Terpenoids have interesting pharmacological properties, including antiviral, antibacterial, anti-inflammatory, cholesterol synthesis inhibition, and anticancer effects. Saponins have antibacterial properties, suppress fungi, and shield plants from insect damage. Lipoprotein-lowering saponins are antioxidant, antiviral, anti-carcinogenic, and rumen fermentation manipulators (Hainil et al., 2023).

Table 2. Secondary metabolite of *C. racemosa*

No.	Secondary Metabolites	Presence
1.	Alkaloids	+
2.	Flavonoids	+
3.	Fucoidan	+
4.	Phenolic	+
5.	Phytosterol	+
6.	Tannins	+
7.	Terpenoids	+
8.	Saponins	+

Reproduced from (Palaniyappan et al., 2023)

Analysis of the mineral content showed that calcium had the highest mineral content. According to Khairy and El-Sheikh (2015), sodium, potassium, and calcium are the minerals commonly found in seaweeds. The highest mineral content was calcium which ranged from 149.66-168.64 mg/100 g and the lowest mineral content was magnesium (2.21-2.90 mg/100 g). The results of the analysis showed that mineral content increased with decreasing water content. According to Agoreyo et al. (2011), minerals are not damaged by heat treatment and exhibit very low volatility. The increase in mineral content was caused only by a decrease in the water content of the material. According to Tuteja and Sopory (2008), calcium is a macro element that is very important for plants and acts as a second messenger in the message-delivery pathway, and its concentration increases with stress signals. Calcium is a chemical signal under abiotic stress conditions in plants. Tuteja and Mahajan (2007) also reported that many physiological stimuli stimulate increased Ca^{2+} ion concentrations, such as light, touch or friction, pathogenic elicitors, plant hormones, and abiotic stresses, including high salinity, cold temperatures, and drought. The mineral contents of *C. racemosa* are shown in Table 3.

Table 3. Mineral

No.	Mineral	Fresh (mg/100g)	Semi-Dried (mg/100g)	Dried (mg/100g)
1.	Ca	149.66	163.99	168.64
2.	Fe	9.52	9.95	10.13
3.	K	77.59	86.77	92.50
4.	Mg	2.21	2.56	2.90
5.	Na	13.05	13.85	14.00

Reproduced from (Palaniyappan et al., 2023)

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Table 5. Amino Acid

No.	Amino Acid Compound	Fresh (%)	Semi-Dry (%)	Dried (%)
1.	Aspartic acid	28.99	28.71	73.11
2.	Glutamic acid	32.53	31.46	78.75
3.	Serine	13.73	13.83	32.79
4.	Histidine	1.10	1.21	5.91
5.	Glycine	13.60	8.51	27.74
6.	Threonine	13.12	12.42	31.61
7.	Arginine	13.59	12.21	33.57
8.	Alanine	13.73	19.82	45.03
9.	Tyrosine	18.25	15.89	40.85
10.	Methionine	2.52	2.25	10.38
11.	Valin	13.22	12.01	31.18
12.	Phenylalanine	13.80	13.06	33.97
13.	Ileucine	9.78	8.83	22.69
14.	Leucine	19.90	19.09	49.57
15.	Lysine	8.33	2.89	9.67

Reproduced from (Sanjaya et al., 2016)

Eight fatty acids from semi-dry samples and four fatty acids from dry samples. The dominant PUFA from fresh and dry samples was α-linoleic acid (9.74% and 16.76%, respectively), while that from semi-dried samples was arachidonic acid (10.69%). Blažinareported(that 2009) Reported the most dominant unsaturated fatty acid in *C. racemosa* was α-linoleic acid. The fat content of *C. racemosa* was very low (1.13-2.32% db), but their fatty acids had the potency to contain unsaturated fatty acids, which are essential for the body. According to Farid et al. (2013), seaweed has a very low fat content, but is rich in long-chain unsaturated fatty acids. In the green seaweed group (Chlorophyceae), the main unsaturated fatty acid was C20:5ω3 and the main saturated fatty acid was C16:0. The results in Table 5 show an increase in the relative percentage of the main fatty acids in dry seaweed. This is due to the large amount of fatty acid impurities present in fresh and semi-dried seaweed (Souza et al., 2009). The fatty acid contents of *C. racemosa* are presented in Table 4.

Table 4. Fatty Acid

No.	Fatty Acids	Fresh (%)	Semi-Dried (%)	Dried (%)
1.	Palmitic acid	15.79	11.53	38.41
2.	Linoleic acid	8.42	12.24	12.39
3.	α-Linoleic acid	9.74	8.64	16.76
4.	Arachidonic acid	7.13	10.69	3.31
5.	Oleic acid	3.10	2.40	7.58

Reproduced from (Sanjaya et al., 2016)

The protein content in *C. racemosa* was dominated by glutamic and aspartic acids. They are amino acids that play a large role in the taste of food and have a strong impact on taste (Lewis, 1962; Gunlu & Gunlu, 2014; Santoso & Yoshie, 2004). The amino acid content of dried *C. racemosa* was higher than that of fresh, and fresh and semi-dried *C. racemosa* were unstable because the moisture content of the material was still high (53-90% wb).

Sulfated polysaccharides are polyanionic linear macromolecular compounds that contain sulfate groups. The SPs fraction was isolated from the *C. racemosa* extract collected from the Gujrat Coast and analyzed for sugar content. The results showed that the main sugars were galactose, glucose, arabinose, and xylose. Sugar is widely distributed in–9-11 hemiester sulfate groups. Sulfation and methylation occur in O-6 galactose and O-3 arabinose (Da Matta et al., 2011). This polysaccharide is branched and contains 1,3- and 1,3,6-linked galactose, 1,3,4-linked arabinose, 1,4-linked glucose, and terminal- and 1,4-linked xylose residues (Ragasa et al., 2015). Research has demonstrated that Racemosin C, an alkaloid present in the green alga Caulerpa racemosa, has anti-inflammatory properties. Specifically, it suppresses the activity of protein tyrosine phosphatase 1 B (PTP1B), which plays a detrimental role in regulating insulin and leptin signaling. Such inhibition can result in increased insulin and leptin activity, which may diminish inflammation and enhance metabolic well-being (Dissanayake et al., 2022). Furthermore, racemose C has been investigated for prospective therapeutic use, particularly in relation to inflammatory disorders. This intervention modulates the signaling

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E-ISSN: 2580-8303

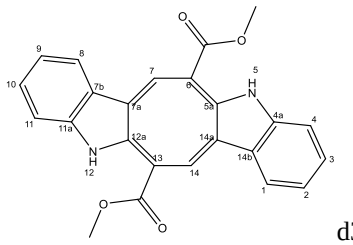
pathways that play a critical role in regulating inflammatory responses, including the NF-κB pathway (Souza et al., 2020). Squalene has been shown to decrease the synthesis of inflammatory mediators, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and prostaglandin E2 (PGE2) in RAW macrophages stimulated by lipopolysaccharide (LPS) (Fernando et al., 2018).

Table 6. Alkaloids and terpenoids compound

No.	Compound	Presence	Reference
Alkaloid			
1.	Caulerchlorin	+	(Liu et al., 2013)
2.	Caulerprenylols A	+	(Liu et al., 2013)
3.	Caulerprenylols B	+	(Liu et al., 2013)
4.	Racemosin A	+	(Liu et al., 2013)
5.	Racemosin B	+	(Liu et al., 2013)
6.	Racemosin C	+	(Liu et al., 2013)
7.	Caulerpin	+	(Ornano et al., 2014)
Terpenoid			
1.	racemobutenolids A, B	+	(Yang et al., 2015)
2.	4,5-dehydrodiodictyonema A	+	(Yang et al., 2015)
3.	α-tocopheroid	+	(Yang et al., 2015)
4.	Squalene	+	(Ragasa et al., 2015)

Environmental factors that affect seaweed growth include temperature, salinity, pH, sunshine, physiological conditions, and CO_2 availability. This is because of different adaptation strategies. The different physiological adaptive properties of seaweeds affect the number of unique structural centers in secondary metabolites, including alkaloids, quinones, polyketides, polysaccharides, cyclic peptides, diterpenoids, glycerol, lipids, and flavonoids. Chlorophytian seaweeds are widely distributed in the intertidal zones.

The anti-inflammatory characteristics of caulerpin make it a highly promising candidate for the advancement of innovative therapeutic medications, particularly for the treatment of diverse inflammatory disorders (Souza et al., 2009). The chemical structure of caulerpin is shown in Figure 3.



d3

Figure 3. Structural of Caulerpin as Anti-Inflammatory

Tumor necrosis factor-alpha (TNF-α), interleukin 1β (IL-1β), and interleukin 6 (IL-6) are pro-inflammatory cytokines released by macrophages in response to foreign antigens. These cytokines promote antigen removal and tissue repair by enhancing chemotaxis of mast cells, granulocytes, lymphocytes, and monocytes to the site of injury. However, greater infiltration and activation of these cells increases the possibility of tissue damage because of exaggerated inflammation and its primary symptoms, edema, and pain. During chronic inflammation, an increase in the expression of proinflammatory mediators was observed. iNOS, COX-2, TNF-α, IL-1β, IL-6, and Prostaglandin E2 (PGE2) are primary mediators of inflammation. Changes in cytokine levels may have an impact on cellular reactions and may be related to the anti-inflammatory properties of phytochemicals. NO is another mediator of the inflammatory processes. Inflammation protects against NO levels. However, elevated NO generation also results in cytotoxicity and tissue damage under some clinical circumstances. Cellular responses are governed by a complex network of signaling pathways that govern these processes (Sanniyasi et al., 2023).

T cell activation triggers the production of a diverse range of lymphokines such as interleukin-2 and interferon-γ (IFN-γ). A variety of white blood cells are stimulated to develop, differentiate, and become activated B cells. Overwhelming inflammation gradually ruins the structure of healthy tissue and damages organs. As a result, the demand for safe oral medications with anti-inflammatory properties has increased. Extensive research has been conducted on the qualities of caulerpin, including its antioxidative, anticoagulant, anticancer, anti-inflammatory, and antiviral effects, is one potential contender.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

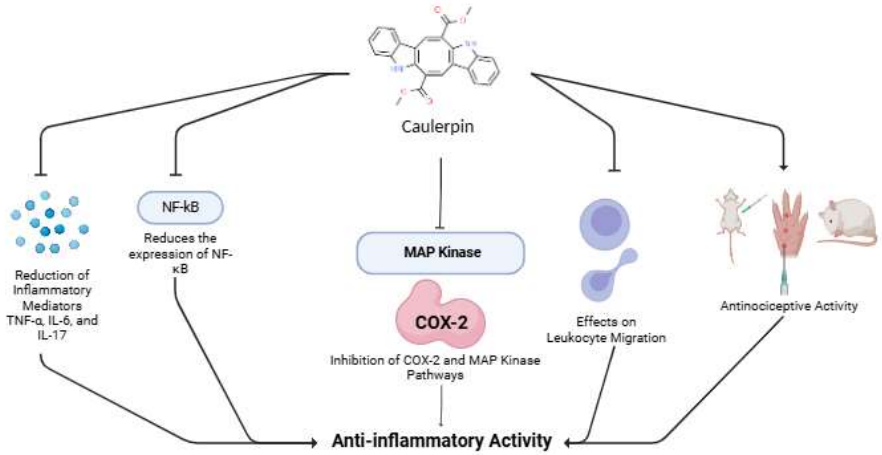


Figure 4. Anti-inflammatory effect of caulerpin

Extensive studies have been conducted on its benefits in malignancies, ischemia, immunological dysfunction, and inflammatory illnesses. Its potential to reduce inflammation may be attributed to a reduction in NF-κB signalling pathway activity (De Souza et al., 2009;Lucena et al., 2018). The function of the mitogen-activated protein kinase (MAPK) cascade in the biological effects of fucoidan has been covered by other studies. Many mammalian cells contain serine/threonine protein kinases, which are members of the MAPK family. The MAPK cascade is involved in gene expression, cell proliferation and differentiation, neuronal survival, and apoptosis, according to numerous studies. Every major MAPK pathway primarily contributes to a specific set of processes. For example, the p38 MAPK pathway controls the production and release of pro-inflammatory mediators; the ERK pathway must be activated for cells to proliferate, survive, and differentiate; and the JNK pathway controls apoptosis (Huang & Ferrell, 1996). Alkaloid Caulerpein substance's mechanism inhibits inflammation with different targets mentioned in the cascade as can be seen in Figure 4.

The anti-inflammatory activities of caulerpine have been thoroughly investigated, and multiple studies have presented comprehensive information on the methodology employed in these tests. The dosage of caulerpine administered orally in in vivo experiments employed different inflammatory models, including carrageenan-induced peritonitis, was 100 µmol/kg. A 100 µmol/kg dose of caulerpine was administered orally to the capsaicin-induced ear edema model (De Souza et al., 2009). In peritonitis and ulcerative colitis models, the efficacious doses of caulerpine were 40 and 4 mg/kg, respectively, administered orally (Schiano et al., 2022). Caulerpin can affect the inflammatory process in many phases, including apoptosis, inhibition of multiple enzymes, and the prevention of lymphocyte adhesion and invasion. The most well-discussed mode of action of caulerpin involves suppression of the NF-κB and MAPK signaling pathways, which lowers the generation of proinflammatory cytokines (Wu et a*l*., 2022), as shown in Figure 3.

Despite extensive reports on the anti-inflammatory properties of caulerpin, several studies have shown an increased generation of pro-inflammatory cytokines. This compound slowed the natural apoptosis of human neutrophils, natural killer cells (NK), and pro-inflammatory cytokines (IL-6, IL-8, and TNF-α). Caulerpin sourced from Fucus vesiculosus facilitates several immunological responses, including Th1 immunity, memory T-cell generation, antigen-induced antibody production, and dendritic cell maturation. Additionally, Caulerpin may stimulate the immune system. Souza et al. (2020) showed how Caulerpin interacts with "toll-like receptors" (TLRs), increasing the expression of MHC molecules and the synthesis of chemokines and cytokines. Increased activity of innate and specialized immune cells is an outcome. The innate immune system and chemicals that bind to TLR to activate the NF-κB signaling cascade are known to have toll-like receptors. Caulerpin improves the immune response by binding to TLR-2 and TLR-4 but not TLR-5 (Huang & Ferrell, 1996).

CONCLUSION

This systematic review shows that Caulerpa is a genus of green seaweed that has been proven to have anti-inflammatory activity across various plant parts and types of herbal medicinal preparations used in experimental settings. The purported anti-inflammatory effect of sea grapes may be attributed, in part, to their capacity to impede the accumulation of pro-inflammatory cytokines at the site of inflammation.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

ACKNOWLEDGMENT

This research was supported by International Research Collaboration Top Over #500 at year 2024 and also the author would like to thank for the support given from the Master Program of Pharmaceutical Sciences Faculty of Pharmacy, Univeritas Airlangga.

AUTHOR CONTRIBUTIONS

Conceptualization, R.W.; Methodology, R.W., S., S.D.; Software, E.W.P., I.S.; Validation, R.W., S., S.D.; Formal Analysis, E.W.P., I.S.; Investigation, E.W.P., I.S.; Resources, E.W.P., R.W.; Data Curration; E.W.P., I.S.; Writing - Original Draft, E.W.P.; Writing - Review & Editing, R.W., S., S.D.; Visualization, E.W.P., I.S.; Supervision, R.W., S., S.D.; Project Administration, R.W., S., S.D., I.S.; Funding Acquisition, R.W., S.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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P-ISSN: 2406-9388
E-ISSN: 2580-8303

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia
Pharmacy and Pharmaceutical Sciences Journal
Published by Faculty of Pharmacy Universitas Airlangga
OPEN ACCESS
E-ISSN 2580-8303
P-ISSN 2406-9388

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia Vol. 11 No. 2 August 2024, 174-183
DOI: 10.20473/jfiki.v11i22024.174-183
Available online at https://e-journal.unair.ac.id/JFIKI/

Adverse Drug Reactions Reporting Profile in Tertiary Referral Hospital: A Retrospective Pharmacovigilance Study in Indonesia

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Submitted: 20 June 2024
Revised: 17 August 2024
Accepted: 31 August 2024

Abstract

Background*: Pharmacovigilance is administered to several pharmacological classes of drugs worldwide. However, there are still insufficient data regarding the prevalence and general characteristics of drug reactions, especially in developing countries.* ***Objective****: This study aimed to determine the prevalence and characteristics of ADRs, including the pharmacological class involved, and report and classify the clinical manifestations associated with ADRs.* ***Methods****: This retrospective study was based on patient ADR reports during observation. Prevalence, patient demographics, and other data were evaluated using descriptive statistics.* ***Results:*** *Of 773 reports that met the inclusion criteria, most were doctors (80.6%), followed by pharmacists (18.7%). Of the total cases, 430 (55.6%) occurred in the women. Most suspected ADRs occurred in the 19-60 years age group (583; 75.4%). The highest incidence of ADR was observed in patients using antineoplastic agents (19.5%), systemic antibacterials (16.4%), or antihypertensives (12.5%). The majority of clinical manifestations were gastrointestinal disorders (41.7%), and approximately 309 (40%) ADR cases continued with antagonists/antidotes. Approximately 62% of the patients who experienced ADRs recovered.* ***Conclusion:*** *Antineoplastic, systemic antibacterial, and antihypertensive drugs appeared to be the most common drugs used for suspected ADR cases in this hospital. ADR reporting has been running well, but not all healthcare workers have participated actively. Hopefully, the results of this research will contribute to the upcoming strategies for pharmacovigilance activities in this hospital and other healthcare facilities to improve the quality and quantity of ADR reporting and increase the safety of medication usage.*

Keywords*: adverse drug reaction, adverse drug reaction reporting, ADR reporting, pharmacovigilance*

How to cite this article:
Sari, C. M. & Suprapti, B. (2024). Adverse Drug Reactions Reporting Profile in Tertiary Referral Hospital: A Retrospective Pharmacovigilance Study in Indonesia. *Jurnal Farmasi dan Ilmu Kefarmasian Indonesia*, 11(2), 174-183. http://doi.org/10.20473/jfiki.v11i22024.174-183

P-ISSN: 2406-9388
E-ISSN: 2580-8303

INTRODUCTION

Adverse drug reactions (ADR) are drug-related problems that require special attention from health care workers. The increasing frequency and severity of ADRs are related to the worsening health status of patients, a significant increase in the healthcare burden due to prolonged hospitalization periods, and the need for additional therapy to treat the complaints and symptoms experienced by patients (Giardina et al., 2018). ADR monitoring guidelines for healthcare workers state that ADR is an undesirable response to drugs that occur at the usual doses in humans for the purpose of prevention, diagnosis, disease therapy, or to modify physiological functions (BPOM RI, 2012).

According to various studies, ADR is an important cause of morbidity and mortality in healthcare facilities. A systematic review including many studies around the world and research in other countries showed that approximately 10% of hospital admissions are related to ADRs (Yadesa et al., 2021). Moreover, ADRs are suspected to be one of the main causes of death and escalation in healthcare costs (Montastruc et al., 2021). However, the development of drugs and several new therapeutic agents makes ADR monitoring a necessity that should be considered daily to evaluate the safety of distributed drugs worldwide (Montastruc et al., 2021).

During preclinical and clinical trials in humans, selected subjects were used with certain strict criteria and limited samples in a completely different setting with daily clinical practice, so sometimes it did not adequately describe the drug's safety profile in humans because the ADRs detected in these phases were likely common ADRs with a high frequency of occurrence. Chronic toxicity, potential drug interactions, and drug safety in special groups (children, pregnant or breastfeeding women, and geriatrics) are very difficult to determine in the development and research phase before the drug receives marketing authorization (Tadge et al., 2023). Therefore, ADR reporting is a crucial tool for detecting the possibility of serious and rare ADRs associated with therapeutic agents, so that patients will receive better intervention earlier, prevent further medical injury and harm, and avoid the emergence of greater risk problems in drug use. Moreover, it can be used as a consideration for drug regulation, policies for withdrawals and distribution permits, and changes to the safety information listed on drug packaging (BPOM RI, 2019; Tadge et al., 2023).

Based on Indonesia's National Pharmacovigilance Data Center, from January to early December 2023, 11,084 ADR reports were received from various healthcare facilities and pharmaceutical industries in Indonesia, but it was thought that there are still many more underreported ADRs (BPOM RI, 2023). In contrast, approximately 21, 336 drugs have been registered over the last five years in Indonesia (BPOM RI, 2022). Ideally, ADR reporting should be performed for all drugs distributed and circulating in Indonesia. However, ADR reporting was not mandatory for healthcare workers because a voluntary reporting system was adopted, which was manually sent using an ADR reporting form or digitally entered on the E-MESO website. Therefore, the number of reports is still quite small compared to the total number of distributed drugs. In line with this, a systematic review analyzed 37 studies conducted in different countries and found that the rate of underreporting of ADRs exceeded 90% in many cases, showing that widespread and significant underreporting of ADRs is a global problem and affects all types of ADRs (Al Meslamani, 2023).

In a study on ADR prevalence worldwide, 85% of reports came from developed countries such as the United States, England, France, Germany, Canada, and Australia (Aagaard et al., 2012). In developing countries, including Indonesia, various studies have been conducted on the ADR of several pharmacological classes, such as chemotherapeutic agents (Melani, Darmawan and Raharjo, 2019), anti-diabetic (Yosmar, Inanta and Sari, 2018), anti-hypertensive (Indriani, Rokhmah and Shania, 2022), anti-tuberculosis (Rini, Ikawati and Perwitasari, 2014), anti-retroviral (Pertiwi, Wardani and Wedayani, 2021), analgesic-anti-inflammatories (Permata and Azmi, 2024), and cardiovascular drugs (Almasdy et al., 2018). However, there is insufficient information available regarding ADR prevalence and characteristics in healthcare facilities, particularly tertiary referral hospitals that manage complex multidisciplinary cases involving polypharmacy with diverse therapeutic classes and high-risk medications. Therefore, a retrospective pharmacovigilance study was conducted using ADR reports. This study aimed to ascertain the prevalence and characteristics of ADRs in hospitalized and ambulatory patients, including the pharmacological classes involved, and document and categorize the clinical manifestations associated with ADRs.

MATERIALS AND METHODS

Study design

This retrospective study was based on ADR reports at 2 years and 10 months from January 2021 to October

P-ISSN: 2406-9388
E-ISSN: 2580-8303

2023 at the Saiful Anwar General Hospital, Malang, East Java, Indonesia.

Instrument and data analysis

Data were obtained from inpatient ADR reports during the observational period. ADR reports were collected manually in yellow and digitally using an internal ADR reporting link. The inclusion criteria were completeness of ADR reports, including patient demographics, manifestations of ADR, suspected drugs, chronology of events, and outcome of ADR.

The data obtained included the number of reports per month, reporters, demographic characteristics of the patient (age, sex), history of disease and comorbidities (if any), history of previous drug allergies (if any), main diagnosis, number of drugs received when experiencing ADR, drugs suspected, ADR clinical manifestations, patient follow-up, and outcome. Other medications used by patients (if any) were also included in the report. The actions taken to treat ADR are grouped into four categories: continuing the drug with an antagonist/antidote, continuing the drug without the antagonist/antidote, stopping the drug with the antagonist/antidote, and stopping the drug without the antagonist/antidote.

The main diagnoses and clinical manifestations of ADRs were grouped using the Medical Dictionary for Regulatory Activities (MedDRA)® system and classified according to System Organ Class (SOC). Drugs suspected to cause ADR are categorized using the Anatomical Therapeutic and Chemical (ATC) group (2^{nd} level) (Giardina et al., 2018). Patient demographics and ADR reporting data were evaluated using descriptive statistics.

RESULTS AND DISCUSSION

During the observation period, there were 773 cases reported as ADRs, 178 (0.68%) in 2021, 301 (0.95%) in 2022, and 294 (0.92%) in 2023. The number of reports was still small compared to the total number of patients, whereas previous research stated that out of 3695 episodes of hospital stay, approximately 15% of inpatients experienced at least one ADR during their inpatient period (Davies et al., 2009). This is in line with the relatively low number of ADR reports in Indonesia, as in other developing countries (Al-Worafi et al., 2017). However, the total number of reports received by the Indonesian National Pharmacovigilance Center has significantly increased. In 2022, the number of national ADR reports reached more than 10,000 from healthcare facilities all over Indonesia, an increase of 53% compared to the average number of reports for the past five years (BPOM RI, 2023). This is probably a positive sign of underreporting, which was a limitation of the spontaneous ADR reporting system implemented in Indonesia because it was estimated that only 6-10% of ADRs were reported from the actual number. A systematic review examined factors that influence ADR reporting among healthcare workers. The results showed that the socio-demographic characteristics of healthcare workers did not significantly influence ADR underreporting, but several other factors that mattered were the wrong assumption (only serious ADRs need to be reported), apathy (delayed reporting, lack of interest in reporting), complacency (the assumption that all drugs must be safe and well-tolerated), fear of being thought strange if reporting a predictable ADR, and feelings of insecurity (feeling that it is almost impossible to determine whether a drug is the suspected cause of a specific ADR). In addition, the absence of reporting obligations and confidentiality is another reason for low ADR reporting rates (García-Abeijon et al., 2023).

In this study, the largest number of ADR reporters was dominated by residents and physicians (80.6%), followed by pharmacists (18.7%) and other healthcare workers, such as nurses and midwives (0.7%). According to the ADR Reporting Guidelines in Indonesia, all healthcare workers are allowed to report ADR (BPOM RI, 2012). Residents and doctors were the most frequently reported ADRs. This is probably because of the obligation to report ADRs as academic assignments in some medical residency programs. The second most frequent reporters were pharmacists, especially ward pharmacists and ambulatory pharmacists. This is in line with their competence in monitoring the safety and efficacy of patients' medications, but more reports should be collected due to the availability of clinical pharmacists in each hospital ward. The nurses and midwives were the least frequently reported. According to previous research, barriers for nurses to report ADRs include lack of time and heavy workload, unawareness of the reporting procedure, insecurity to make the wrong report, and fear of being accused (Adu-Gyamfi et al., 2022). Furthermore, it has been found that the knowledge and implementation of pharmacovigilance among healthcare workers is quite low; therefore, continuous socialization regarding this matter is urgently needed (Wangge and Akbar, 2016).

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Table 1. Characteristics of study subjects

Characteristics	Number of Cases (n=773)	% Percentage
Age Group (years)		
0-18	31	4.0
19-60	583	75.4
> 60	159	20.6
Sex		
Males	343	44.4
Females	430	55.6
Main Disease Categories[a]		
Infections and infestations	181	23.4
Benign, malignant and unspecified neoplasms	179	23.2
Renal and urinary disorders	102	13.2
Blood and lymphatic system disorders	87	11.3
Immune system disorders	62	8.0
Gastrointestinal disorders	24	3.1
Hepatobiliary disorders	24	3.1
Psychiatric disorders	22	2.8
Hypertensive	16	2.1
Endocrine disorders	14	1.8
Cardiac disorders	13	1.7
Vascular disorders	13	1.7
Musculoskeletal	12	1.6
Nervous system disorders	6	0.8
Respiratory, thoracic and mediastinal disorders	6	0.8
Reproductive system and breast disorders	6	0.8
Eye disorders	2	0.3
Skin and subcutaneous tissue disorders	2	0.3
Metabolism and nutritional disorders	1	0.1
Dental Impaction	1	0.1
Comorbidities[b]		
Geriatric	159	25.7
Hypertension	109	17.6
Infection	79	12.8
Renal impairment	72	11.6
Cardiovascular disorders	52	8.4
Diabetes mellitus	47	7.6
Myelosuppression	20	3,2
Hypoalbumin	16	2.6
Electrolyte imbalance	16	2.6
Malignancy	12	1.9
Autoimmune	10	1.6
Previous history of drug/food allergies	8	1.3
Hepatic impairment	7	1.1
Blood disorders	6	1.0
Underweight	5	0.8
Hyperthyroid	1	0.2
Total	**619**	**100%**
Number of Drugs Taken		
≤ 4	722	93.4
5-9	51	6.6
≥ 10	0	0
Total ADR Reports obtained	**773**	

[a] Main disease: a diagnostic which caused a patient received medication suspected for ADR

[b] Comorbidities: any medical condition other than the main disease

P-ISSN: 2406-9388
E-ISSN: 2580-8303

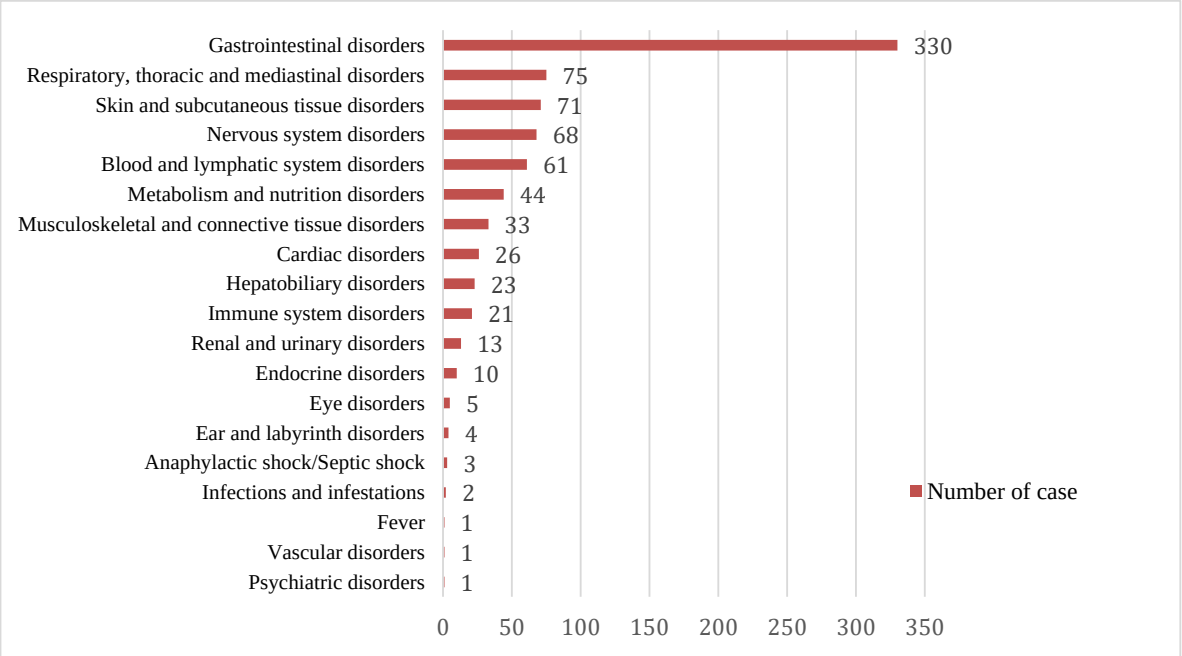

Figure 1. Clinical Manifestation of Suspected ADRs classified by SOC according to MedDRA®

Patient demographic characteristics are presented in Table 1. Of all cases, 430 cases of ADR were experienced by women, and the rest were men (343); therefore, women tended to experience more ADRs than men (55,6% vs 44.4%). This is in line with global post-marketing surveillance data on spontaneous reports, which indicates that women, especially in their reproductive years, have more ADRs than men (Watson et al., 2019). Previous studies have also stated that women have an approximately two-fold higher risk of ADRs than men. Several studies have also reported the existence of a specific pattern and relationship between sex, pharmacokinetic-pharmacodynamic parameters, and ADR incidence. In general, women have a lower body weight and organ size but a higher percentage of body fat, which affects the absorption and distribution of drugs. The larger the volume of distribution (Vd), the more likely it is that the drug will be found in body tissue. Research has shown that 86 types of FDA-approved drugs result in increased drug levels and longer drug elimination times in women than in men, making them a greater potential for ADR incidence (Zucker and Prendergast, 2020). Another study in Indonesia found that female patients were more likely to experience ADR to oral hypoglycemic drugs than were male patients (Yosmar et al., 2018).

The largest age group exposed to ADR was adults (19-60 years), which is in line with previous studies (Gupta et al., 2017; Keche et al., 2021). Geriatrics aged > 60 years were in the second position with 159 cases (20.6%), followed by pediatrics with 31 cases (4%), as shown in Table 1. Approximately a quarter of the geriatric patient population admitted to the hospital experienced at least one type of ADR during their period of hospitalization (Yadesa et al., 2021). Various physiological changes occur in geriatrics, including changes in the pharmacokinetic and pharmacodynamic responses to drugs inside the body, making them more susceptible to ADR (Yadesa et al., 2021). Reduced organ perfusion also implies deprivation of liver function, causing a decline in the hepatic clearance of certain drugs. In addition, along with the aging process, kidney function and muscle mass decrease, so the glomerular filtration rate decreases even though serum creatinine levels are within the normal range (Corsonello, Pedone, and Incalzi, 2010). Apart from physiological changes, various degenerative diseases in geriatric patients could trigger polypharmacy in their therapeutic management, which was also associated with a higher risk of ADR in this age group. On the other hand, the occurrence of ADR in geriatric patients could reduce patient compliance and detain the expected therapeutic outcomes, resulting in a higher burden and cost in healthcare services (Yadesa et al., 2021).

The top five main diagnoses were infectious diseases (23.4%), malignancies (23.2%), kidney and urinary tract disorders (13.2%), blood and lymphatic system disorders (11.3%), and immune system disorders (8%). Approximately 23.4% of patients presented with hypertension as a comorbidity, 17% had infections, 15.5% had kidney impairment, 11.2% had other cardiovascular disorders, and 10.1% had diabetes mellitus. Ferner and Aronson (2019) stated that diseases can affect the absorption, distribution, metabolism, and

P-ISSN: 2406-9388
E-ISSN: 2580-8303

elimination of drugs, particularly those related to kidney and hepatic impairment. Higher drug concentrations can occur due to reduced hepatic metabolism and renal elimination, leading to a higher chance of ADR manifestation (Ferner and Aronson, 2019). Furthermore, the influence of other diseases and conditions remains poorly explored. Of all the patients, 93.4% received 1–4 medications during the hospitalization period. The concurrent use of medication and drug–drug interactions is well established and is an important cause of avoidable ADRs (Ferner and Aronson, 2019). Therefore, it is highly recommended that healthcare providers monitor any potential or major drug-drug interactions.

Most ADR were gastrointestinal disorders (330, 41.7%), followed by respiratory tract disorders (75, 9.5%), skin and subcutaneous tissue disorders (71, 9%), neurological disorders (68, 8.6%), and blood and lymphatic system disorders (61, 7.7%) (Figure 1). Among all ADR reports, antineoplastic agents (19.5%) were in the first rank of suspected drugs, followed by systemic antibacterials (16.4%), antihypertensives (12.5%), analgesics/anti-inflammatory drugs (9.6%), and antituberculosis drugs (8.9%) (Figure 2). The top five classes of suspected drugs for ADR were in line with the pharmacovigilance data of 2022 in Indonesia, which mentioned the top 10 suspected drugs for ADR, namely antituberculosis, systemic antibacterial, and antineoplastic drugs (BPOM RI, 2023). Aagaard et al. (2012), who examined ADR patterns reported worldwide over the last 10 years (2000-2009), also found that in developed countries, the highest prevalence of ADR was found in antineoplastic and immune system-related drugs, whereas in developing countries, the highest prevalence of ADR was found in systemic antibacterials (Aagaard et al., 2012). Other studies have reported that antibacterials contribute to 33-68% of ADR incidents (Keche et al., 2021). However, chemotherapeutic agents are known to cause potentially serious ADR. For example, in a study conducted on more than thousand chemotherapy patients in France, almost half experienced ADR (Ingrand et al., 2020).

In 40% (309) of ADR cases, the suspected drugs were continued with antagonists/antidotes, such as urticaria and diarrhea manifestation due to afatinib. Afatinib was continued with antihistamines and supportive therapy for diarrhea. In 246 (31.8%) cases, the drugs were stopped without antagonists/antidotes, for example, in toxic optic neuropathy due to linezolid toxicity in drug-resistant tuberculosis patients. In another case, prolonged QT interval was suspected due to Levofloxacin, Bedaquiline and Clofazimin. The drugs were discontinued, the patient's heart rhythm was monitored periodically, and the anti-tuberculosis regimen was changed without any addition of antagonists/antidotes. Furthermore, in 143 (18.5%) cases, the suspected drugs were stopped with antagonists/antidotes, such as in bleeding manifestations due to Warfarin and Clopidogrel, the drugs were stopped, and the patients were given Vitamin K injection as a warfarin antagonist and Tranexamic Acid as an antifibrinolytic. However, in 75 (9.7%) patients, the drugs were continued without antagonists/antidotes. In these cases, patients generally showed improvement without specific antagonist/antidote or the symptoms improved with dose reduction, so the drug could be continued with consideration of greater benefits, for example, in constipation cases due to bortezomib injection in multiple myeloma patients and hypokalemia due to furosemide. actions taken during the follow-up of ADRs are shown in Figure 3.

Regarding ADR outcomes, most patients (482; 62%) recovered, while the remaining patients (163; 21.1%) recovered with residual symptoms, had not recovered yet (131; 13.1%), or had unknown outcomes (15; 1.9%) because the patients were moved to another ward or the data were incomplete. Unfortunately, 1.5% (12 cases) of patients died due to progression of the main disease and poor prognosis (Figure 4).

This study was conducted retrospectively using ADR report data history; therefore, the limitation of this study was that only the available data archives with all of their limitations could be analyzed. Most ADR reports collected lacked details describing the chronology of ADR occurrence, making causality analysis difficult. In fact, some improvements and adjustments were required for the internal reporting links so that the ADR reports collected would be more complete and reliable; thus, they could be analyzed comprehensively in the future. We hope this article adds to the information on pharmacovigilance data in Indonesia, particularly data from tertiary hospitals. In addition, it is hoped that healthcare workers as professional care providers will take an active role in detecting and reporting ADR incidence to collect more drug post-marketing surveillance data and to enhance drug safety monitoring in Indonesia.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

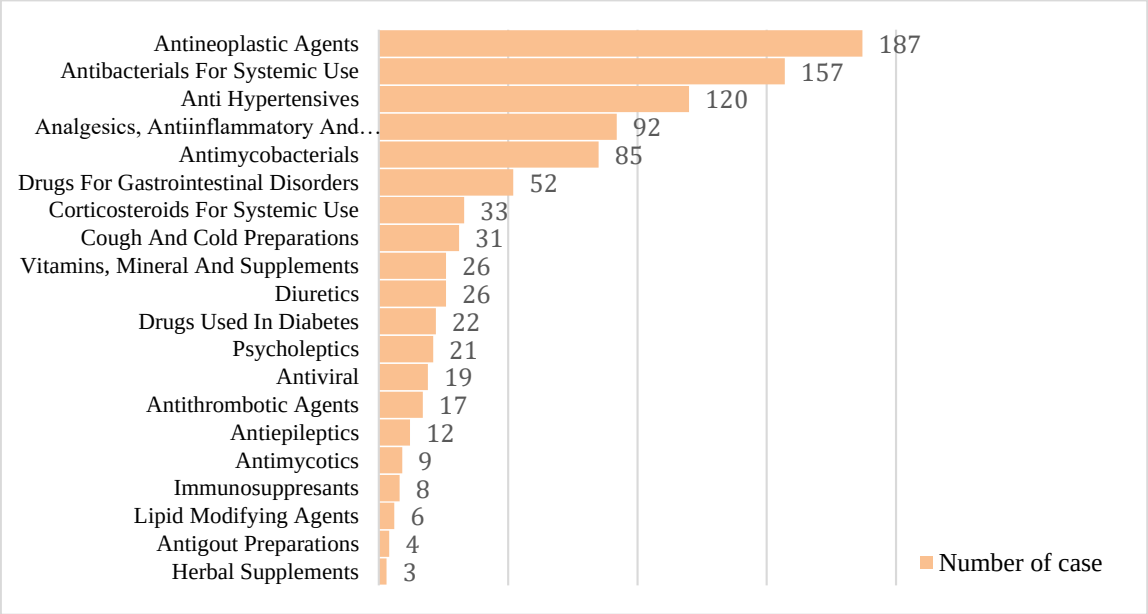

Figure 2. Drug Classes Suspected for ADRs classified by ATC code 2nd level

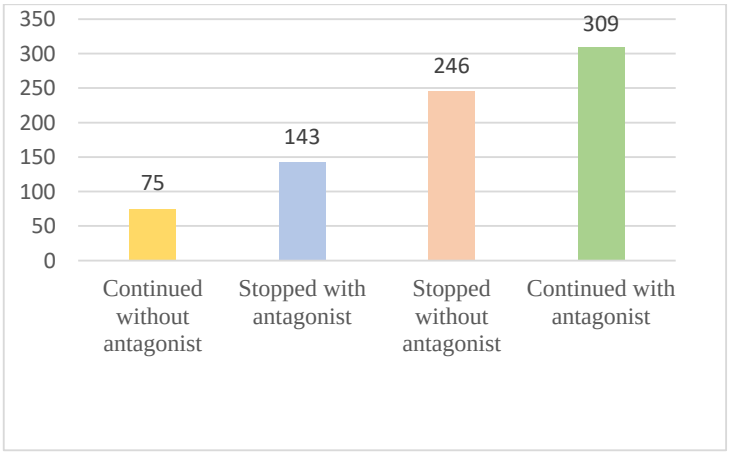

Figure 3. Follow up to suspected ADRs

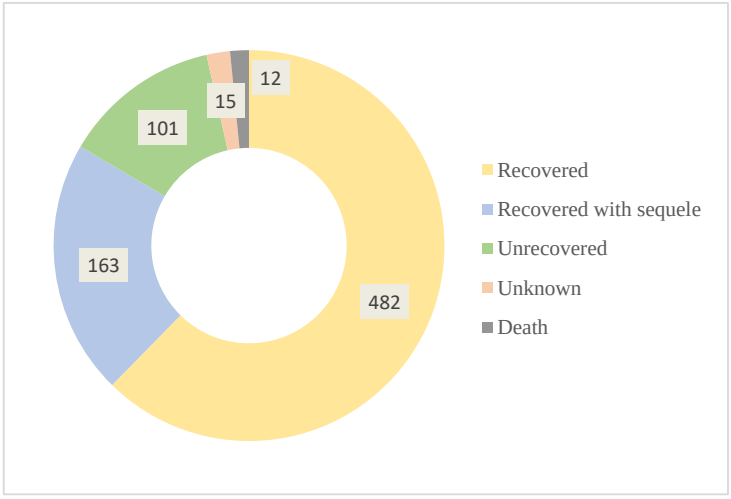

Figure 4. Output of Suspected ADRs

P-ISSN: 2406-9388
E-ISSN: 2580-8303

CONCLUSION

Antineoplastic, systemic antibacterial, and antihypertensive drugs appeared to be the most common drugs for suspected ADR in this hospital. ADR reporting has been running well, but not all healthcare workers have participated actively. Most ADRs manifest as gastrointestinal, respiratory, or subcutaneous skin disorders. Hopefully, the results of this research will contribute to upcoming strategies for pharmacovigilance activities in this hospital and other healthcare facilities to improve the quality and quantity of ADR reporting, especially in Indonesia, to increase the safety of medication usage.

ACKNOWLEDGMENT

The authors are grateful to the ADR Reporting Team and the Head of the Pharmacy Department at Dr. Saiful Anwar General Hospital for permission to use the data provided in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, C.M.S., B.S.; Methodology, C.M.S.; Software, C.M.S.; Validation, B.S.; Formal Analysis, C.M.S.; Investigation, C.M.S.; Resources, B.S.; Data Curration; B.S.; Writing - Original Draft, C.M.S.; Writing - Review & Editing, B.S.; Visualization, C.M.S.; Supervision, B.S.; Project Administration, C.M.S., B.S.; Funding Acquisition, C.M.S., B.S.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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P-ISSN: 2406-9388
E-ISSN: 2580-8303

Yosmar, R., Inanta, N.P. and Sari, Y.O. (2018) 'Studi Prospektif Adverse Drug Reactions (ADRS) Obat Hipoglikemik Oral Terhadap Pasien Diabetes Mellitus Tipe 2 di Suatu Rumah Sakit, Padang', *Jurnal Sains Farmasi & Klinis*, 5(3), pp. 169–175. Available at: http://jsfk.ffarmasi.unand.ac.id

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P-ISSN: 2406-9388
E-ISSN: 2580-8303

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia
Pharmacy and Pharmaceutical Sciences Journal
Published by Faculty of Pharmacy Universitas Airlangga
OPEN ACCESS
E-ISSN 2580-8303
P-ISSN 2406-9388

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia Vol. 11 No. 2 August 2024, 184-191
DOI: 10.20473/jfiki.v11i22024.184-191
Available online at https://e-journal.unair.ac.id/JFIKI/

An ABC-VEN Analysis for Outpatient Medicines Use in the Department of Internal Medicine at Universitas Airlangga Teaching Hospital

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Submitted: 29 June 2024
Revised: 17 August 2024
Accepted: 31 August 2024

Abstract

***Background**: Application of the ABC-VEN method in evaluating drug planning can increase efficiency and ensure optimal medicine availability and stable access to medications. **Objective**: To analyze ABC-VEN combinations to examine the profile of medicine use in the internal medicine department. **Methods**: This was an observational study with retrospective prescription data from outpatients in the Internal Medicine Department from January to March 2020. Collected data included the type, number of medicines, and medicine prices. Patients undergoing chemotherapy and retroviral therapy for HIV were excluded from the study. Subsequently, an ABC-VEN analysis was performed. **Results**: Of 4,242 prescription samples, 188 types of medicines were used. Based on the drug use evaluation with ABC analysis, category A contained 23 items (12.17%), category B contained 35 items (18.52%), and category C contained 130 items (69.31%). The ABC analysis for investment value found that category A contained eight items (4.23%), category B contained 22 items (11.64%), and 158 items (84.13%). Based on the VEN analysis, Group V had six medicine items, Group E had 152 medicine items, and Group N had 30 medicine items. The ABC-VEN investigation showed that there were eight, 151, and 29 items of medicines in Categories I, I, and III, respectively. **Conclusion**: Although there are medicines that are highly used, their investment value is quite low. The use of the ABC-VEN method to evaluate medicine use is crucial for organizing and controlling the medicine supply.*

***Keywords**: ABC-VEN analysis, drug usage, investment, internal medicine department*

How to cite this article:
Norachuriya, Z., Suprapti, B., Ratri, D. M. N., Nugroho, C. W. & Safari, Y. (2024). An ABC-VEN Analysis for Outpatient Medicines Use in the Department of Internal Medicine at Universitas Airlangga Teaching Hospital. *Jurnal Farmasi dan Ilmu Kefarmasian Indonesia*, 11(2), 184-191. http://doi.org/10.20473/jfiki.v11i22024.184-191

P-ISSN: 2406-9388
E-ISSN: 2580-8303

INTRODUCTION

Inventory planning is a management system designed to determine how many items need to be ordered, when to order, and how many items are stored in the inventory (Fahriati et al., 2021). In developing countries, including Indonesia, the largest component of hospital spending is the budget for medicines, which accounts for approximately 40– 50% of the total cost (Karauan, 2022). However, the available funds do not always match needs, so the procurement of medicines needs to be economical to minimize expenses. Therefore, efficient and cost-effective budgeting is necessary to balance supply expenditure with drug needs (Deressa et al., 2022). Healthcare providers arrange the distribution of medicines according to their needs. In these situations, medical availability is essential to ensure that patients take them as prescribed.

Furthermore, medicine serves as a mediator between patients and health care providers to promote public confidence in this service (Rahem et al. 2021). The availability of the medicine stock is important. Therefore, it is necessary to maintain sufficient stock levels to ensure that the supply chain is not disrupted (Mfizi et al. 2023).

Several inventory management techniques have been used to analyze medicine use in pharmacy services (Mani et al., 2018). The most compelling analysis used in material management is the ABC analysis, according to Pareto's law, which states that as much as 80% of the overall value can represent 20% of the number of products (Antonoglu et al., 2017). This analysis classifies medicine use categories into three categories: category A, with a percentage of 10-20% representing 70-80% cumulative value (cost); category B, with a percentage of 10–20% representing 15–20% depicting a cumulative value; and category C, the percentage covering 60–80% of items representing 5–10% cumulative value (Migbaru et al., 2016).

The method used to help determine the priority level of medicine purchases and maintain the amount of medicine storage uses the VEN analysis. The VEN analysis is classified into three categories: vital, essential, and non-essential (Sharma et al., 2018). Vital classes (V) are medicines that are life-saving, consumed regularly, and must always be in stock; essential classes (E) are medicines in cases that are non-life-saving; and non-essential classes (N) are medicines in the therapy class for mild illnesses (Deressa. et al., 2022).

ABC and VEN analyses, when applied separately, are sometimes inadequate because of their limitations. Therefore, combining the ABC-VEN method is highly recommended to overcome this limitation, and medications should be split into groups. ABC and VEN analyses have successfully encouraged the employment of every technique to enhance others (Mohammed et al., 2020). Group I (AV, BV, CV, AE, and AN) includes vital medicines and medicines with high investment values. Group II consisted of medications included in categories E and B (BE, CE, BN), while those included in group III were medicines that were non-essential and had low prices (CN). Category III consists of a group of non-essential (desirable) goods and a group of affordable goods (CN) (Devarajan et al., 2016). Category I medicine must be constantly observed and managed; periodic inspections are necessary for Category II, but not Category III (Deressa et al., 2022).

ABC-VEN analysis, which stands for "Always, Better, Control-Vital, Essential, Non-Essential," provides a deep understanding of medicine management by identifying essential medicines, measuring consumption, and effectively controlling supply. This study aimed to analyze the use of medicines using ABC-VEN analysis in JKN outpatients of the Internal Medicine Department at Airlangga University Hospital.

MATERIALS AND METHODS

Materials

The study data were obtained from the outpatient prescriptions. The Ethics Committee of Airlangga University Hospital reviewed the research methodology and decided that it was ethically approved based on the Ethics Certificate Review Number 002/KEP/2022.

Method

This observational study used retrospective prescription data from outpatients at the Internal Medicine Department Universitas Airlangga Teaching Hospital from January to March 2020. The study was conducted at the Outpatient Pharmacy Installation at Airlangga University Teaching Hospital with samples of all prescriptions. The data collected included the type, number of drugs, and drug price. Chemotherapy and retroviral therapy for HIV were excluded. ABC-VEN analysis was performed for the amount of drug use and drug investment values in the ABC group. Group A had a cumulative value of 80%, Group B had a value of 15%, and Group C had a value of 5%. The analysis was continued using ABC-VEN. Determination of whether a drug is in the vital (V), essential (E), or non-essential (N) category is carried out through discussion by internal medicine specialists, pharmacists, and pharmacy faculty members. Group V consists of pharmaceuticals that are necessary to save human lives;

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Group E consists of vital drugs that address the underlying cause of the illness; and Group N advocates the use of medications so that minor issues can be better handled by action or therapy. The VEN and ABC expenditure value data were cross-tabulated to form a matrix. The matrix was further subdivided into three groups: group I was the priority group (AV, AE, AD, BV, CV), group II was the primary group (BE, CE, BD), and group III was the extra group (CD).

RESULTS AND DISCUSSION

ABC analysis

ABC value usage

The results of this study show that the total number of prescriptions that met the inclusion criteria was 4,242, with a total of 188 items of medicines, which will then be carried out by ABC-VEN analysis. ABC analysis based on medicine use values is presented in Table I. For group A, there were 23 items of medicines (12.17%) of the total medicines used for three months in the Internal Medicine Department, with a total use of 321,645 pcs (80.94%) of the total use. Group B contained 35 medicines (18.52%) of the total items used, with a total use of 56,022 pcs (14.10%). In group C, there were 130 medicines (69.31%) of the total medicines used, with a total usage of 19,732 pcs (4.87%).

ABC investment

For three months, ABC's investment analysis of medicine use in the Internal Medicine Department showed that eight items of medication (4.23%) were in group A, where the investment represented 80.50% of the total medicine investment. This is because in group A, there is insulin, where this therapy is recommended (Perkeni, 2021) as a combination therapy in cases of diabetes and has a high investment value. Group B, with an investment value of 14.55%, contained 22 medication items (11.64%). At the same time, Group C comprised 158 medication items (95.05%) and represented 4.95% of all drug investment values.

VEN analysis

The results of the VEN analysis are presented in Table II. The Internal Medicine Department utilized six items, or 3.18% of the medicine items included in the critical group medications. Of the total number of medications administered, 152 (80.95%) were in the essential group. Thirty medical items (15.87%) were assigned to the non-essential group.

ABC and VEN combination analysis

According to Table III, the Internal Medicine department used eight items (12.08%) out of all the drugs in Category I over a three-month period. The cost of medicine was 80.50% of the overall cost. Of the total number of medications, 151 (84.60%) were included in Category II, with an investment value of 18.97%. There were 29 medications in Category III (3.32%) of all the drugs, with 0.53% of the total cost of medicine use.

Medicine classification according to the ABC analysis on medicine use in the neurology department showed that group A, or medicine with the highest use value, were antihypertensive and antidiabetic medicine groups. Antihypertensives belong to group B, or medicines with modest utility. Vitamins and supplements are classified as class C pharmaceuticals or low-use pharmaceuticals. Class A medications require regular monitoring to prevent pharmaceutical shortages caused by excessive use (Fahriati et al., 2021). Nonetheless, medicines in group B had a moderate utility value, and group C medicines could not be disregarded since patients still required them to support their treatment demands (Damayanti et al., 2024).

In this study, the medicines in Group A, which had the highest investment value, included medicines to treat diabetes and hypertension. Regular use of medications with lengthy treatment durations, such as diabetes treatment, is a major cause of investment in group A. This is because insulin therapy units have a high value and diabetes has the highest incidence (PeuPatty et al., 2022). Medicine for liver disease, adjuvant analgesics, and antihypertensives had medium-scale investment values in group B in this study, where internal medicine patients typically had multiple comorbidities. Although the prevalence of liver disease is lower than that of hypertension, the cost of utilizing ursodeoxycholic acid is the highest in class B owing to its 25 times higher unit value than that of metformin. Other clinics, including the Cardiovascular Department, also frequently prescribe antihypertensive medications. The findings of this study are directly related to those of Ab Rahman's research from 2022, which discovered that despite no differences in the average number of medications taken, the therapy for individuals with diabetes was significantly more complex than that for those with hypertension. Group C comprises food supplements, antisecretory drugs, and oral antidiabetic drugs, all of which have a minimal investment value. (Schulman-Rosenbaum, 2023).

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Table 1. The result of ABC analysis medicine application data and the amount of investment value data from January until March 2020

	Usage Amount				Investment Cost			
	Number of Items (n)	**Percentage of items (%)**	**Top 5 Pharmaceutical Product**	**Amount of Cost (%)**	**Number of Items (n)**	**Percentage of items (%)**	**Top 5 Pharmaceutical Product**	**Amount of Cost (%)**
A	23	12.17	Metformin 500 mg Acarbose 100 mg Glimepirid 2 mg Nifedipine Simvastatin 20 mg	80.94	8	4,.3	Insulin Aspartat 100 iu Insulin Aspartat 30% protamine crystallized insulin aspartat 70% Insulin Detemir Nifedipin Insulin Lispro 25%	80.50
B	35	18.52	Sulfasalazine 500 mg Mecobalamin 500 mg Paracetamol 500 mg Insulin Aspartat 100 iu Domperidon 10 mg	14.10	22	11.64	Urosodeoxycholic acid 250 mg Gabapentin 300 mg Candesatan 16 mg Gabapentin 100 mg Metformin 500 mg	14.55
C	130	69.31	Diazepam 2mg Curcuma FCT 20 mg Betahistine 6 mg Gliquidon 30 mg Amoxicillin 500 mh	4.97	158	84.3	Glimepirid 3 mg Omeprazole 20 mg Probiotik lactobacillus acidophilus Amlodipin 10 mg Cilostazol 100 g	4.95
Total	**188**	**100.00**		**100.00**	**188**	**100.00**		**100.00**

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Table 2. VEN Analysis Result for January to March 2020

Group	Number of Item (n)	Percentage of items (%)	Amount of Drug (pcs)	Top 5 Pharmaceutical Product	Amount of Cost (%)
V	6	3.18	6,459	Insulin Aspartat 100 iu Insulin Detemir Insulin Aspartat 30% protamine crystallized insulin aspartat 70% Insulin Glulisine 100 iu	1.63
E	152	80.95	374,359	Insulin Glargine Metformin 500 mg Acarbose 100 mg Glimepirid 2 mg Nifedipin	94.20
N	30	15.87	16,581	Simvastatin 20 mg Vit. Bcomp Mecobalamin 500 mg Vit. B1 Curcuma FCT 20 mg Vit. B6	4.17
Total	**188**	**100.00**	**397,399**		**100.00**

Table 3. Distribution of medicines to Group I, II, and III

Group	Number of Item (n)	Percentage of items (%)	Amount of Drug (pcs)	Amount of Costs (%)
I	8 (AV, AE, AN, BV, CV)	12.08	47,987	80.50
II	151 (BE, CE, BN)	84.60	336,219	18.97
III	29 (CN)	3.32	13,192	0.53
Total	**188**	**100.00**	**397,399**	**100.00**

Based on the usage and investment values, different results were obtained from the ABC analysis. There were 23 medicines in Group A based on use values and eight medicine items based on investment value. Group B comprises 35 medicines valued for use and 22 medication items valued for investment. Group C comprises 130 medication products valued for use and 158 medication items valued for investment. The findings of this investigation are similar to those of Deressa et al. 'sresearch from 2022, with minor differences: Group A had 13.74% of medicine items, Group B had 18.18% of medicine items, and Group C had 68.08%. Research by Suprapti et al. (2022) in cardiology clinics A, B, and C in Indonesian teaching hospitals provided additional support for this data; the percentages were 7.45 %, 9.58 %, and 82.97 %, respectively. These discrepancies may result from several variables, including variations in research methodology, location and time, and classification and definitional frameworks.

VEN analysis is used in drug categorization to categorize medications based on their degree of criticality. AVEN analysis was performed for all 181 medication items. Insulin was a class V drug in this study and is a necessary medication for treating diabetes in individuals with both DM 1 and DM 2. Treating acute hyperglycemia and optimizing treatment therapy are two benefits of insulin in the treatment of diabetes (Maifitriani et al., 2020). Although they are utilized for situations that are severe but not life-threatening, class E medications are also used for disorders of lower severity. Owing to the use of substitute medications, unavailability in this category is accepted for two–three days (Al-Najjar et al., 2020). Oral antidiabetic and hypertension medications were among the medications used in the essential group. Class N medications are used to treat mild ailments and are the least important. Vitamins and supplements are classified as medicines in this category.

Taking needs and costs into account, ABC-VEN analysis can assist in identifying medication groups that require intensive monitoring and control. According to Pilankar et al. (2014), the ABC-VEN matrix works better and provides an approach for managing pharmaceutical drug inventories. We focused on eight drug items and their related costs, which totaled 80.50% and belonged to Category I (AV, BV, CV, and AE) for strict supervision guidelines, thanks to the resultant matrix that was produced after an examination of the ABC-VEN combo. Three of the 151 drug items in

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Category II (AE, BE, and CE) accounted for 18.97% of total drug expenses. When these drugs are bought in bulk, management complexity is reduced, there are no capital limits, transportation costs are low, and ordering charges are avoided with modest storage costs (Devnani et al., 2010; Anand et al., 2013). Because Category II is the Drug most frequently used in Internal Medicine Clinics and is a member of the CE group, it is important to keep an eye on drug procurement to preserve medication supply. One type of medication in this category was metformin 500 mg. The extra category (CN) comprised 29 drug products, accounting for 0.53% of the total drug expenses in this study. According to Anand et al. (2013), there are no substantial financial restrictions, and it is possible to order these medications three or four times a year to save money. Vitamins and supplements are a class of extra-category pharmaceuticals that have the greatest number of applications.

The present study's findings are consistent with research carried out at The Millennium Medical College at Saint Paul Hospital (Ethiopia) between 2013 and 2014 and 2015–2016. In that study, the three drug categories represented over 85%, 12%, and less than 1% of the total amount spent on pharmaceuticals annually over three years (Legese, 2017). This might be a result of the high number of diabetes mellitus patients that the nation's internal medicine departments have seen.

These findings differ from those of research carried out in tertiary care neuropsychiatric hospitals in India, where results showed that items in group I accounted for 33.8% of pharmaceutical spending, group II items for 60% of pharmaceutical expenditure, and category III items for 6.2% of pharmaceutical expenditure, or 92.33% of annual pharmaceutical costs (Khurana et al., 2013). The current study differs from another one by Nigah et al. (2010), wherein 22.09% of pharmaceutical expenditure is absorbed by Category I, which accounts for 74.21% of pharmaceutical expenditure, 22.23% of pharmaceutical spending is absorbed by Category II, which accounts for 23.28% of pharmaceutical expenditure, and Category III absorbs 3.56% of pharmaceutical expenditure. Numerous variables, including variations in hospital levels, healthcare facilities, pharmaceutical goods used, and budgets at individual healthcare facilities, may have contributed to this discrepancy.

The limitation of this study is that it was conducted in a single department that provides outpatient services. Overall, service data are required for the planning and procurement of hospital pharmacies. For this purpose, a similar analysis must be conducted for other services.

CONCLUSION

There was a discrepancy between the results of ABC analysis based on drug use and investment value. Drug items are frequently used, but their investment value is quite low. Using ABC-VEN analysis helps improve pharmacy management, especially in the hospital's planning and procurement of drugs.

AUTHOR CONTRIBUTIONS

Conceptualization, B.S.; Methodology, B.S., D.M.N.R.; Software, Z.N.; Validation, B.S., D.M.N.R., C.W.N.; Formal Analysis, Z.N., Y.S.; Investigation, Z.N., Y.S.; Resources, Z.N., Y.S.; Data Curration; Z.N., Y.S.; Writing - Original Draft, Z.N., Y.S.; Writing - Review & Editing, B.S., D.M.N.R., Y.S.; Visualization, Z.N., Y.S.; Supervision, B.S., D.M.N.R., C.W.N.; Project Administration, B.S., D.M.N.R., C.W.N.; Funding Acquisition, B.S.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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P-ISSN: 2406-9388
E-ISSN: 2580-8303

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P-ISSN: 2406-9388
E-ISSN: 2580-8303

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia
Pharmacy and Pharmaceutical Sciences Journal
Published by Faculty of Pharmacy Universitas Airlangga
OPEN ACCESS
E-ISSN 2580-8303
P-ISSN 2406-9388

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia Vol. 11 No. 2 August 2024, 192-203
DOI: 10.20473/jfiki.v11i22024.192-203
Available online at https://e-journal.unair.ac.id/JFIKI/

Potential of *Graptophyllum pictum* Leaf Decoction as an Immunomodulator: Modulation of Macrophage Phagocytosis and Lymphocyte Proliferation

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Submitted: 29 June 2024
Revised: 17 August 2024
Accepted: 31 August 2024

Abstract

***Background**: Red pudding leaves (Graptophyllum pictum) are commonly used by the Lebong community in Bengkulu as an immune system-enhancing drink containing flavonoids, glycosides, saponins, tannins, and triterpenoids. **Objective**: This study aimed to evaluate the potential of red pudding leaves as immunomodulatory agents in vitro and assess their total flavonoid content. **Methods**: The extraction method employed was a decoction, and the flavonoid content was measured using the TLC method by calculating the resulting Rf value and utilizing the LC-MS technique. The total flavonoid content was quantified using a colorimetric method, and immunomodulatory activity was assessed based on the phagocytosis capacity, phagocytosis index, and lymphocyte proliferation. **Results**: The results showed that red pudding leaf contained flavonoid compounds based on the LC-MS method in the form of trans-3-Indoleacrylic acid, schaftoside, adenine, corymboside, fraxetin and 4-coumaric acid. The total flavonoid content obtained at a concentration of 7.5% amounted to 74.937 mg QE/g; at a concentration of 15%, it amounted to 75.483 mg QE/g; and at a concentration of 30%, it amounted to 97.825 mg QE/g. All red pudding leaf infusion concentrations increased macrophage phagocytosis activity and lymphocyte cell proliferation. **Conclusion**: In conclusion, red pudding leaves show potential for development as an alternative beverage to enhance the immune system.*

***Keywords**: flavonoids, immunomodulators, lymphocytes, macrophages, red pudding leaves*

How to cite this article:
Irdanita, A., Natasya, S., Alifah, Y. & Winanta, A. (2024). Potential of *Graptophyllum pictum* Leaf Decoction as an Immunomodulator: Modulation of Macrophage Phagocytosis and Lymphocyte Proliferation. *Jurnal Farmasi dan Ilmu Kefarmasian Indonesia*, 11(2), 192-203. http://doi.org/10.20473/jfiki.v11i22024.192-203

INTRODUCTION

The immune system serves as the body's defense mechanism against external foreign substances, including parasites, bacteria, viruses, fungi, and other tumor cells (Kalsum, 2017). External support is necessary to enhance the defence capabilities of the immune system. Immunomodulators are biological compounds that influence or regulate the immune system by stimulating, modulating, or suppressing both innate and adaptive immune responses. Besides synthesized drugs, immunomodulators can come from natural ingredients, such as plants (Lestari, 2021).

Several studies related to the role of herbal plants as immunomodulators can explain the various effects that can be caused by herbal administration on the immune system. Herbal plants can affect T cells, mast cells, and exert anticancer and antimicrobial effects. Several active ingredients in herbal plants, such as flavonoid polysaccharides, are thought to enhance the immune system. The role of herbal plants as immunomodulatory agents can be immunostimulatory or immunosuppressive (Lestari 2021).

Macrophages play an important role in the immune system. Macrophages produce cytokines that play a role in various wound healing processes and antigen presentation. Monocytes are produced by the spinal cord and migrate through blood vessels to turn into monocytes and differentiate into macrophages (Wolska et al., 2019). Macrophages are phagocytic cells that play a major role in the defense against pathogen or microorganism attacks through phagocytosis mechanisms, which play an important role in adaptive and innate immune responses (Abbas et al., 2012). Phagocytosis is the ability of macrophages to phagocytise latex particles. Macrophage phagocytosis is used as the standard for a person's health or immunity. Macrophage activity in the phagocytosis of latex particles can be measured using two parameters: phagocytosis index (PI) and phagocytosis capacity (PC) (Hartini et al., 2013).

Proliferation is the process of mitotic cell division, a biological function of the body. Lymphocytes are part of the adaptive immune response that can recognize pathogens for the first time and increase the specific immune response when exposed to repeated exposure. Lymphocytes play a role in a specific immune response (T cells) for the body's defense against viruses, bacteria, and parasites. The lymphocyte proliferation response is used as a reference for describing lymphocyte function and the immune status of the human body (Meilandani & Makiyah, 2015).

The use of herbal plants as traditional medicine is often utilized by Indonesians. Traditional medicinal plants are a combination of natural ingredients derived from generations that have been used as treatments based on experience. Red Pudding Leaf is an ornamental plant commonly utilized by the residents of Bengkulu Province, especially in the Lebong district, as a traditional medicine (Permenkes RI, 2016). The factors used in this study were based on previous studies. The results showed that red pudding leaves were positive for flavonoids, alkaloids, steroids, tannins, and saponins after phytochemical screening using UV-Vis spectrophotometry. The ethanol extract of red pudding leaves in this study showed antibacterial activity. This was caused by the high content of secondary metabolites (Fauzi et al., 2016). The difficulty of health facilities in this area has led people in Lebong Regency to use boiled red pudding leaves as a first alternative for treating bleeding or bruising. Testing the effectiveness of red pudding leaves on wound healing in mice showed that administration of red pudding leaf extract at concentrations of 10% and 15% had a good wound healing effect on mice (Tukiran et al., 2014). Testing the effectiveness of red pudding leaves on wound healing in rats showed that red pudding leaf extract at concentrations of 10% and 15% had a good wound healing effect on rats (Andiyani et al., 2018).

Previous research related to phytochemical tests have shown that this plant contains non-toxic alkaloids, steroids, flavonoids, glycosides, calcium oxalate, saponins, tannins, formic acid, and fat. (Tukiran et al., 2014). Previous research on testing the total flavonoid content of ethanol extracts showed that it had high flavonoid levels of 402.88 mg / 100 g QE. There is a correlation between the flavonoid content in red pudding leaf extract and its capacity to diminish free radicals. As flavonoid content increases, so does its effectiveness in reducing free radicals (Rustini & Arianti, 2017). Furthermore, in the antioxidant testing of red pudding leaves, the results of red pudding leaf extract in ethanol have an IC50 value; therefore, it can be stated that red pudding leaf extract has the strongest antioxidant effect. In the anti-inflammatory test, it was found that 10% red pudding leaf extract produced the highest number of fibroblast cells (167.25 %) (Sartika & Indradi, 2021). To advance this research, additional studies will be conducted to explore the potential of red pudding leaf decoction as an herbal remedy to enhance the immune system.

The research conducted will discuss whether the *G. pictum* extract has flavonoid compounds based on the

P-ISSN: 2406-9388
E-ISSN: 2580-8303

TLC method, Next, the study will determine the total flavonoid content of the *G. pictum* extract and investigate whether this extract exhibits immunomodulatory activity based on macrophage cell function and lymphocyte cell proliferation.

MATERIALS AND METHODS

Materials

Red pudding (*Graptophyllum pictum*) leaves were collected from Lebong, Bengkulu, China. Subsequently, plant determination tests were conducted at the Faculty of Biology, Ahmad Dahlan University, Yogyakarta. The selected red pudding leaves were separated and aerated without direct sunlight for several days until the leaves had dried completely. The dried leaves were then ground with a blender until they became a powder.

Extraction

An infusion of red pudding leaves at a concentration of 7.5% was made by putting 7.5 grams of dried red pudding leaf powder into a pot and then adding distilled water until all the dried red pudding leaf powder became wet. After standing for 10 min, 100 ml of water was added to the container. The mixture was heated for 15 min starting at a temperature of 90 °C while stirring. Subsequently, the infusion results were obtained. The process was carried out with the same thing at concentrations of 15% (15 g of red pudding leaves) and 30% (30 g of red pudding leaves) (Hamdan, 2017).

Analysis of compound with TLC method

The sample was dissolved in 70% ethanol, and the sample was dotted in the stationary phase (silica gel GF254 plate) to identify the compound. The TLC plate was sprayed with the FeCl3 reagent. The spots were detected using UV 254 nm, UV 366 nm, and visible light. To obtain the color reaction of flavonoid compounds, the silica plate was sprayed with ammonia and then left for 15 min to observe the color of the spots that appeared. The RF value was then calculated based on the TLC results. The Rf value was used to identify the content of chemical compounds in the TLC method by calculating the distance of spot displacement(Munawaroh et al., 2018).

Compound content test LC-MS method

Secondary metabolites in the red pudding leaf infusion were analyzed by liquid chromatography-mass spectrometry (LC-MS) using a Thermo Scientific Vanquish UHPLC Binary Pump coupled with a Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer. Chromatographic separation was achieved on an Accucore™ Phenyl-Hexyl analytical column (100 mm × 2.1 mm, 2.6 µm) with a mobile phase gradient of MS-grade water containing 0.1% formic acid (A) and MS-grade methanol with 0.1% formic acid (B) at a flow rate of 0.3 mL/min. Mobile phase B was initially set to 5% and gradually increased to 90% over 16 min, maintained for 4 min, and returned to the initial conditions, completing a 25-minute run. The column was held at 40 °C with a 3 µL injection volume. Data were acquired in the full MS/dd-MS^2 mode for untargeted screening using both positive and negative ionization. Nitrogen was utilized as the sheath, auxiliary, and sweep gases, with settings of 32, 8, and 4 units, respectively. The spray voltage was 3.3 kV, the capillary temperature was 320 °C, and the auxiliary heater was maintained at 30 °C. Scans ranged from 66.7–1000 m/z with a resolution of 70,000 for full MS and 17,500 for dd-MS^2. Instrument settings and tuning were managed with XCalibur 4.4 software, with weekly calibration for mass accuracy, ion transfer, and sensitivity using Thermo Scientific Pierce ESI calibration solution (Windarsih et al., 2022).

Total flavonoid level measurement

The maximum absorbance wavelength (λ_max) was determined using quercetin solution prepared at a concentration of 30 µg/mL. A 0.5 mL aliquot of 35 µg/mL quercetin was mixed with 0.1 mL of 10% $AlCl_3$ and 0.1 mL of 1 M sodium acetate in a 5 mL volumetric flask, and distilled water was added to a final volume of 5 mL. After brief incubation at room temperature, the absorbance was measured at λmax. A 500 µg/mL stock solution of quercetin was prepared by dissolving 5 mg of quercetin in 10 mL 70% ethanol, from which dilutions of 15, 20, 25, 30, and 40 µg/mL were prepared. To each dilution, 0.1 mL of 1 M sodium acetate, 1.5 mL of methanol, and 0.1 mL of $AlCl_3$ were added, followed by distilled water to a final volume of 5 mL. Incubation was conducted at room temperature for 30 min, and absorbance readings were recorded at 421.5 nm using a UV-Vis spectrophotometer. For sample analysis, 5 mg of freeze-dried red pudding leaf extract was dissolved in 5 mL of distilled water to yield a 10,000 µg/mL solution. A 0.5 mL aliquot was mixed with 1.5 mL of methanol, 0.1 mL of 10% $AlCl_3$, and 0.1 mL of 1 M sodium citrate, with distilled water added to reach 5 mL. The mixture was incubated at room temperature for 30 min, and the absorbance was measured at 421.5 nm (Ipandi et al., 2016). Each sample was analyzed in triplicate, and the average absorbance was used to calculate the flavonoid concentration based on a calibration curve, expressed as quercetin equivalents (mg QE/g extract) (Ahmad et al., 2017).

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Immunomodulatory activity test

Isolation and incubation of macrophage cells

Macrophages were isolated from male Balb/c mice (2–3 months old) following euthanasia by chloroform inhalation. The mice were positioned supine and their abdominal areas were disinfected with 70% ethanol. A small incision is made to expose the peritoneum. A total of 10 mL of RPMI 1640 medium was injected into the peritoneal cavity, and after a 5-minute wait with gentle shaking, macrophages were released into the medium. Peritoneal fluid was collected and centrifuged at 2000 rpm for 10 min, and the supernatant was discarded. The cell pellet was resuspended in 3 mL RPMI medium with 10% FBS, yielding a suspension of 2.5 × 10^6 cells/mL. Cells were seeded onto a 24-well plate, each well containing a coverslip and 200 µL of cell suspension (5 × 10^5 cells). Following a 30-minute settling period, the cells were incubated in a 5% CO_2 incubator at 37°C, washed three times with 250 µL of complete medium, and incubated for an additional 2 h. After washing twice with RPMI, 1 mL complete RPMI medium was added to each well, followed by a 24-hour incubation (Munawaroh et al., 2018).

Macrophage phagocytosis assay

24 hours after the cells were cultured, the medium was removed using a pipette so that only macrophages remained on the coverslip. The medium was removed using a drop pipette and the cells were washed twice with RPMI-1640. Latex (200 µL/well) was added to a sample concentration series (62.5, 125, 250, and 500 µg/mL extract) and LPS as a positive control, and three replicates were performed and incubated for 2 h in a 5% CO_2 incubator at 37 °C. The cells were then washed 3 times with PBS. The samples were then dried at room temperature for 30 s and fixed using methanol. The coverslip was then allowed to dry, and the methanol was removed. The cover slips were stained using 10 % (v/v) Giemsa for 20 min, washed with distilled water, and then the culture wells were removed and dried at room temperature. Using a 100 × magnification light microscope, observations were made on 100 macrophage cells were observed, and the number of macrophages that could phagocytose latex was counted using a microscope. SFA values were calculated using the amount of latex per 100 macrophages and phagocytosis capacity for the macrophage phagocytosis activity parameter (Munawaroh et al., 2018).

$$\text{Phagocytosis Index (IP)} = \frac{number\ of\ phagocytized\ latex}{\text{number of activated macrophages}(100)}$$

$$\text{Phagocytosis Capacity (KF)} = \frac{number\ of\ phagocytizing\ macrophages}{number\ of\ macrophages\ counted(100)} \text{ x } 100\%$$

Isolation of lymphocyte organs

Lymphocyte cells were isolated from the spleens of mice because the spleen is a primary secondary lymphoid organ containing T and B cells and serves as a key site for the immune response to antigens (Abbas et al., 2017). The lymphoid organs were rinsed three times with PBS, after which 10 mL of RPMI medium was added to the spleen tissue. The resulting cell suspension was transferred to a centrifuge tube, adjusted to a volume of 15 mL, and centrifuged at 2000 rpm for 10 min. To lyse the erythrocytes within the pellet, 1 mL of ammonium chloride was added and thoroughly mixed, followed by a 5-minute centrifugation at 2000 rpm. The supernatant was discarded and the lymphocytes were resuspended in 1 mL of complete RPMI medium. Cells were counted using a hemocytometer and further diluted with complete RPMI to achieve a final cell density of 1.5 × 10^6 cells/mL (Hertiani, 2010).

Lymphocyte cells (1.5 x 106/mL) of 100µL were distributed into 96-well microplate wells and incubated for 48 h in an incubator with 5% CO_2 flow at 37 °C.One hundred µL of the sample extract was added to a concentration series of 62.5, 125, 250, and 500 µg/ml. LPS was used as a positive control. Next, 10 µL of 5 mg/mL MTT solution was added to each well. The cells were incubated for 4 h at 37°C. The reaction was halted by adding 50 µL of stop reagent in 0.001 N HCl. Incubation was continued for 24 h at room temperature, and the results were measured using an ELISA reader at a wavelength of 550 nm (Hertiani, 2010). The proliferation stimulation index (IS) was calculated using a microplate reader and the absorbance was measured at 550 nm (Sumardi et al., 2013).

$$\text{Stimulation Index (IS)} = \frac{\text{Absorbance (Sample} - \text{Control Medium)}}{\text{Absorbance (Normal Control} - \text{Medium Control)}}$$

Data analysis

The results of the data obtained were then processed by statistical analysis using SPSS to assess whether there were significant differences between the treatment groups. This analysis aimed to determine if there were significant differences between the independent variables.

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RESULTS AND DISCUSSION

Decoction

The decoction method was chosen because it has been empirically used in the community. The obtained decoction was then *freeze-dried* to obtain the water extract. The purpose was to remove water by sublimation at 0 °C. This method avoids the loss of compounds and damage to compounds due to the heating process (Reubun et al., 2020). From this process, the yields of the infusion extracts at 7.5% concentration were 0.466%, 15% was 0.44% and 0.263%. From this process, the yield of infuse extract at 7.5% concentration was 0.466%, 15% was 0.44% and 0.263%.

Analysis of compound content by TLC method

Based on the results of the TLC method using the Rf values. The Rf value was used to identify the content of chemical compounds with spots. The results showed yellow spots with an Rf value of 0.98 on the quercetin standard. The Rf of freeze-dried red pudding leaves at 7.5%, 15% concentration is 0.62, and 30% concentrations was 0.60, 0.62, and 0.91, respectively. Rf value obtained for the sample spot at a concentration of 30% was close to that of the standard spot of quercetin. The compounds in the red pudding leaves are thought to contain quercetin compounds, as determined by TLC. Red pudding leaf infuse extract is thought to contain flavonoids characterized by the appearance of a yellow spot color under UV light at 254 nm. At a UV light wavelength of 366 nm, no visible spots appeared due to a less clear light spectrophotometric lamp. In the quercetin standard solution, brownish-yellow spots were observed under visible light as well as under UV light at 254 nm and 366 nm. The presence of flavonoid compounds was confirmed by the visual greenish color observed with UV light at 254 nm.

LC-MS analysis of red pudding leaf decoction

Compound identification using LCMS yielded 171 compounds contained in the red pudding leaves. The compounds identified were assumed to be flavonoid compounds, with seven compounds having potential as immunomodulatory agents.

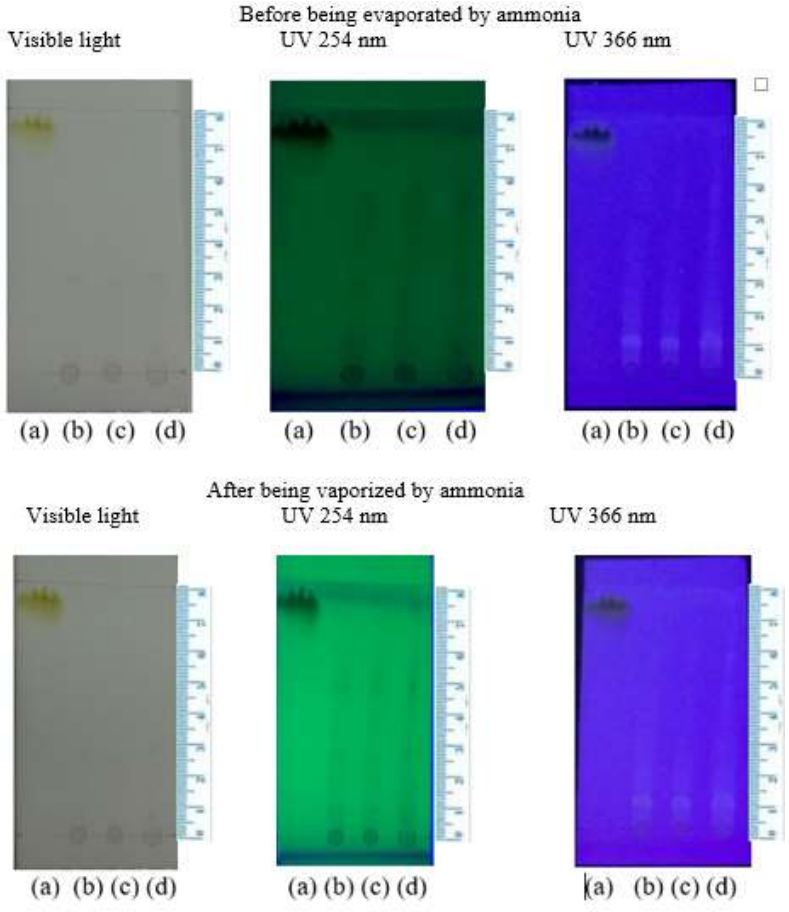

Figure 1. TLC profiles of quercetin (marked with arrow) standard (a), 7.5% red pudding leaf extract (b), 15% red pudding leaf extract (c), 30% red pudding leaf extract (d)

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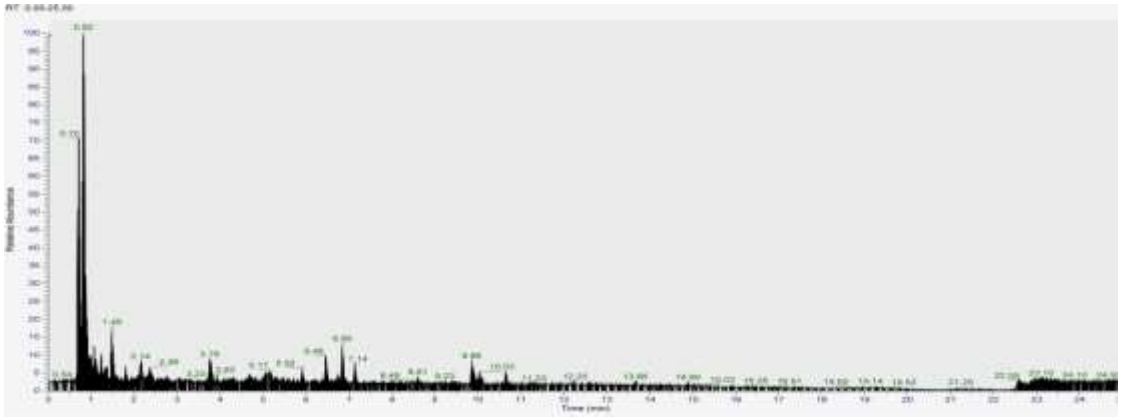
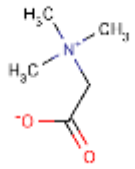
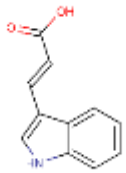
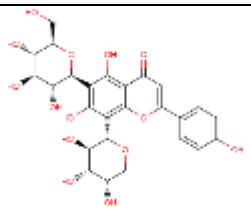
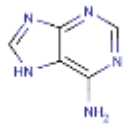
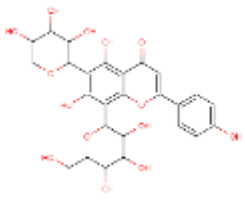
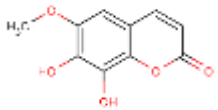
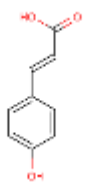


Figure 2. Chromatogram of red pudding leaves using LC-MS method

Table 1. Identification and detection results of flavonoid compounds using LC-MS

Name of compound	Chemical formula	Retention time (Rt)	Composition (%)	Structure
Betaine	$C_5 H_{11} N O_2$	0.828	58	
trans-3-Indoleacrylic acid	$C_{11} H_9 N O_2$	2.363	1,8	
Schaftoside	$C_{26} H_{28} O_{14}$	5.177	1,3	
Adenine	$C_5 H_5 N_5$	0.901	1,02	
Corymboside	$C_{26} H_{28} O_{14}$	5.304	0,59	
Fraxetin	$C_{10} H_8 O_5$	1.328	0,48	
4-Coumaric acid	$C_9 H_8 O_3$	4.29	0,22	

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As shown in Table 1, the compound contained in the infusion extract of red pudding leaves was betaine, with the highest content of 58%, which had a retention time of 0.828 with peak mass. *Betaine* and *trimethylglycine* are stable and non-toxic natural substances found in plants, animals, and microorganisms (Arumugam et al., 2021) and possess osmoprotective properties that are crucial for the immune, cardiovascular, nervous system, and kidneys (Ghasemi & Nari, 2020).

Wlodarska et al. (2018) indicates that *trans-3-Indoleacrylic acid* could enhance the function of the intestinal epithelial barrier and diminish inflammatory responses. Certain species of Peptostreptococcus produce indoleacetic acid metabolites that positively affect intestinal epithelial barrier function and reduce inflammation mediated by immune cells (Wlodarska et al., 2017).

Schaftoside is a flavonoid classified as a low-molecular-weight phenolic compound and a secondary metabolite. It is a flavonoid found in various Chinese herbal medicines including Eleusine indica, Rhizoma arisaematis, Lysimachia christinae Hance, Glycyrrhiza uralensis, and Dendrobium nobile (Zhou et al., 2019). A previous study by Yang Yi et al. (2018), involving proteomic analysis and cytokine assays, demonstrated that schaftoside also modulates the immune response and inflammation in host cells. Schaftoside exhibits safety and favorable pharmacokinetic properties, making it a promising candidate for the prevention and treatment of COVID-19 (Yi et al., 2022).

Adenine is a purine nucleoside produced by dephosphorylation of adenine nucleotides. *Adenine* markedly reduced lipopolysaccharide-induced release of inflammatory cytokines, including TNF-α, IL-1β, and IL-6, in THP-1 cells. The anti-inflammatory action of adenine may be linked to an increase in intracellular AMP, catalyzed by adenine phosphoribosyltransferase, which in turn activates AMPK (Wu et al., 2019).

Corymboside is a flavonoid compound that forms four hydrogen bonds and hydrophobic interactions with caspase 3 proteins. Previous research by Cristina *et al.* (2021) was related to the activity of corymboside in Phaleria macrocarpa (Scheff.) extract as a potential anti-cancer agent showed that corymboside had the highest TP53 expression enhancer and anticarcinogenic activity (Pa = 0.941 and 0.872, respectively) (Christina et al., 2021).

Fraxetin is a coumarin derivative extracted from the traditional medicinal plant Fraxinus rhynchophylla and is a key component in various herbal and dietary supplements. Previous studies investigating the impact of fraxetin on neuroinflammation following microglia-induced ischemic stroke have demonstrated that fraxetin effectively suppressed the expression of pro-inflammatory cytokines, including inducible nitric oxide synthase, tumor necrosis factor-α, interleukin-1 beta, and interleukin-6 in LPS-activated microglia. (Deng et al. 2022).

Cumaric acid (CA) is a secondary metabolite of phenol. Zhao et al. (2016) demonstrated that coumaric acid can inhibit the NF-kB and MAPK signaling pathways by blocking LPS-induced inflammatory cytokines. As a result, p-coumaric acid shows promise as an immunosuppressive agent for the treatment of autoimmune inflammatory diseases including rheumatoid arthritis (Kilani-Jaziri et al., 2017).

Total flavonoid measurement

The total flavonoid assay yielded a linear regression equation, $y = 0.0107x + 0.1188$, with an R^2 value of 0.9865. An R2 value close to 1 indicated a relationship between the concentration of the quercetin standard and the absorption value. The average total flavonoid content of red pudding leaf infusion extract at each concentration can be obtained through a linear regression equation, such as at a concentration of 7.5% of 74.937 mg QE/g, at a concentration of 15% of 75.483 mg QE/g, and at a concentration of 30% of 97.835 QE/g. The mean value was 82.75 mg QE/g, with a standard deviation of ± 13.06. Flavonoids are secondary metabolites widely present in several herbal plants. Flavonoids have immunostimulatory and immunosuppressive properties. Flavonoid compounds can boost the body's immune system and fight infection attacks from bacteria, viruses, fungi, or other types of microbes.

Immunomodulatory assay

Phagocytosis refers to the ability of macrophages to phagocytose latex particles. Macrophage phagocytosis is used as the standard for immunity. Macrophage activity in the phagocytosis of latex particles can be measured using two parameters: phagocytosis index (PI) and phagocytosis capacity (PC) (Hartini et al., 2013). Phagocytosis data were obtained by calculating the amount of latex phagocytosed before and after treatment with red pudding leaf infusion extract. From these data, the phagocytosis index and phagocytosis capacity were obtained, which shows that red pudding leaf extract has the ability to increase phagocytosis activity by increasing the value of phagocytosis capacity and phagocytosis index from several concentration series when compared to control cells.

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Immunomodulatory activity test results showed that the sample significantly increased the phagocytic activity of macrophages with LPS cell control. The 500 µg/mL concentration showed the highest activity, with a phagocytosis index of 1 1.701 ± 0.76, and a phagocytosis capacity of 94.833% ± 2.26.

Figure 3 (a) shows control cells without treatment, while Figure (b) shows cells treated with red pudding leaf infusion extract. Macrophages treated with red pudding leaf infusion extract phagocytose less latex than macrophages treated with red pudding leaf infusion extract.

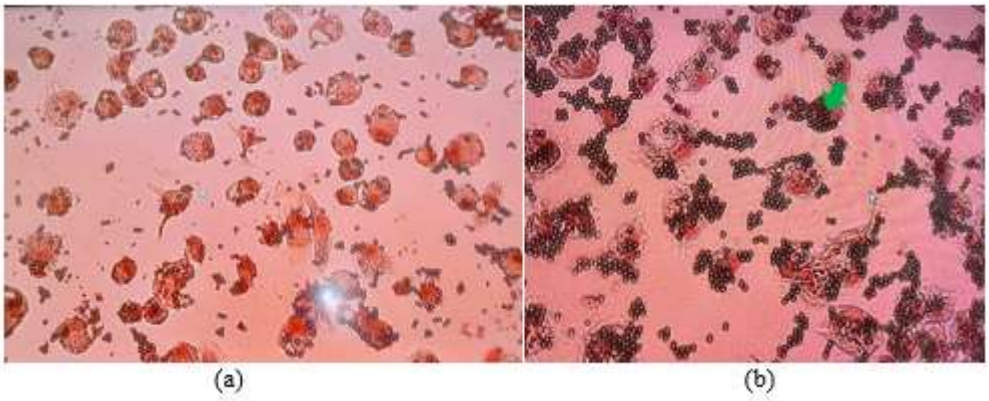

Figure 3. Comparison of the phagocytic activity of macrophages in control cells (a) and those treated with red pudding leaf infusion extract (b) at 100 × magnification. *The blue arrow reveals macrophage cells, and green arrow describes latexs wich is phagocyte by macrophage

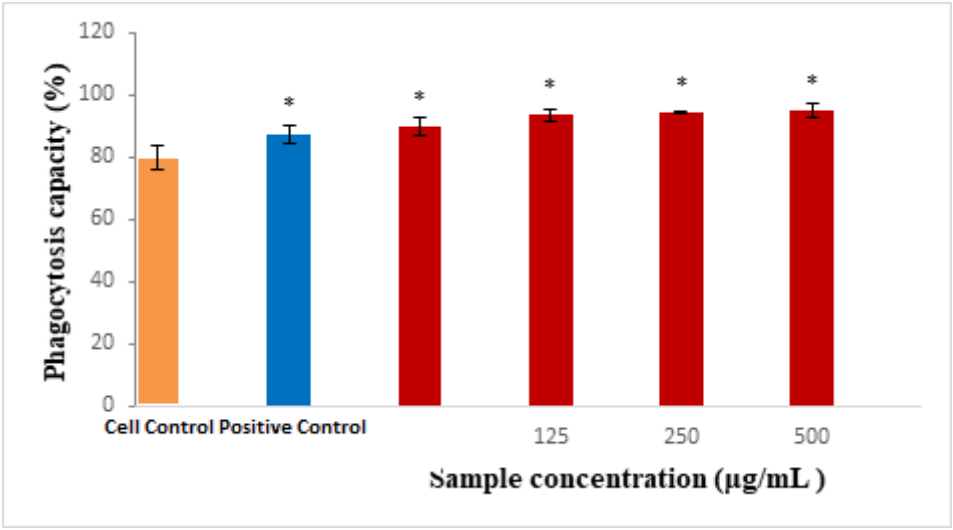

Figure 4 (a). Phagocytosis capacity profile. Phagocytosis capacity (%) at 100x magnification (mean±SD, n=3, α=0.05) *indicates a significant difference (P < 0.05) between the treatment and control groups. Cell control was macrophage cells with no treatment and treatment with LPS as a positive control

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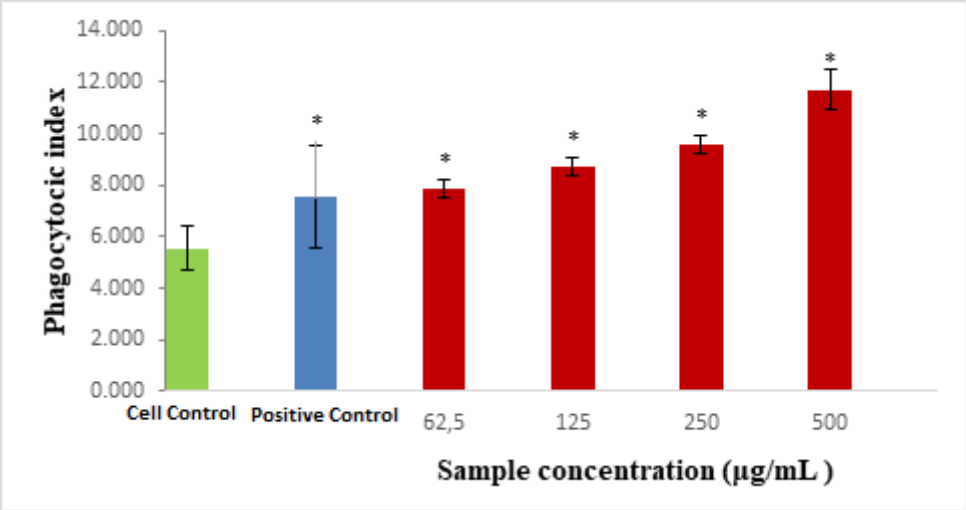

Figure 4 (b). Macrophage phagocytosis index profile at 100x magnification (mean±SD, n=3, α=0.05). *indicates a significant difference (P < 0.05) between the treatment and control groups. Cell control was macrophage cells with no treatment and treatment with LPS as a positive con control

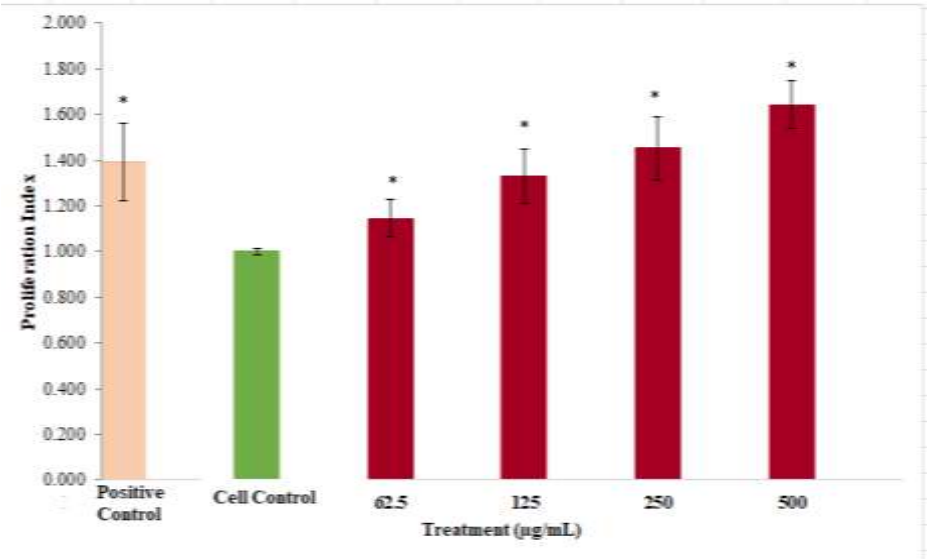

Figure 5. Lymphocyte proliferation activity at 100x magnification (mean±SD, n=3, α=0.05). * indicates a significant difference (P<0.05) between the treatment and control groups. Cell control was macrophage cells with no treatment and treatment with LPS as a positive con control

The results of this study (Figures 4 a and b) show that red pudding leaf extract can increase the phagocytic activity of macrophages characterized by an increase in the phagocytic capacity and phagocytosis index of several concentration series when compared to control cells. Compounds with IF>1 values are grouped as immunostimulant compounds, which means that these substances can increase or stimulate the body's immune system, whereas compounds with IF<1 values are grouped as immunosuppressant compounds, which means that these substances can sensitize the body's immune system (Kresno, 2007). Flavonoids have properties as immunostimulants and immunosuppressants. Flavonoid compounds can boost the body's immune system and fight infections by bacteria, viruses, fungi, or other microbial species (Erjon, 2022). Flavonoids act as immunomodulators by increasing the activity of IL-2 and lymphocyte proliferation. Additionally, flavonoids can activate NK cells, leading to stimulation of IFN-γ production. IFN-γ is the primary macrophage-activating cytokine among Macrophage Activating Cytokines (MAC), which plays a crucial role in the destruction of bacteria as part of cellular non-specific immunity (Abbas et al., 2012). The data analysis results indicated that each concentration exhibited a significant difference.

Proliferation is the process of mitotic cell division which is a biological function of the body. Lymphocytes are part of the adjuvant immune response that can recognize pathogens for the first time and increase the

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specific immune response if exposed repeatedly (Meilandani & Makiyah, 2015).

The lymphocyte proliferation assay was performed using the colorimetric method. The microtetrazolium (MTT) assay was used to determine the amount of potential possessed by natural ingredients using a microplate reader that will read the absorbance of formazan, which is generated from the reduction process by the enzyme succinate dehydrogenase found in the mitochondria of living cells (Amir & Murcitro, 2017).

The results depicting lymphocyte proliferation activity at different concentrations indicated that the administration of red pudding leaf infusion extract yielded a proliferation index <2. Generally, a stimulation index for lymphocyte proliferation between 2 and 3 is considered weakly positive, whereas an index value >3 is regarded as positive, especially if more than one concentration is obtained (Winanta et al., 2023). The results of this study show that red pudding leaf extract can increase lymphocyte proliferation activity marked by an increase in the value of the lymphocyte proliferation stimulation index from several concentration series when compared to the control cell. This suggests that a higher concentration leads to greater presence of flavonoid compounds. Flavonoids enhance lymphocyte proliferation by increasing IL-2 levels. IL-2 plays a crucial role in the proliferation of T lymphocytes, and antigen-stimulated T lymphocyte proliferation is regulated by the interplay between IL-2 and differentiation of B lymphocytes and Natural Killer (NK) cells (Ulfah et al., 2017). According to Makiyah and Wardhani (2017), flavonoids can boost lymphocyte proliferation, as evidenced by an increase in the diameter of the white pulp and area of the germinal center. The data analysis results indicated significant differences among the concentrations.

CONCLUSION

Red pudding leaf infusion extract (*G. pictum*) based on phytochemical screening of TLC and LC-MS methods contains flavonoid secondary metabolite compounds in the form of trans-3-Indoleacrylic acid, schaftoside, adenine, corymboside, fraxetin, and 4-coumaric acid. The highest average value of total flavonoids in *G. pictum* infusion extract was obtained at a concentration of 30%, with a value of 97.825 mg QE/g compared to the other two concentrations at 7.5% and 15%. The red pudding leaf infusion extract showed immunostimulant activity through macrophage phagocytosis and lymphocyte proliferation methods with an increase in phagocytic capacity value and the highest macrophage phagocytosis index at a concentration of 500 µg/ml (IF = 11.701 ± 0.761; % KF = 94.833 ± 2.268) compared to the cell control and positive control, but did not affect lymphocyte cell proliferation in vitro.

ACKNOWLEDGEMENTS

This research was funded by the Ministry of Education, Culture, Research, and Technology (KEMEDIKBUDRISTEK), and we would like to thank Adi Kurniawan, a staff member of the Culture Cell, for his assistance in facilitating our research.

AUTHOR CONTRIBUTIONS

Conceptualization, A.I.; Methodology, A.I.; Software, S.N.; Validation, A.I.; Formal Analysis, S.N.; Investigation, A.I.; Resources, S.N.; Data Curration; Y.A.; Writing - Original Draft, A.I.; Writing - Review & Editing, A.I.; Visualization, Y.A.; Supervision, A.I.; Project Administration, A.W.; Funding Acquisition, A.W.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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P-ISSN: 2406-9388
E-ISSN: 2580-8303

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia
Pharmacy and Pharmaceutical Sciences Journal
Published by Faculty of Pharmacy Universitas Airlangga
OPEN ACCESS
E-ISSN 2580-8303
P-ISSN 2406-9388

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia Vol. 11 No. 2 August 2024, 204-218
DOI: 10.20473/jfiki.v11i22024.204-218
Available online at https://e-journal.unair.ac.id/JFIKI/

Network Pharmacology Approach to *Acalypha indica* L. and *Plumbago zeylanica* L. As Anti-Rheumatoid Arthritis Candidates

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Submitted: 1 March 2024
Revised: 15 August 2024
Accepted: 31 August 2024

Abstract

Background*: Rheumatoid arthritis (RA) is a chronic autoimmune disease that can reduce quality of life. Currently, the goal of therapy is to achieve remission and prevent joint damage and disability. Acalypha indica L. and Plumbago zeylanica L. are known to be involved in rheumatoid pathogenesis.* ***Objective****: This study aimed to determine the compounds in Acalypha indica L. and Plumbago zeylanica L. that correlate with target proteins and anti-rheumatoid arthritis mechanisms.* ***Methods****: Plant compound data were collected from the KNApSAcK and IMPPAT databases, target protein data were collected using the KEGG pathway, validated using UniProt, and protein-protein interactions were analyzed using STRING. Target protein prediction using SwissTarget Prediction and SEA. Visualization of network pharmacology profiles using Cytoscape software based on the correlation between plant compounds and target proteins.* ***Results****: Acalypha indica L., which correlates with target proteins, contained quinine, gallotannin, 1,4 benzoquinone, chrysin, and kaempferol. For Plumbago zeylanica L., the compounds were vanillic acid, cinnamic acid, plumbagin, isoaffinetin, isoorientin, isovitexin, methylnaphthazarin, l-tryptophan, beta-sitosterol, stigmasterol, ficusin, suberosin, and quercetin 3-ol-rhamnoside.* ***Conclusion****: Network pharmacology visualization results showed that both Acalypha indica L. and Plumbago zeylanica L. correlated with disease target proteins in their respective rheumatoid arthritis signaling pathways.*

Keywords*: Acalypha indica L. cytoscape, network pharmacology, Plumbago zeylanica L., rheumatoid arthritis*

How to cite this article:
Afriliza, D., Herowati, R. & Indrayati, A. (2024). Network Pharmacology Approach to *Acalypha indica* L. and *Plumbago zeylanica* L. As Anti-Rheumatoid Arthritis Candidates. *Jurnal Farmasi dan Ilmu Kefarmasian Indonesia,* 11(2), 204-218. http://doi.org/10.20473/jfiki.v11i22024.204-218

P-ISSN: 2406-9388
E-ISSN: 2580-8303

INTRODUCTION

Rheumatoid arthritis is a chronic autoimmune disease that causes inflammation in the synovium and cartilage as well as damage to joints and bones through a variety of inflammatory mediators (Ono et al., 2016). Typical symptoms of rheumatoid arthritis include wrist, knee, and finger discomfort and swelling. The typical symptoms of rheumatoid arthritis include wrist, knee, and finger discomfort and swelling. This illness can lower the quality of life and cause death. The incidence increases with age, particularly in women, owing to factors related to hormonal balance. It peaks between 40 and 60 years of age (Amalia et al., 2021). Apart from the aforementioned symptoms, symptoms that are often experienced include stiffness in the morning for >30 min, fatigue, fever, and weight loss (Bullock et al., 2019). (Bullock et al., 2019). The activation of monocyte cells, such as immune cells, macrophages, and synovial fibroblasts, which subsequently generate antigen-activated CD4+ T cells, is one of the many environmental and genetic variables that contribute to the disease (Hu et al., 2019). The primary mediators of rheumatoid arthritis, interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α), are subsequently produced as a result of CD4+ T cell activation (Mateen et al., 2016).

Currently, treatment aims to achieve remission or low disease activity; it must also prevent systemic manifestations, joint damage, and disabilities (Burmester & Pope, 2017). Conventional and biological disease-modifying antirheumatic medications (DMARDs) and Janus kinase (JAK) inhibitors are used to treat and halt the progression of rheumatoid arthritis (Schwinghammer et al., 2021). Methotrexate, leflunomide, and sulfasalazine are examples of DMARD that can have harmful adverse effects. It was reported that approximately 20%-30% of rheumatoid arthritis patients stopped using methotrexate within the first year of therapy because they could not tolerate the side effects induced by methorexate, but the potential side effects could persist for 5 years (Huang et al., 2017). The side effects of these drugs are what cause many sufferers to switch to herbal remedies which have fewer side effects (Amalia et al., 2021). Botanical drugs from traditional Chinese medicine have been used to treat rheumatoid arthritis since ancient times. The use of decoction with more than one herb is a common practice, especially in traditional Chinese medicine (Hong et al., 2017)

Acalypha indica L. with the compound kaempferol based on research (Pan et al., 2018), kaempferol suppresses migration, invasion, MMP expression in rheumatoid arthritis FLS (fibroblast-like synovyocites). Kaempferol has been shown to increase the reduction in lipopolysaccharide (LPS) levels (lipopolysaccharide) of chondrogenic markers and reduce the expression levels of MMP3 and MMP13. This shows that kaempferol in rheumatoid arthritis FLS reduced the production of MMP1, MMP3, MMP9, and MMP13.

Plumbago zeylanica L. contain the compound cinnamic acid which is based on research (Zhou et al., 2023) the combination of mangiferin and cinnamic acid reduces joint inflammation and bone erosion by suppressing NLRP3 inflammasome activation by inhibiting NF-κB via TLR4/PI3K/AKT signaling. This results in decreased release of IL1B and IL-18, downregulation of caspase-1, and modulation of pyroptosis GSDMD (Gasdermin D). Vanillic acid compounds, based on research conducted by Thilertdecha et al. (2019), reduced COX-2 expression and NF-κB activation, which in turn led to lower levels of TNF-α and IL-2.

In this case, network pharmacology, which integrates systematic treatment with scientific information, is new in drug discovery. This method incorporates an in silico technique by constructing a network of "protein-active substance/disease-gene" to ascertain the mechanism of the synergistic therapeutic action of traditional medications. Network pharmacology techniques are used to determine active substances, potential targets, and signaling pathways (Noor et al., 2022).

Based on data from previous research, this study was intended to confirm and determine the molecular correlation. This research will carry out an analysis using a network pharmacology approach on *Acalypha indica* L. and *Plumbago zeylanica* L. on rheumatoid arthritis target proteins as anti-rheumatoid arthritis drug candidates. Screening was carried out on *Acalypha indica* L. and *Plumbago zeylanica* L. to determine the compounds found in *Acalypha indica* L. and *Plumbago zeylanica* L. as well as the proteins found in the compounds of *Acalypha indica* L. and *Plumbago zeylanica* L., then continued to look for disease target proteins in the KEGG pathway via the signaling pathway of rheumatoid arthritis in the form of T-cell receptor, Th17 cell differentiation, Toll-like receptor, osteoclast differentiation, VEGF, leukocyte migration, cyclooxygenase, and lipoxygenase which will eventually form network pharmacology visualization.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

MATERIALS AND METHODS

Materials

The materials used in this study was compounds from *Acalypha indica* L. and *Plumbago zeylanica* L. were obtained from KNApSAcK and IMPPAT. The target proteins of RA were obtained from the KEGG database. Protein-protein interactions were obtained from STRING. *Acalypha indica* L. and *Plumbago zeylanica* L. compounds with anti-rheumatoid arthritis activities were obtained from PubChem. Target protein prediction was performed using SwissTargetPrediction and SEA.

Tools

The tools used in this study were a set of ACER Aspire 5 with Intel(R) Core (TM) i3- 1115G4 processor specifications, 8.0 Giga Byte RAM, 233 Giga Byte SSD hard disk, Cytoscape 3.10, KNApSAcK, IMPPAT, PubChem, SwissTargetPrediction, SEA, KEGG, Uniprot, and STRING.

Method

Data collection on plants compounds

Compound data for *Acalypha indica* L. and *Plumbago zeylanica* L. were collected from several databases, including KNApSAcK (http://www.knapsackfamily.com/KNApSAcK_Family/) and IMPPAT (https://cb.imsc.res.in/imppat/). These databases provide smiles from each compound.

Data collection of rheumatoid arthritis target proteins

A rheumatoid arthritis target search was performed using KEGG (KEGG PATHWAY Database (Genome.jp)). Subsequently, target gene names were standardized and invalid targets were eliminated using the UniProt database (https://www.uniprot.org/); only target genes marked as "Reviewed (Swiss-Prot)" and "Homo sapiens" were chosen from UniProt to guarantee prediction accuracy (Deng et al., 2020).

Analysis of protein-protein interactions

STRING database was used to analyze protein-protein interactions (https://STRING-db.org/). “Homo sapiens” was choosen with an interaction score >0.9. (Huang et al., 2020).

Identification of the biological activity of compounds

Identification of biological compound activity data was performed using PubChem (https://pubchem.ncbi.nlm.nih.gov/). The data obtained are sorted based on activity; if it is not active, it is eliminated.

Target proteins prediction via SwissTargetPrediction and SEA

Using the SwissTargetPrediction database (http://www.swisstargetprediction.ch/), SMILES of *Acalypha indica* L. and *Plumbago zeylanica* L. compounds were used to acquire targets using a reverse pharmacophore-matching approach. For this reason, targets with probability ≥0.5 were chosen (Noor et al., 2022b). SwissTragetPrediction accurately predicts bioactive target molecules based on a combination of 2D and 3D similarity measures with known ligands (Gfeller et al., 2014). The function of the Similarity Ensemble Approach (SEA) is to identify pharmacological relationships between molecular targets based on similarity set ligands (Achenbach et al., 2011). In the SEA database, the existing data are sorted by the maximum Tc section, selected by a value ≥ 0.5.

Visualization using cytoscape

Compound-target networks were constructed using the candidate compounds and potential targets. The network was constructed using Cytoscape 3.10. In this bilateral network, the nodes present compounds and potential targets, and the edges present the compound–target or interactions (Huang et al., 2017).

RESULTS AND DISCUSSION

Data collection on plants compounds

Data collection on plant compounds was obtained from several databases including KNApSAcK and IMPPAT, from these two plants, where *Acalypha indica* L. contains 23 compounds, while *Plumbago zeylanica* L. contains 48 compounds.

Data collection of rheumatoid arthritis target proteins

Collection of target proteins involved in the pathophysiology of rheumatoid arthritis was carried out using the KEGG pathway database, there will be a picture of the rheumatoid arthritis signaling pathway. In the picture there are several signaling pathways, namely T-cell receptor, Th17 cell differentiation, Toll-like receptor, Osteoclast differentiation, VEGF, Leukocyte migration. Then in the signaling pathway there is a rheumatoid arthritis target protein. Validation of proteins from the KEGG pathway using UniProt, to obtain universally validated protein names.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

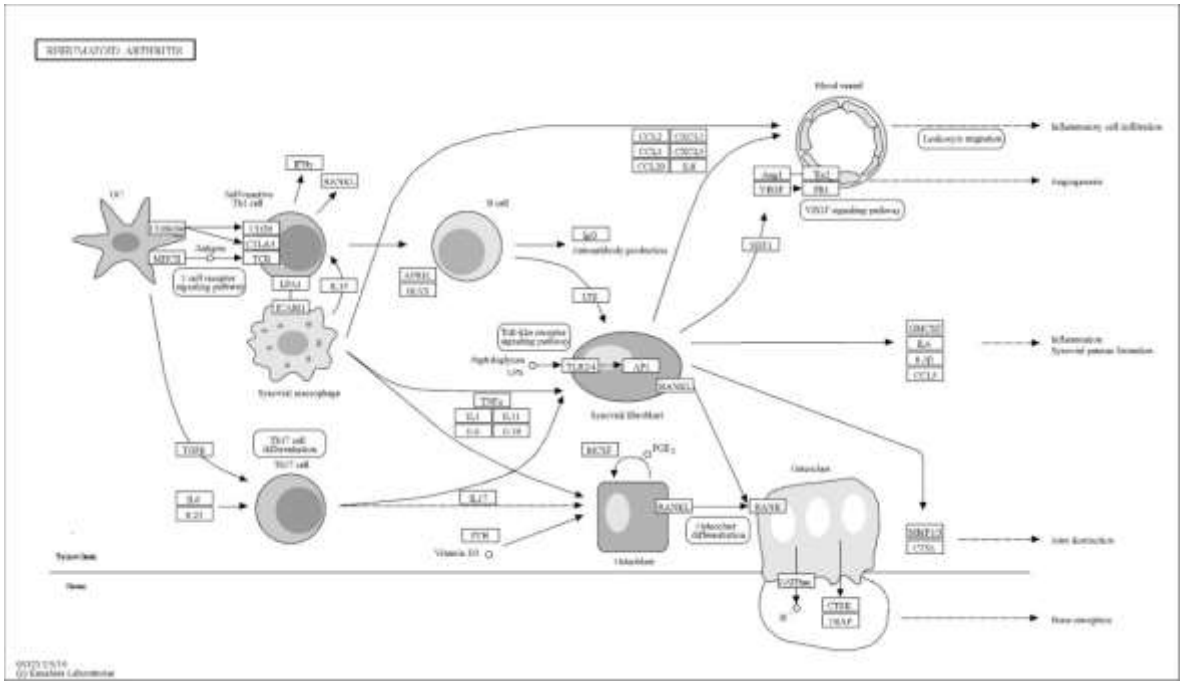

Figure 1. Signaling pathway rheumatoid arthritis from KEGG pathway

Analysis of protein-protein interactions

STRING is an early effort web server that attempts to differentiate protein-protein interactions primarily through wide coverage, user-friendliness, and a constant scoring system (Szklarczyk et al., 2019). Combination scores above 0.9 can be taken, scores below 0.9 will be eliminated. The sorting data obtained 307 protein-protein interactions with a combination score above 0.9. Based on the results of STRING, it was found that proteins from rheumatoid arthritis bind to each other. These proteins have many nodes, which are circle-shaped images that show the proteins present. Apart from that, there are also colorful lines called edges that represent protein-protein associations. It provides a score for every protein-protein interaction that is maintained in STRING. This score (the weight of the edges in each network) represents the trust score and is scaled between zero and one. This is an estimation of the likelihood that in the presence of supporting data, a given interaction is biologically significant, distinct, and repeatable. Authenticity and type of evidence determine which 'evidence channels' the supporting evidence for each encounter. Seven distinct channels were constructed, assessed, and benchmarked (Szklarczyk et al., 2017).

Each interaction is computed as a combination and a final trust score based on the seven channels; this so-called combination score serves as the benchmark when creating networks or organizing and filtering interactions. When there is evidence of many channels contributing to the engagement score, in addition to a high score, it is a positive indication of support (Szklarczyk et al., 2017). The results were sorted based on a combination score ≥ 0.9. This is because the higher the combination score for a protein, the more the interaction between proteins is based on the number of studies that have been conducted.

Identification of the biological activity of compounds

The compound data from KNapSAck and IMPPAT were used to search for biological activity using PubChem. The data obtained is sorted based on activity, if it is not active it will be eliminated. From the identification results using PubChem, we found that the five active compounds in *Acalypha indica* L. were quinine, kaempferol, 1,4-benzoquinone, gallotannin, and chrysin. There are 13 active compounds in Plumbago zeylanica L., including vanillic acid, plumbagin, isoaffinetin, isoorientin, isovitexin, methylnaphthazarin, l-tryptophan, cinnamic acid, beta sitosterol, stigmasterol, ficusin, suberosin, quercetin3-o-l-rhamnoside.

Target proteins prediction via SwissTargetPrediction and SEA

This analysis was used to determine the level of similarity between bioactive compounds and rheumatoid arthritis target proteins. In SwissTargetPrediction, the data that are sorted probability data where only values ≥ 0.5 are taken. In the SEA database, a maximum Tc value ≥ 0.5 is selected.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Table 1. Prediction of metabolit target proteins in *Acalypha indica* L. with SwissTargetPrediction and SEA

Plant	Compound	Sources	Target Proteins	Prediction		
				PubChem	SwissTargetPrediction	SEA
***Acalypha indica* L.**	*Quinine*	(IMPPAT: Indian Medicinal Plants, 2023a)	IFNG	√		
	Kaempferol	(IMPPAT: Indian Medicinal Plants, 2023a)	NFKB1	√		
			MMP1	√		
			MMP2		√	
			MMP9	√		
			ALOX12	√	√	√
			ALOX15	√	√	√
			ALOX5	√	√	√
			SYK		√	
	1,4 benzoquinone	(IMPPAT: Indian Medicinal Plants, 2023a)	CASP1	√		√
			CCR6	√		
	Gallotannin	(IMPPAT: Indian Medicinal Plants, 2023a)	JUN	√		
			HSPD1	√		
			BCL2L1	√		
			LCK	√		
	Chrysin	(IMPPAT: Indian Medicinal Plants, 2023a)	PGF	√		
			ALOX12	√	√	√
			ALOX15	√	√	√
			CBR1	√	√	

Table 2. Prediction of metabolit target proteins in *Plumbago zeylanica* L. with SwissTargetPrediction and SEA

Plant	Compound	Sources	Target Proteins	Prediction		
				PubChem	SwissTargetPrediction	SEA
***Plumbago zeylanica* L.**	*Vanillic acid*	(IMPPAT: Indian Medicinal Plants, 2023b)	CXCL12			√
			ALOX5		√	√
	Plumbagin	(KNApSAcK Core System, 2023)	EGFR	√		
			EP300	√	√	√
			XPO1	√		
			HSPD1	√		
	Isoaffinetin	(KNApSAcK Core System, 2023)	IL2			√
	Isoorientin	(KNApSAcK Core System, 2023)	IL2			√
			ALOX5	√	√	√
	Isovitexin	(KNApSAcK Core System, 2023)	IL2			√
	Methylnaphthazarin	(IMPPAT: Indian Medicinal Plants, 2023b)	BCL2L1	√		
	L-tryptophan	(KNApSAcK Core System, 2023)	CTSL			√
			MMP1			√
			MMP2			√
			MMP3			√
			MMP9			√
	Cinnamic acid	(IMPPAT: Indian Medicinal Plants, 2023b)	MMP1			√
			MMP2			√
			MMP9			√

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Beta sitosterol	(IMPPAT: Indian Medicinal Plants, 2023b)	FGF2			√
Stigmasterol	(KNApSAcK Core System, 2023)	FGF2			√
Suberosin	(KNApSAcK Core System, 2023)	XPO1			√
Ficusin	(KNApSAcK Core System, 2023)	NFKB1	√	√	√
Quercetin 3-o-1 rhamnoside	(KNApSAcK Core System, 2023)	ALOX5		√	√

The results obtained from SwissTargetPrediction and SEA showed that there are several compounds that pass the sorting ≥ 0.5, but some that pass the sorting do not match the rheumatoid arthritis target protein. Only a few compounds from *Acalypha indica* L. and the gout leaf plant are compatible with rheumatoid arthritis target proteins.

Visualization using cytoscape

Network pharmacology visualization was created using Cytoscape software using data from STRING, PubChem, SwissTargetPrediction, and SEA. Pharmacology networks contain nodes and edges. Nodes contain target proteins and interacting compounds that are connected via edges (connecting lines). The visualization of two plants, *Acalypha indica* L. and *Plumbago zeylanica* L., where the nodes were differentiated by color and shape for compounds from *Acalypha indica* L. were yellow with an elliptical shape, while compounds from *Plumbago zeylanica* L. were green and diamond-shaped with orange for target proteins.

As shown in Figure 2, the *Acalypha indica* L. compound, quinine, correlated with the target protein IFNG. This molecule is the primary inflammatory cytokine that marks the Th1 lineage in addition to other CD4+ T subsets. CD8+ T cells secrete IFNG to control infection and are composed of CD4+ T helper 1 (Th1) cells. It is involved in intracellular invasion, inflammation, and autoimmune diseases, suggesting that IFNG produced by Th1 cells is involved in the pathogenesis of rheumatoid arthritis (Peng et al., 2020).

Quinine specifically inhibits autophagy, prevents the activation of MHC II antigens, and increases endosomal pH, which inhibits Toll-like receptors, which are included in the cytokine production pathway (Song & Fields, 2020). IFNG, TNF, IL-1, and IL-6 are examples of pro-inflammatory cytokines that are reduced in production and blocked by suppressing T cell responses (dos Reis Neto et al., 2020). A mechanism that interferes with the production of inflammatory cytokines is the ability to interfere with the synthesis of GMP-AMP signaling (cGAS). cGAS is an important component of the cGAS signaling stimulator of the IFNG gene required for the type I IFN response of immune cells, giving it a critical role in the activation of pro-inflammatory responses in autoimmune diseases (Nirk et al., 2020).

Table 3. Predicted correlation of *Acalypha indica* L. with rheumatoid arthritis signaling pathway based on KEGG

Compound	Target protein code	Name of the main protein	RA Pathway
Quinine	IFNG	CD4 CSF2 IFNG IL1B	*T-cell receptors*
Kaempferol	NFKB1	IL18	*Toll-like receptors*
	MMP1	MMP1 MMP3	*Osteoclast differentiation*
	MMP2	MMP1 VEGFA	*Osteoclast differentiation*
	MMP9	MMP1 MMP3 VEGFA	*Osteoclast differentiation*

Compound	Target protein code	Name of the main protein	RA Pathway
	ALOX12		*Lipoxygenase*
	ALOX15		*Lipoxygenase*
	ALOX5		*Lipoxygenase*
	SYK	IGH	*Osteoclast differentiation*
1,4-benzoquinone	CASP1	CASP1	*Toll-like receptors*
		IL1B	
		IL18	
	CCR6	CCL20	*Leukocyte migration*
Gallotannin	JUN	JUN	*Toll-like receptors*
	HSPD1	TLR4	*Toll-like receptors*
	BCL2L1	CTSK	*Osteoclast differentiation*
		CTSL	
	LCK	CD28	*T-cell receptors*
Chrysin	PGF	FLT1	*VEGF*
	ALOX12		*Lipoxygenase*
	ALOX15		*Lipoxygenase*
	CBR1		*Cyclooxygenase*

Table 4. Predicted correlation of *Plumbago zeylanica* L. with rheumatoid arthritis signaling pathway based on KEGG

Compound	Target protein code	Name of the main protein	RA Pathway
Vanillic acid	CXCL12	CCL20	*Leukocyte migration*
	ALOX5		*Lipoxygenase*
Plumbagin	EGFR	ANGPT1	*VEGF*
	EP300	JUN	*Toll-like receptors*
	XPO1	TNFSF13	*T-cell receptors*
	HSPD1	TLR4	*Toll-like receptors*
Isoaffinetin	IL2	CSF2	*Th 17 cell differentiation*
		IFNG	
		IL1A	
		IL1B	
		IL15	
		IL16	
		IL17A	
Isoorientine	IL2	CSF2	*Th 17 cell differentiation*
		IFNG	
		IL1A	
		IL1B	
		IL15	
		IL16	
		IL17A	
	ALOX5		*Lipoxygenase*
Isovitexin	IL2	CSF2	*Th 17 cell differentiation*
		IFNG	
		IL1A	
		IL1B	
		IL15	
		IL16	
		IL17A	
Methylnaphthazarin	BCL2L1	CTSK	*Osteoclast differentiation*
		CTSL	
L-tryptophan	CTSL	CTSK	*Osteoclast differentiation*
		CTSL	
	MMP1	MMP1	*Osteoclast differentiation*
		MMP3	
	MMP2	MMP1	*Osteoclast differentiation*
		VEGFA	

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	MMP3	MMP1 MMP3	*Osteoclast differentiation*
	MMP9	MMP1 MMP3 VEGFA	*Osteoclast differentiation*
Cinnamic acid	MMP1	MMP1 MMP3	*Osteoclast differentiation*
	MMP2	MMP1 VEGFA	*Osteoclast differentiation*
	MMP9	MMP1 MMP3 VEGFA	*Osteoclast differentiation*
Beta sitosterol	FGF2	TEK	*VEGF*
Stigmasterol	FGF2	TEK	*VEGF*
Ficusin	NFKB1	TNF IL18	*Toll-like receptors*
Suberosin	XPO1	TNFSF13	*T-cell receptors*
Quercetin 3-ol-rhamnoside	ALOX5		*Lipoxygenase*

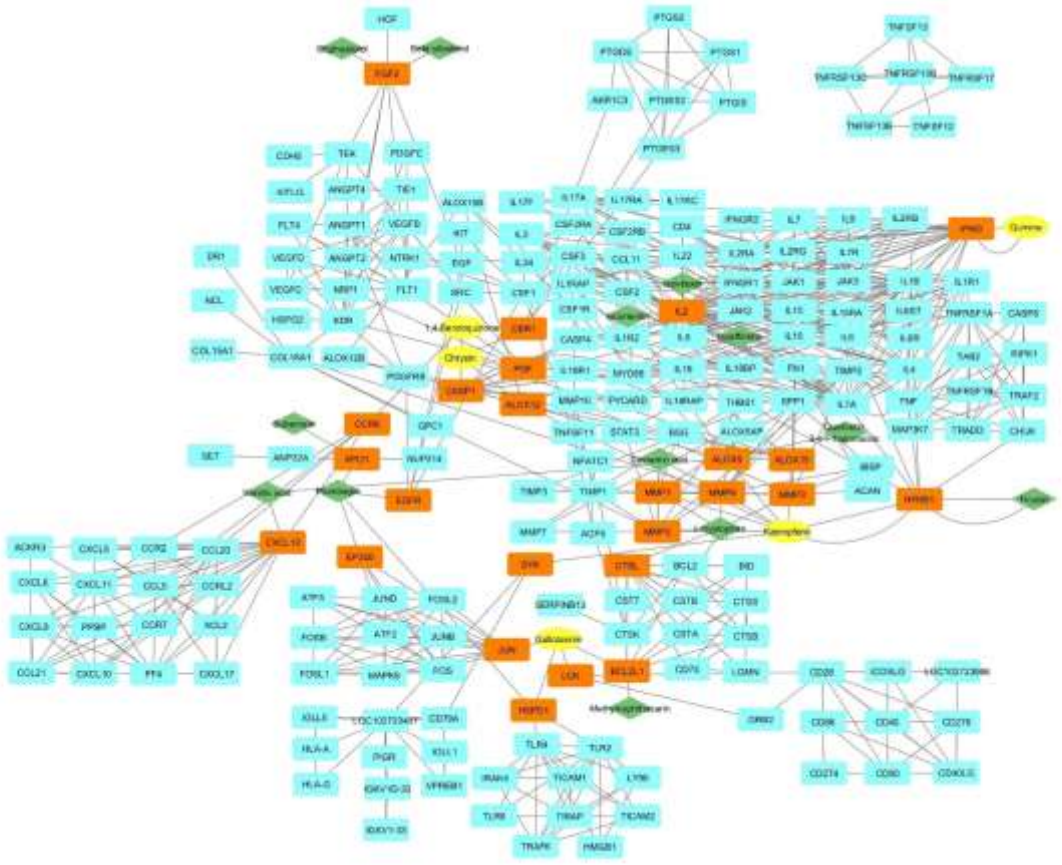

Figure 2. Visualization network pharmacology of *Acalypha indica* L. (yellow) and *Plumbago zeylanica* L. (green) compounds correlated with target proteins (orange)

The above image shows that the target protein NFKB1 correlates with two compounds originating from *Acalypha indica* L. and *Plumbago zeylanica* L.. For *Acalypha indica* L. with the compound kaempferol and *Plumbago zeylanica* L. with the compound ficusin. The family of inducible transcription factors known as NF-κB is involved in several immune system functions (Hayden & Ghosh, 2014).

NF-κB controls the activation, differentiation, and effector activity of inflammatory T-cells. Recent studies have shown that NF-κB plays a role in regulating inflammasome activation. The main inflammatory mediator of rheumatoid arthritis is NF-κB, which has

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been shown to be activated in the synovial tissue of patients with rheumatoid arthritis. The pathogenesis of rheumatoid arthritis involves a variety of cell types, including innate immune cells such as monocytes/macrophages, T cells, B cells, and synovial fibroblasts. NF-κB mediates the activation of pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6, in monocytes/macrophages. Numerous cytokines can trigger NF-κB in fibroblasts and innate immune cells, which in turn triggers the release of more inflammatory cytokines and chemokines, which in turn promotes the recruitment of more inflammatory immune cells and the expansion of inflammation. Specifically, individuals with rheumatoid arthritis frequently have elevated serum levels of TNF family B cell-activating factors, which are linked to deregulated NF-κB activation. Consequently, NF-κB functions in several cell types to mediate the pathogenesis of rheumatoid arthritis (Liu et al., 2017).

Kaempferol is a flavonoid, and one study examined the possible anti-rheumatoid arthritis effect of kaempferol on synovial tissue after knee arthroplasty. Administration of kaempferol suppressed the expression of NF-κB, MAPK, COX-2, PGE2, MMP3, and MMP1, these results indicating an anti-rheumatic effect of kaempferol on synovial tissue (Behl et al., 2022). Previous studies have shown that ficusin compounds exhibit low cytotoxicity against chondrocytes over a range of doses. Ficusin suppressed chondrocyte proliferation at a concentration of 100 μM. Nevertheless, research on the immunomodulatory effects of these compounds in RA is lacking (Pai et al., 2021).

As shown in Figure 2, kaempferol and cinnamic acid correlated with MMP1, MMP2, and MMP9, and l-tryptophan correlated with MMP1, MMP2, MMP3, and MMP9. Rheumatoid arthritis is one of the diseases for which matrix metalloproteinase (MMP) is implicated in the pathogenesis. MMP is strongly linked to the development of RA because it frequently results from abnormally increased MMP levels, which induce synovial joint lesions. It is also recognized that MMPs cause permanent damage to the tendons, bones, and cartilage in joints. Tissue inhibitors of MMP (TIMP) have been shown to ameliorate rheumatoid arthritis; hence, MMP is a significant therapeutic target for rheumatoid arthritis (Li et al., 2022).

Kaempferol inhibits migration, invasion, and MMP expression in rheumatoid arthritis FLS. Kaempferol has been shown to lower MMP3 and MMP13 expression levels as well as lower LPS levels of chondrogenic markers. This indicates that kaempferol in rheumatoid arthritis FLS reduces the production of MMP1, MMP3, MMP9, and MMP13 (Pan et al., 2018). These chemicals, such as cinnamic acid, have a propensity to displace hydrogen atoms and donate electrons from aromatic phenolic rings to transform them into free radicals. Thus, it absorbs free radicals and functions as a reducing agent. It can activate different endogenous antioxidant pathways, leading to an increase in antioxidant enzyme levels (Behl et al., 2022). Anti-inflammatory role due to inhibitory effect on the NF-κB signaling pathway (Ruwizhi & Aderibigbe, 2020). Previous studies have shown that cell invasion and migration of synovial fibroblasts can be considerably decreased by MMP inhibition, and cinnamic acid suppresses the expression of MMP1, MMP2, and MMP3 (Liu et al., 2020).

Kaempferol was correlated with the target protein SYK. The cytoplasmic protein tyrosine kinase Spleen tyrosine kinase (Syk) is a member of the Src family of non-receptor tyrosine kinases (Deng et al., 2016). Patients with rheumatoid arthritis have increased levels of pSyk in the peripheral blood B cells. In antibody-induced arthritis, depleting Syk from neutrophils was useful in preventing joint inflammation, and injecting Syk siRNA directly into the joint stopped the disease progression (Deng et al., 2016). In another study, kaempferol reduced the increased levels of Syk autophosphorylation induced by Myc-Syk overexpression. Kaempferol also decreased Syk-induced NF-κB-mediated luciferase activity, suggesting that kaempferol can directly suppress Syk at the enzyme and associated functional levels. Kaempferol blocked the catalytic activity of IRAK1 and IRAK4, suggesting that the protein tyrosine kinases Src and Syk were suppressed and that these enzymes were directly targeted (Kim et al., 2015).

Three compounds, isoaffinetin, isoorientin, and isovitexin, were correlated with the IL2 target protein. T-cell activation and proliferation are stimulated by IL-2, an autocrine growth factor, and cytokines generated by Th1 lymphocytes. Clinical research has shown a correlation between serum IL-2 level and RA disease activity. It has been shown that IL-2 has both an indirect suppressive effect and a direct stimulatory effect in the CIA model. As both early and late treatment with IL-2 exacerbated CIA in mice treated with anti-IFNG Ab, it was determined that the suppressive action was not directly mediated by IFNG. It has been discovered that the IL-2/anti-IL-2 monoclonal antibody immune complex inhibits murine CIA. According to current research, CD8+ T cells are the main source of IFNG, which activates monocytes/macrophages, synovial

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fibroblasts, and CD4+ T cells. IFNG, which is produced by monocytes/macrophages, promotes osteoclastogenesis and causes joint damage in rheumatoid arthritis (Kondo et al., 2021).

Isoaffinetin, this compound from *Plumbago zeylanica* L. shows therapeutic activity such as rheumatoid arthritis (Bharadvaja, 2017). In vitro experiments using LPS-stimulated mouse macrophage RAW 264.7, demonstrated the strong anti-inflammatory effects of isoorientin, a specific inhibitor of COX-2. Isoorientin effectively reduced carrageenan-induced inflammatory rat paw edema Inactivation of NF-κB and downregulation of pro-inflammatory gene expression, including COX-2, iNOS, and TNFα, mediates this effect (Anilkumar et al., 2017). Isovitexin exhibits a range of pharmacological properties, including anti-inflammatory, antioxidant, and antineoplastic effects. Isovitexin is known to suppress the NF-κB and MAPK pathways in macrophages (Zhang et al., 2021).

β-Sitosterol and stigmasterol compounds were correlated with FGF2. The only bone-resorptive cytokine that has been shown to be highly expressed in the synovial fluid of patients with rheumatoid arthritis is correlated with the extent of joint destruction is basic FGF2. It is well known that via binding to the receptor (FGFR), FGF2 stimulates osteoclastogenesis and promotes bone resorption by binding to the receptor FGFR (Zhao et al., 2020).

Beta-sitosterol is a bioactive phytosterol having antioxidant and anti-oxidant effects-inflammation. VEGF expression was decreased by beta-sitosterol in kidney tissue. Beta-Sitosterol Inhibits VEGFR2 Production and Activation. Previous research has also shown that beta-sitosterol has an anti-angiogenic function by inhibiting VEGF or inflammatory cytokine expression. This suggests that beta-sitosterol acts on the VEGF pathway to treat rheumatoid arthritis (Qian et al., 2022).

Stigmasterol exerts antipyretic, anticancer, and anti-inflammatory effects. In the research carried out (Ahmad Khan et al., 2020), showed the results that stigmasterol improved clinical severity in CIA mice compared to controls. The therapeutic effect is associated with a reduction in joint destruction and an improvement in histological changes. By downregulating the expression of NF-κB and p38MAPK in joints, stigmasterol treatment also markedly inhibited the expression of pro-inflammatory mediators (TNFα, IL6, IL-1β, iNOS, and COX-2) and boosted the expression of anti-inflammatory cytokines (IL10) (Ahmad Khan et al., 2020).

The compound 1,4-benzoquinone was correlated with the target protein, CASP1. Gasdermin D protein is cleaved by caspase-1, triggering pyroptosis, a pro-inflammatory form of dead cells and pro-IL-1β and pro-IL-18 interleukins in their active cytokine forms (Caruso et al., 2022).

1,4-benzoquinone also known as para-benzoquinone(Jing et al., 2021) showed that celastrol a methylated triterpenoid quinone, has anti-rheumatoid arthritis effects, where the secretion of IL-1β and IL-18 in mouse serum induced by complete Freund's adjuvant (CFA) and THP-1 cell supernatant was decreased (Jing et al., 2021).

This suggests that CCR6 may be downregulated upon effector/memory T cell infiltration because of the inflammatory environment of rheumatoid arthritis joints (Schutyser et al., 2003).

The target protein JUN correlates with gallotannin, and in rheumatoid arthritis, VCAM-1 production is induced by IL-18, which is activated by AP-1. AP-1 functions as a signaling molecule that triggers the production of VCAM-1, mostly through p-38/MAPK, instead of epithelial cell NF-κB. Therapeutically, AP-1 impairs cell migration and invasiveness and prevents pannus development in rheumatoid arthritis joints. Inflammatory disorders, cartilage degradation, leukocyte infiltration, eicosanoid synthesis, and antioxidant effects are all caused by AP-1 inhibition. In addition, AP-1 inhibition can minimize synovial expansion and hyperplasia (Le Rossignol et al., 2018)

Two compounds, gallotannin and plumbagin, correlate with the same target protein, HSPD1. Serum HSP60, also known as HSPD1, is elevated in patients with inflammatory conditions, such as colitis, diabetes, and acute lung injury. According to previous reports, HSP60 antibodies balance cytokines toward anti-inflammatory responses and prevent colitis and arthritis in mice. Furthermore, HSP60 triggers an inflammatory cascade by activating macrophages through TLR4 (Huang et al., 2020).

Gallotannin and methylnaphthazarin correlate with BCL2L1, the BCL-2 family of proteins known to be involved in promoting or inhibiting apoptosis. The mitochondrial apoptotic pathway requires the presence of two important pro-apoptotic multi-domains, BAX and BAK, for its execution phase. Common anti-apoptotic proteins that support cell survival include BCL2, BCL-xL (gene/transcript name BCL2L1), MCL1, BCL2A1, and BCL-W (Loo et al., 2020).

Gallotannin compounds correlated with LCK, Four gene biomarkers (LCK, MS4A1, CXCL13, and IGHM) had good predictive ability for rheumatoid

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arthritis. Studies show that LCK regulates initiation of TCR signaling, T cell development, and homeostasis (Ao et al., 2023)

Chrysin is correlated with PGF target protein. Patients with rheumatoid arthritis have higher levels of VEGF expression in their serum and synovial fluid, which correlates with CRP in connection with radiological abnormalities in the hands and feet. VEGF interacts with one or two receptor tyrosine kinases, VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2). VEGFR-1, also known as fms-related tyrosine kinase 1 (FLT-1), triggers the production of pro-inflammatory cytokines that contribute to inflammation in rheumatoid arthritis patients. VEGFR-1 plays a core role in pathological angiogenesis during rheumatoid arthritis, which is mediated by VEGF and placental growth factor (PGF). Upregulation of FLT-1 expression was positively correlated with VEGF and PGF concentrations. This causes hyper-responsiveness and increased production of specific pro-inflammatory cytokines in rheumatoid arthritis. Animal models of rheumatoid arthritis using antibodies against FLT-1 have shown suppression of angiogenesis and inflammatory joint damage. This suggests that selective reduction of pathological angiogenesis and inflammatory responses in patients with active rheumatoid arthritis may be attainable by suppressing FLT-1 (Paradowska-Gorycka et al., 2017).

Chrysin compounds correlated with two target proteins, namely ALOX12 and ALOX15, also correlated with CBR1, while kaempferol correlated with three target proteins, ALOX5, ALOX12, and ALOX15.

Vanillic acid is associated with CXCL12. One of the primary sources of chemokine motif CXC ligand 12 (CXCL12), which is essential for the migration and activation of inflammatory cells into synovial tissue, is stromal cells. The natural receptor for CXCL12 is CXC receptor 4 (CXCR4). The chemokine CXCL12 mediates T cell and B cell migration and activation in immune cells and may contribute to the immunological response against rheumatoid arthritis. Joint synovial cells produce and secrete CXCL12. Apoptosis and chondrocyte destruction can result from articular chondrocytes secreting different inflammatory agents when CXCR4 and CXCL12 are activated (Peng et al., 2020).

The plumbagin compound correlated with the target protein EGFR. Serum and joint epidermal growth factor receptor (EGFR) concentrations were significantly higher in rheumatoid arthritis. The EGFR inhibitor erlotinib was shown by Swanson et al. to mitigate antigen-induced arthritis in mice and decrease synovitis, pannus development, cartilage loss, and bone erosion, suggesting that EGFR may be a potential target for rheumatoid arthritis treatment (Yuan et al., 2013).

Plumbagin has been linked to the pathogenesis of fibrosis, inflammation, transition from epithelial to mesenchymal, and promotion of extracellular matrix deposition. It is connected to EP300 (Rubio et al., 2023).

Plumbagin and suberosin, which correlate with the same target protein XPO1. XPO1 is a novel candidate for targeted therapy in rheumatoid arthritis. These genes were primarily enriched in intercellular communication and fungal immune-related pathways, including tight junction formation, Th17 cell differentiation, cell-leukocyte adhesion, focal adhesion, cytokine-mediated regulation of signaling pathways, and regulation of interleukin 2 production. This was revealed by GO and KEGG pathway enrichment analyses of HRG (Birga et al., 2022).

l-Tryptophan was correlated with the target protein CTSL. Three compounds correlate to one target protein, namely isoorientin, quercetin 3-ol-rhamnoside, and vanillic acid. These three compounds were correlated with the same target protein, ALOX5. In this study, quercetin 3-o-l-rhamnoside correlated with the inflammatory lipoxygenase signaling pathway.

Quercetin inhibits LPS-induced TNF-α and IL-8 production generated by LPS in macrophages and lung A549 cells. It has been reported to inhibit LPS-induced TNF-α mRNA levels and IL-1α expression. Quercetin also inhibits inflammatory lipoxygenase (LOX) and cyclooxygenase (COX) (Shorobi et al., 2023).

In research conducted by Anilkumar et al., 2017, isoorientin has been shown to decrease inflammation in mice with air sac models. Additionally, Western blot analysis has revealed the expression of inflammatory proteins COX-2, TNFα, IL-1β, iNOS, and 5-LOX. Carrageenan significantly raised the expression of COX-2, TNFα, IL-1β, iNOS, and 5-LOX; however, isoorientin treatment reduced the expression of these proteins.

In the network pharmacology visualization results of the two plants, it was found that several compounds from the two plants had the same correlation with the rheumatoid arthritis target protein. There are also three compounds that correlate with only one target protein and there are compounds that have many correlations with several target proteins. It can be seen that compounds from the two plants correlate with the target proteins of various rheumatoid arthritis signaling pathways. If these plants are used together, it is expected that they will have an effect in accordance with the intended protein target or signaling pathway.

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E-ISSN: 2580-8303

CONCLUSION

In summary, the network pharmacology results of the two plants Acalypha indica L. and Plumbago zeylanica L. showed a correlation between each compound and the target proteins of rheumatoid arthritis in different signaling pathways. The results of this screening can be used to determine whether compounds from the two plants have a correlation with various signaling pathways in rheumatoid, which can be used for further research.

ACKNOWLEDGEMENTS

The authors would like to thank the Master Program of Pharmaceutical Science, Faculty of Pharmacy, Universitas Setia Budi.

AUTHOR CONTRIBUTIONS

Conceptualization, D.A., R.H., A.I.; Methodology, D.A., R.H., A.I.; Software, D.A., R.H., A.I.; Validation, D.A., R.H., A.I.; Formal Analysis, D.A., R.H., A.I.; Investigation, D.A., R.H., A.I.; Resources, D.A., R.H., A.I.; Data Curration; D.A., R.H., A.I.; Writing - Original Draft, D.A., R.H., A.I.; Writing - Review & Editing, D.A., R.H., A.I.; Visualization, D.A., R.H., A.I.; Supervision, D.A., R.H., A.I.; Project Administration, D.A., R.H., A.I.; Funding Acquisition, D.A., R.H., A.I.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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P-ISSN: 2406-9388
E-ISSN: 2580-8303

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia
Pharmacy and Pharmaceutical Sciences Journal
Published by Faculty of Pharmacy Universitas Airlangga
OPEN ACCESS
E-ISSN 2580-8303
P-ISSN 2406-9388

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia Vol. 11 No. 2 August 2024, 219-229
DOI: 10.20473/jfiki.v11i22024.219-229
Available online at https://e-journal.unair.ac.id/JFIKI/

Potential Combination of Dayak onion *Eleutherine palmifolia* L. and Chrysanthemum flower *Chrysanthemum indicum* L. as Anti-aging: Network Pharmacology Approach

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Submitted: 18 April 2024
Revised: 12 August 2024
Accepted: 31 August 2024

Abstract

Background*: An anti-aging agent is a preparation or product used to inhibit skin aging. Aging occurs because of cell damage caused by free radicals. Therefore, anti-aging agents, which contain antioxidants to inhibit oxidative stress caused by free radicals, are needed. Dayak onion (eleutherine palmifolia (L) Merr.) and chrysanthemum flowers (Chrysanthemum indicum L.) have high antioxidant content, which has the potential to inhibit aging.* ***Objective:*** *To determine the anti-aging potential of dayak onion and chrysanthemum flower compounds* ***Methods:*** *Network pharmacology was performed using GeneCards (https://www.genecards.org) to obtain target genes, Cytoscape v3.10.1 software (https://cytoscape.org), DisGeNET (https://www.disgenet.org), STRING (https://www.string-db.org/), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) PATHWAY (https://www.genome.jp/kegg/pathway.html).* ***Results:*** *Based on Network Pharmacology, dayak onion and chrysanthemum flowers showed that 14 of the 200 target proteins were involved in biological processes and signaling pathways for premature aging syndrome.* ***Conclusion:*** *The combined compound from dayak onion and chrysanthemum flower has anti-aging activity due to seven bioactive components in the hydroxycinnamic acid group. This compound influences biological processes and longevity by regulating the CAD, SIRT1, and TP53 signaling pathways. Therefore, the combination of dayak onion and chrysanthemum flowers has potential as an anti-aging agent.*

Keywords*: anti-aging, dayak onion, chrysanthemum flower, longevity regulating pathway, network pharmacology*

How to cite this article:
Fitriyani, Ningrum, S. E. S. & Mutiah, R. (2024). Potential Combination of Dayak onion *Eleutherine palmifolia* L. and Chrysanthemum flower *Chrysanthemum indicum* L. as Anti-aging: Network Pharmacology Approach. *Jurnal Farmasi dan Ilmu Kefarmasian Indonesia,* 11(2), 219-229. http://doi.org/10.20473/jfiki.v11i22024.219-229

P-ISSN: 2406-9388
E-ISSN: 2580-8303

INTRODUCTION

An anti-aging agent is a preparation or product used to prevent aging. Aging is a complex biological process characterized by structural changes and skin elasticity, appearance of wrinkles, rough skin, dry skin, and changes in pigmentation (Kumalasari & Prihandiwati, 2019). Aging is caused by environmental factors such as sunlight, air humidity, temperature, cigarette smoke, and air pollution (Nailufa & Najih, 2020). Aging occurs because of cell damage caused by free radicals (Rahmadiani & Hasanah, 2019). The main cause of aging is extrinsic factors due to exposure to UV rays from the sun, which contain free radicals; therefore, Indonesian people are prone to this problem owing to the influence of the tropical climate (Firdayeni & Sari, 2022).

In the human body, oxidation or combustion processes, inflammation, excessive physical activity, and pollution exposure lead to the dynamic formation of free radicals. The overproduction of free radicals can harm macromolecules (lipids, carbohydrates, and nucleic acids) and cells, resulting in aging and degenerative illnesses (Zhang et al., 2020). Enzymatic and non-enzymatic antioxidants protect the body against free radical damage by removing excess ROS and preventing aging. Thus, anti-aging products contain antioxidants that inhibit the oxidative stress caused by free radicals (Kumalasari & Prihandiwati, 2019).

Dayak onion (eleutherine palmifolia (L) Merr.) is a typical Central Kalimantan plant originating from America, containing secondary metabolite compounds of the flavonoid group, naphthoquinone group, and their derivatives (eleutherin, eleutherol, eleutherinol, eleutherionin, eleuthoside B, and eleuthoside A), as well as the polyphenol group (oxyresveratrol) (Muti'ah et al., 2020). According to Pramiastuti et al. (2021), dayak onions have secondary metabolites with IC50 values for flavonoids, alkaloids, tannins, saponins, phenolics, and steroids or triterpenoids with antioxidant or anti-free radical properties. According to Novaryatiin et al. (2019), dayak onions contain alkaloids, flavonoids, quinones, polyphenols, saponins, steroids, monoterpenoids and tannins. The flavonoid group in dayak onion has the ability to transform to produce antioxidant activity that inhibits free radicals (Mokoginta et al., 2020). The useful part and the part used in this research is dayak onion bulbs.

Chrysanthemum flower (*Chrysanthemum indicum* L.) is a shrub or semi-shrub and sub-tropical plant originating from Japan and North China which is very popular among people because it has aesthetic value and has a variety of colors, and is used as an ingredient in traditional medicine (Sembiring et al., 2021). Chrysanthemum flower are generally only used as cut flowers, even though these flowers contain high levels of antioxidants including flavonoids, tannins, terpenoids, alkaloids, steroids and saponins (Puspita et al., 2023). Chrysanthemum flowers have antioxidant, anti-inflammatory, antineoplastic, antidiabetic, antibacterial, and lipid-lowering properties, and contain flavonoids in the form of quercitrin, myricetin, and luteolin 7-glucoside (Hartanto et al., 2021). According to Marlina and Widiastuti (2021), the main secondary metabolite in chrysanthemum flowers is pyrethrin (12.66 %). Chrysanthemum flowers have the potential for anti-aging because they contain active components, such as essential oils, terpenoids, flavonoids, and phenolic acids, as well as being a source of quercitrin and myricetin. The major flavonoid components included 7-O-β-D-gulocoside and linarin such as phenolic acids from chlorogenic acid, 3,5-di-O-Caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid, and 4, 5-di-O-caffeoylquinic (Wanita, 2022).

Dayak onions and chrysanthemums can potentially be used as anti-aging agents based on their antioxidant content. This is because aging can be overcome by administering natural antioxidant compounds. Flavonoids can prevent the occurrence of Reactive Oxygen Species (ROS), protect the skin from damage, and inhibit specific skin aging enzymes (Kumalasari & Prihandiwati, 2019). Processing dayak onion and chrysanthemum flowers, considering their use as anti-aging agents, has never been studied. This development will have an impact on two things, namely, the cultivation and development of dayak onion and chrysanthemum flowers, as well as anti-aging.

MATERIALS AND METHODS

Tools

The ingredients used in this research for network pharmacology tests are GeneCards (https://www.genecards.org) to obtain target gens, Cytoscape v3.10.1 software (https://cytoscape.org), DisGeNET (https://www.disgenet.org), STRING (https://www.string-db.org/), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) PATHWAY (https://www.genome.jp/kegg/pathway.html).

Method

Network pharmacology test

Network pharmacology uses an integrated network of biological systems and computer analysis technology

P-ISSN: 2406-9388
E-ISSN: 2580-8303

to determine the active components and mechanisms of an active ingredient with target proteins (Tjandrawinata et al., 2022). This method combines network biology with polypharmacology, based on the effectiveness of highly selective compound target proteins, capable of identifying compounds and disease targets from large amounts of data and understanding their mechanisms and pathways of activity including exploring the basic pharmacological effects of a compound on disease and its mechanisms (Zhou et al., 2020). Network pharmacology was used to investigate the molecular mechanisms of dayak onion and chrysanthemum flowers, which play an important role in inhibiting free radicals that cause aging. The computational approach in this method can accommodate large and fast data, as well as promising results for the study of active ingredients, such as dayak onion and chrysanthemum flower (Syahrir et al., 2016).

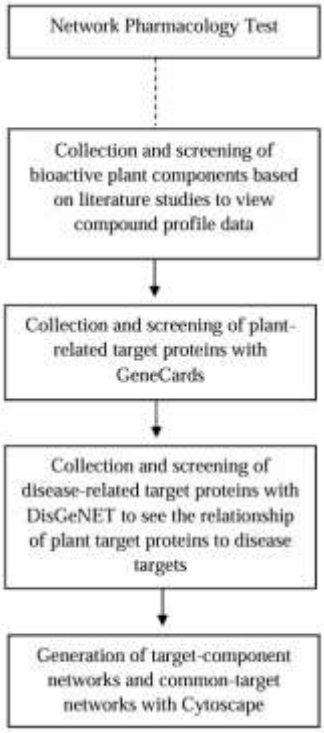

Figure 1. Network pharmacology test flow

Collection and screening of bioactive components of dayak onion and chrysanthemum flower

The bioactive compound components of dayak onion and chrysanthemum flower were obtained based on the results of a literature study from other scientific research indexed by Google Scholar with the keywords "*Chrysanthemum indicum* compound" and "*Eleutherine palmifolia* compound." The components selected were those with high levels and dominated based on comparative literature studies.

Collection and screening of target proteins related to dayak onion and chrysanthemum flower

Target proteins and genes related to anti-aging were obtained from GeneCards (Permatasari et al., 2021). GeneCards is a searchable integrative database that provides comprehensive, easy-to-use information on all annotated and predicted human genes. The knowledge base automatically integrates data, including genomic, transcriptomic, proteomic, genetic, clinical, and functional information. The targets that yield results from GeneCards are restricted to those with a relevance value of ≥10.00, as this value is deemed to satisfy the database requirements (Tjandrawinata et al., 2022). Target protein association data are then associated with diseases from the DisGeNET database, which contains data on genes and variants associated with human diseases.

Collection and screening of disease-related target proteins and creation of target networks

Next, we explored the target genes associated with anti-aging using DisGeNET. DisGeNET is a method for collecting disease target data by searching a database that contains information about the connections between proteins and disease targets (Rosyadah et al., 2017). The next step is to create a target network related to dayak onion and chrysanthemum flowers that are collected into a target-component network, which is then visualized through a similarity network using Cytoscape v3.10.1 (Qomariasih et al., 2016). The target proteins and bioactive components of dayak onion and chrysanthemum flower are represented as "nodes" and the interactions between the two proteins as "edges." The more important the proteins that are the targets of a component, the more that component can be designated as an important component (Tjandrawinata et al., 2022).

Creation of a protein-protein interaction network (PPI network) and enrichment analysis

Using the (STRING) platform, Gene targets at the intersection of the active ingredient and disease were selected for further analysis using the STRING platform. PPI network analysis utilized gene ontology (GO) functional annotations, enrichment of protein pathways by the Kyoto Encyclopedia of Genes and Genomes (KEGG), and their functions in signal transduction. PPI networks were constructed using common target proteins with a minimum interaction score of 0.400 (Tjandrawinata et al., 2022).

RESULTS AND DISCUSSION

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Collection and screening of bioactive components of Dayak onion and Chrysanthemum flower

Based on the results of a literature study using the Google Scholar search engine with the keywords "Chrysanthemum indicum compound" and "Eleutherine palmifolia compound" the following results were obtained (Tables 1 and 2).

Collection and screening of target proteins related to dayak onion and chrysanthemum flower

Based on the collection and screening of target proteins from the GeneCards database with a relevance value ≥10.00, 260 target proteins from various types of compounds were obtained, as shown in Table 3.

Table 1. Bioactive components of dayak onion

Compound Name	Molecular Type	Ppubchem CID	References
Naphthoquinone Naphthalene	Quinones	931	(Kamarudin et al., 2021; Muti'ah et al., 2020; Narko et al., 2017)
Oxyresveratrol	Polyphenols	5281717	(Muti'ah et al., 2020; Qureshi & Javed, 2022; Wahdaningsih et al., 2023) (
Isoliquiritegenin	Flavonoids	135031285	(Muti'ah et al., 2020)
Sitosterol	Steroids	3084097	(Saputra et al., 2016)

Table 2. Bioactive components of chrysanthemum flower

Compound Name	Molecular Type	Ppubchem CID	References
Quercitrin;	Flavonoids	5280343	Chen et al., 2021; Hartanto et al., 2021; Marlina & Widiastuti, 2021; Wanita, 2022; Click or tap here to enter text. Wang et al., 2019; Yuan et al., 2020; Zhou et al., 2023)
Myricetin;		5281672	
luteolin 7-glucoside;		5280637	
Apigenin;		5280443	
Luteolin;		5280445	
Kaempferol;		5280863	
Diosmetin		5281612	
1,3-dicaffeoylquinic acid;	Hydroxycinnamic acids	6474640	(Chen et al., 2021; Lin & Harnly, 2010; Jiang et al., 2022; Ma & Wako, 2017; Zhou et al., 2023)
3,4-dicaffeoylquinic acid;		6474309	
1,5-dicaffeoylquinic acid;		122685	
3,5-dicaffeoylquinic acid;		13604688	
1,4-dicaffeoylquinic acid;		12358846	
4,5-dicaffeoylquinic acid;		5281780	
Chlorogenic acid;		1794427	
Caffeic acid		689043	
β-carotene, α-carotene	Carotenoids	5280489, 6419725	(Chen et al., 2021)

Table 3. Target protein screening results

Compound type	Number of Gene Targets	Gifts Range	Relevance Range
Sitosterol	3	44-49	14-23
Naphthoquinone	1	52	11
1,3-dicaffeoylquinic acid	4	13-25	17-29
3,4-dicaffeoylquinic acid	4	14-55	10-22
1,5-dicaffeoylquinic acid	4	51-57	18-26
3,5-dicaffeoylquinic acid	3	13-57	19-29
1,4-dicaffeoylquinic acid	2	55	17-25
4,5-dicaffeoylquinic acid	4	55-57	12-23
Chlorogenic acid	59	13-57	10-40
Caffeic acid	175	11-59	10-108
Total	**260**		

P-ISSN: 2406-9388
E-ISSN: 2580-8303

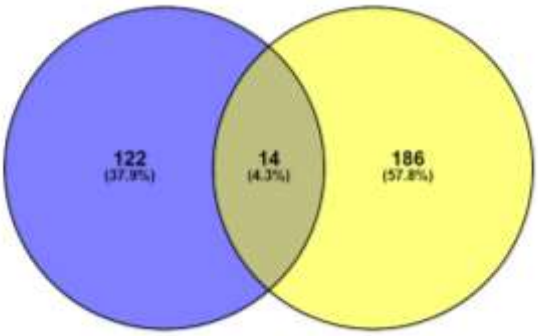

Figure 2. Venn diagram of 200 target proteins of dayak onion and chrysanthemum flower against premature aging syndrome

Collection and screening of disease-related target proteins and creation of target networks

Of the 260 target proteins, there are 60 identical target proteins and 200 different target proteins that are associated with 14 specific target proteins related to anti-aging (Premature Aging Syndrome) through DisGeNET. This network pharmacology study revealed 14 target proteins related to anti-aging, which are the main targets where these proteins lock and interact with each other.

Based on the Venn diagram (Figure 2), there were 14 genes whose interactions were anti-aging. Yellow represents 200 target genes from dayak onion and chrysanthemum flowers, while blue represents 136 target genes for premature aging syndrome. A number of target genes that have anti-aging interactions include APP (*Amyloid-beta A4 protein*), TP53 (*Cellular tumor antigen p53*), SIRT1 (Sirtuin-1), SOD1 (*Superoxide dismutase*), VDR (*Vitamin D3 receptors*), CDKN1A (*Cyclin-dependent kinase inhibitor 1*), BCL2 (B2 cell lymphoma), CAT (Catalase), MAPK1 (*Mitogen-activated protein kinase 1*), NFE2L2 (*Nuclear factor erythroid 2-related factor 2*), POLG (*polymerase subunit gamma-1*), MMP9 (*Matrix metalloproteinase-9*), GUSB (*Beta-glucuronidase*), IL1B (*Interleukin-1 beta*). Next, the target network obtained from the Cytoscape v3.10.1 visualization results obtained protein-target protein interactions, as shown in Figure 3.

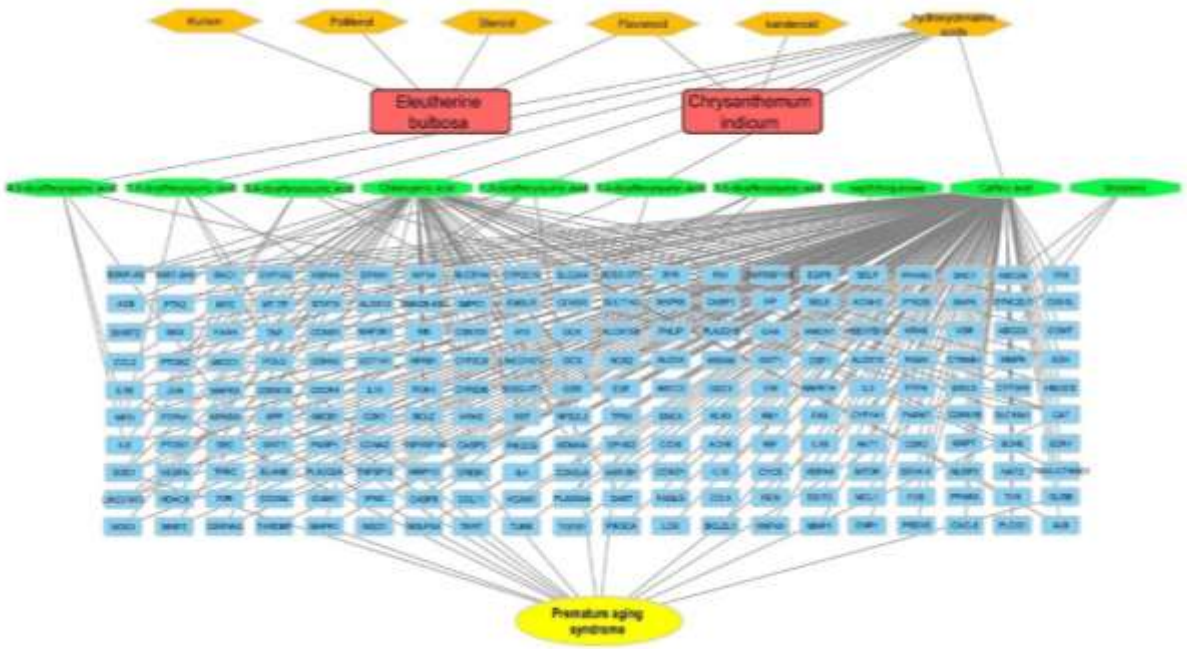

Figure 3. Visual network of dayak onion and chrysanthemum flower (Red: plant name, Orange: compound molecules, Green: Bioactive components, Yellow: Disease, Blue: Target protein)

P-ISSN: 2406-9388
E-ISSN: 2580-8303

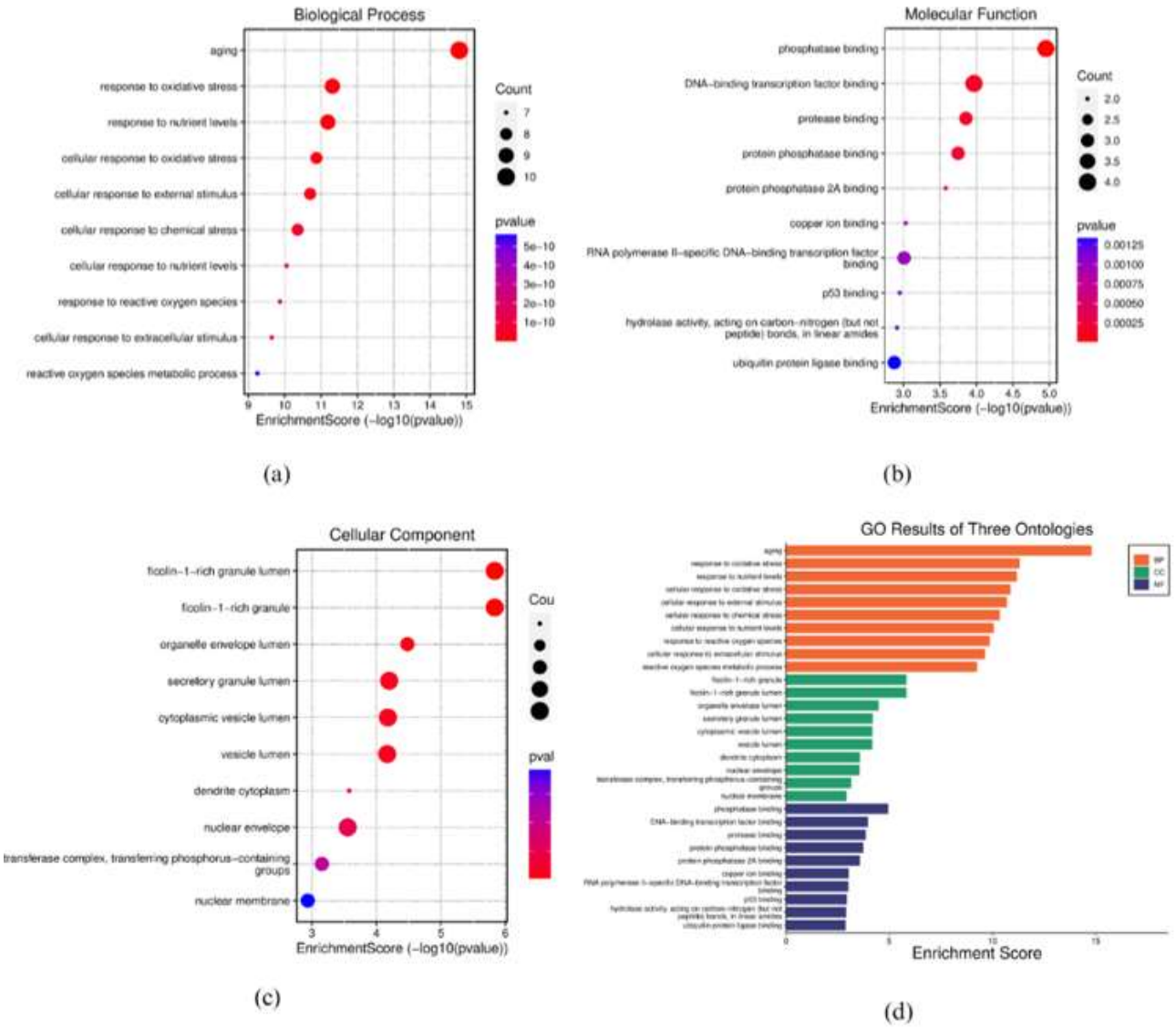

Figure 4. Interaction network of 14 core proteins from STRING database (14 nodes, 48 edges, PPI enrichment p-value: 4.68e-12); (a) 14 target genes interact as aging; (b) 4 target genes involved in anti-aging biological processes

In the visual target network (Figure 2), there were 14 target proteins (APP, TP53, SIRT1, SOD1, VDR, CDKN1A, BCL2, CAT, MAPK1, NFE2L2, POLG, MMP9, GUSB, and IL1B) from seven bioactive components that had target proteins related to anti-aging, namely chlorogenic acid, caffeic acid, 3,4-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, 1,3-dicaffeoylquinic acid, 1,5-dicaffeoylquinic acid, and 1,4-dicaffeoylquinic acid. These active components are some of the important molecules with anti-aging activities, namely hydroxycinnamic acids. According to Cizmarova et al. (2020), hydroxycinnamic acids can influence aging by increasing skin elasticity and providing positive anti-wrinkle effects owing to natural bioactive compounds. Additionally, its antioxidant activity increases collagen production and prevents premature aging. The antimicrobial activity of This compound has also been proven to have anti-wrinkle activity in vivo and is effective against skin problems. Considering that most of the bioactive components belong to caffeic acid, Bastianini et al. (2018) reported that acaffeic acid is a very promising hybrid owing to its higher bioavailability and prolonged antioxidant activity in the skin.

Creation of a protein-protein interaction network (PPI Network) and enrichment analysis

Protein-protein interactions (PPI) are physical or functional interactions between two or more proteins that play key roles in various cellular processes. The results obtained in this study highlight the relationship between various types of proteins in dayak onion and chrysanthemum flowers, which have an anti-aging role. Dayak onion and chrysanthemum flowers contain multiple components that act on multiple targets through

P-ISSN: 2406-9388
E-ISSN: 2580-8303

various mechanisms of action. Through this network pharmacology study, the mechanism of action of dayak onion and chrysanthemum flowers can be described at the molecular level more comprehensively in terms of signaling pathways.

Based on the enrichment analysis of the mechanism of action of dayak onion and chrysanthemum flower as potential anti-aging agents, the results of KEGG analysis identified the target genes that were most associated with the data: TP53 (*cellular tumor antigen p53*), SIRT1 (Sirtuin-1), SOD1(*superoxide dismutase*), and CAT (catalase).

The results of gene ontology analysis showed that the compounds contained in dayak onion and chrysanthemum flower are mainly chlorogenic acid, caffeic acid, 3,4-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, 1,3-dicaffeoylquinic acid, 1,5-dicaffeoylquinic acid, and 1,4-dicaffeoylquinic acid, which are involved in biological processes, molecular functions, and cellular components. In the bubble-shaped enrichment picture, it was found that there are 10 biological processes that have high potential, where aging has the highest significance value, involving as many as 10 genes. This was followed by oxidative stress, nutrient levels, etc., involving genes in the range 7-9 as shown in Figure 4A. In addition, these compounds also affect molecular functions, where the 10 molecular functions with the highest potential were shown by phosphatase binding involving four genes. Next, DNA-binding transcription factor binding, protease binding, and so on, with a range of genes involved from 2-3.5 as shown in Figure 4B. Meanwhile, the highest potential cellular components were ficolin-1-rich granule lumen and rich granules, as shown in Figure 4C. All enrichment scores for biological processes, cellular components, and molecular functions can be seen in the 4D image bar diagram with interpretation of the values presented.

This study showed that dayak onion and chrysanthemum flowers affect the biological processes and signaling pathways of type 2 DM toward TP53 (*cellular tumor antigen p53*) and SIRT1 (Sirtuin-1).

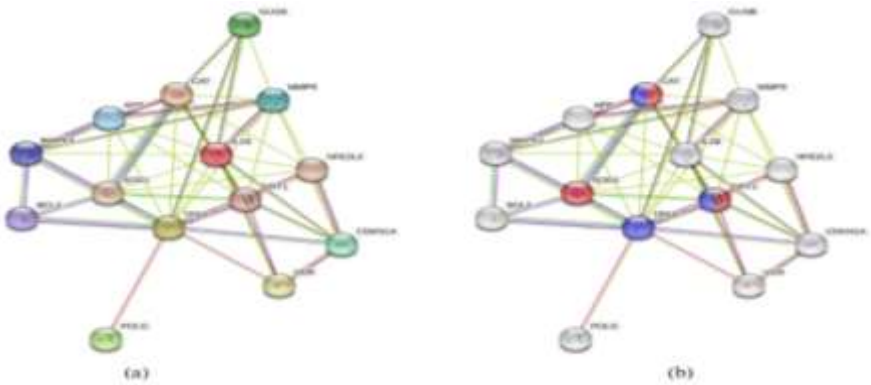

Figure 5. KEGG Gene Ontology and Pathway Enrichment Analysis; (a) Enrichment bubble diagram of 10 biological processes with high potential; (b) Enrichment bubble diagram of 10 molecular functions with high potential; (c) Enrichment bubble diagram of 10 cellular components with high potential; (d) GO bar diagram of enrichment for biological processes, molecular functions, and cellular components

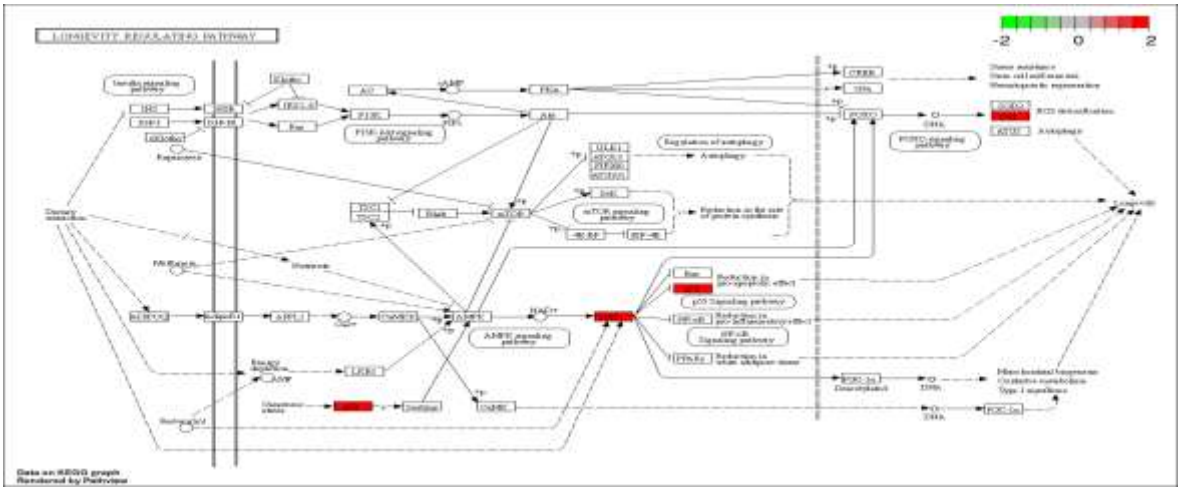

Figure 6. Signaling pathway involving three potential target genes (red marks) in anti-aging activity

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Table 4. Results of the KEGG gene interaction analysis

Pathways	Description	Count in network	Strength	False Discovery Rate	Genes
Hsa04213	Longevity regulating pathway-multiple species	3 of 61	1.84	0.00021	CAT, SIRT1, SOD1
hsa04211	Longevity regulating pathway	7 of 46	1.6	8.70e-09	CAT, SIRT1, TP53

TP53 is involved in cell cycle regulation as a trans-activator that negatively regulates cell division by directing several essential genes. It also functions as a tumor suppressor in many different types of cancers and can induce growth arrest or apoptosis, depending on the physiological state and type of cell. TP53 protein is often called the guardian of the genome because it is an important factor in maintaining genome stability, which induces cell senescence and apoptosis if genome instability occurs, which induces the aging process (Siswanto & Kartiko, 2017). In this case, the TP53 process is anti-aging and prevents or repairs damage at the genomic level.

SIRT1 is a master regulator associated with aging that helps coordinate multiple distinct cellular processes, including the cell cycle, response to DNA damage, metabolism, apoptosis, and autophagy. It also directly connects transcriptional regulation with intracellular energy, as well as modulating chromatin function through histone deacetylation, and can induce changes in histone and DNA methylation, leading to transcriptional repression, which is involved in decisions regarding cellular senescence or apoptosis. In SIRT1, pleiotropic activity is an important marker of cellular aging, as well as in several diseases such as cardiovascular and neurodegenerative diseases, diabetes, and cancer. Generally, with increasing age, SIRT1 levels decrease in the aging liver, whereas there is a simultaneous increase in the accumulation of DNA damage (Grabowska et al., 2017). Vascular aging is accelerated in the liver, heart, kidney, brain, and lung by a decrease in SIRT1 expression in endothelial cells (EC), vascular smooth muscle cells (VSMC), and macrophages. SIRT1 contributes to the inhibition of aging of nucleus pulposus cells, promotion of cell division, inhibition of apoptosis, and inhibition of UV-induced fibroblast aging, as well plays a role in crucial cellular activities including response to stress, metabolism and longevity (cell senesce) (Chen et al., 2020).

CAT protects cells from the harmful effects of hydrogen peroxide and stimulates the proliferation of several cell types, including T cells, B cells, melanoma, mastocytoma, myeloid leukemia, and normal and altered fibroblast cells. CAT significantly contributes to the antioxidant defense of cells by dissolving hydrogen peroxide in oxygen and water. According to its pleiotropic significance, CAT is linked to a reduction or impairment in age-related diseases with a shorter lifetime (Dutta et al., 2022).

CONCLUSION

Based on network pharmacology, dayak onion and chrysanthemum flowers showed that 14 of the 200 target proteins were involved in the biological processes and signaling pathways of premature aging syndrome with target locking and interaction. The 14 target proteins are compound molecules in dayak onion and chrysanthemum flowers, which are included in the seven bioactive components of the hydroxycinnamic acid group. These compounds affect biological processes and signaling pathways of longevity regulators for TP53, SIRT1, and CAT through mechanisms already explained. Therefore, the combination of dayak onion and chrysanthemum flowers has the potential to have anti-aging effects.

AUTHOR CONTRIBUTIONS

Conceptualization, R.M., F., S.E.S.N.; Methodology, R.M., F., S.E.S.N.; Software, R.M., F., S.E.S.N.; Validation, R.M.; Formal Analysis, S.E.S.N.; Investigation, F.; Resources, S.E.S.N.; Data Curration; S.E.S.N.; Writing - Original Draft, S.E.S.N.; Writing - Review & Editing, F.; Visualization, F.; Supervision, R.M.; Project Administration, R.M.; Funding Acquisition, R.M.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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P-ISSN: 2406-9388
E-ISSN: 2580-8303

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia
Pharmacy and Pharmaceutical Sciences Journal
Published by Faculty of Pharmacy Universitas Airlangga
OPEN ACCESS
E-ISSN 2580-8303
P-ISSN 2406-9388

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia Vol. 11 No. 2 August 2024, 230-241
DOI: 10.20473/jfiki.v11i22024.230-241
Available online at https://e-journal.unair.ac.id/JFIKI/

Analysis of Cayenne Pepper Fruit (*Capsicum frutescens*) in Inhibiting HMG-CoA Reductase Activity as a Treatment for Hypercholesterolemia

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Submitted: 6 Maret 2024
Revised: 5 July 2024
Accepted: 31 August 2024

Abstract

Background*: Hypercholesterolemia is a major cause of cardiovascular disease and its incidence continues to increase. Statins are a group of hypercholesterolemic therapies known to trigger various side effects; therefore, statin alternatives need to be investigated. The cayenne pepper (Capsicum frutescens) contains secondary metabolites that inhibit the activity of cholesterol-forming enzymes (HMG-CoA reductase).* ***Objective:*** *The aim of this study was to identify the ability of C. frutescens fruit to inhibit HMG-CoA reductase activity to prevent hypercholesterolemia.* ***Methods:*** *This was a true experimental study using a posttest-only control group design. The independent variables were n-hexane, methanol, and ethanol extracts of C. frutescens fruit, each with a concentration of 0.01%, with HMG-CoA reductase activity as the dependent variable. Enzymatic activity was measured enzymatically using spectrometry.* ***Results:*** *The mean values of % inhibition from n-hexane, methanol, and ethanol extracts of C. frutescens and pravastatin were 95.74%, 104.70%, 100.11%, and 99.27%, respectively. The average specific activities of n-hexane, methanol, and ethanol extracts of C. frutescens and pravastatin were 0.5765, 0.6029, 0.5513, and 0.5716 units/mgP, respectively. There was a significant difference between the sample groups in the inhibition of HMG-CoA reductase activity. HMG-CoA reductase inhibitory activity was highest in the methanol extract, followed by the n-hexane extracts. The activity of these extracts was higher than that of pravastatin alone.* ***Conclusion:*** *The methanol extract showed the best inhibitory activity****.*** *C. frutescens has been shown to have great potential in inhibiting the activity of the enzyme HMG-CoA reductase and preventing hypercholesterolemia.*

Keywords*: Capsicum frutescens, HMG-CoA reductase, hypercholesterolemia*

How to cite this article:
Albar, L. O. M., Tien & Eso, A. (2024). Analysis of Cayenne Pepper Fruit (*Capsicum frutescens*) in Inhibiting HMG-CoA Reductase Activity as a Treatment for Hypercholesterolemia. *Jurnal Farmasi dan Ilmu Kefarmasian Indonesia*, 11(2), 230-241. http://doi.org/10.20473/jfiki.v11i22024.230-241

P-ISSN: 2406-9388
E-ISSN: 2580-8303

INTRODUCTION

Hypercholesterolemia is a non-communicable disease caused by lipid metabolism disorders and is characterized by an increase in total cholesterol levels in the blood. As one of the causes of coronary heart disease (CHD), high cholesterol levels can increase the risk of death by up to three times (Jempormase et al., 2016). Globally, the highest incidence of hypercholesterolemia is in Europe, with a prevalence of 54%, America, with a prevalence of 48%, and Southeast Asia, with a prevalence of 30% (WHO, 2011). Locally, 36 million people, or about 18% of the Indonesian population, suffer from this blood fat disorder. Of that number, 80% of patients die suddenly from a heart attack and 50% have no previous symptoms (Jempormase et al., 2016).

Cholesterol biosynthesis requires the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. This enzyme converts HMG-CoA into mevalonic acid through a series of condensation and rearrangements, and mevalonic acid is then converted into cholesterol (Baskaran et al., 2015).

Statins are cholesterol-lowering drugs that competitively reduce HMG-CoA reductase activity. Inhibition of this enzyme causes a series of reactions that trigger an increase in LDL receptor expression, which increases plasma LDL absorption, thereby decreasing plasma LDL cholesterol levels (Dewi and Merry, 2017). Despite being a guideline for cholesterol-lowering therapy, several studies have shown that long-term use of statins can lead to side effects, including hyperglycemia, leading to a new onset of diabetes mellitus, myopathy, renal failure, neurological side effects such as muscle pain (myalgia) and neurocognitive disorders, and hepatotoxic effects (Rizqi et al., 2014; Farida and Putri, 2016; Ward et al., 2019).

Cayenne pepper (*Capsicum frutescens*) is a shrub that grows widely in Indonesia. This plant is part of the *Plantae* kingdom, *Solanaceae* family, and *Capsicum* genus (Simpson 2010). Several studies have shown the antioxidant effects of *C. frutescens* (Melannisa et al., 2011; Giovedi, 2016; Talitha, 2017) and its ability to inhibit the growth of *Staphylococcus aureus* (Munira et al., 2019; Taolin, 2019; Rahim and Nurmayanti, 2020). A number of studies using in silico docking methods have shown that secondary metabolites contained in *C. frutescens*, including flavonoids, terpenoids, alkaloids, and phenols, have the capacity to competitively reduce the activity of HMG-CoA reductase (Islam et al., 2015; Aqeel et al., 2018; Hariyati et al., 2018; Azmi et al., 2021; Shaik et al., 2020; Mannino et al., 2021). The catalytic activity of the enzyme against substrates in the form of HMG-CoA and NADPH co-substrates to form mevalonate can be prevented (Fridiana et al., 2019; Gesto et al., 2020; Marahatha et al., 2021).

This study aimed to identify the ability of n-hexane, methanol, and ethanol extracts of *C. frutescens* to inhibit HMG-CoA reductase activity and to determine the significant difference in the ability of *C. frutescens* extracts with statin class drugs such as pravastatin. This study can serve as a follow-up to in silico investigations of the numerous secondary metabolites detected in *C. frutescens fruit* with HMG-CoA reductase activity. This study can also serve as a source of scientific information about the *C. frutescens fruit*, enhancing public confidence in its use in everyday life.

MATERIALS AND METHODS

Materials

The material used in this study were *C. frutescens* obtained in Labaha Village, Watopute District, Muna Regency, Southeast Sulawesi. Plant taxonomy was determined at the Research Laboratory, Faculty of Pharmacy, Halu Oleo University, with sample code 017 and letter number 613. a/UN29.18/PP/2024. Other materials used in this research were the HMG-CoA reductase assay kit (Sigma Aldrich, Missouri, USA), ethanol (Merck), methanol (Merck), n-hexane (Merck), Meyer reagent, and Dragendorff reagent , Darmstadt, Germany).

Instrument

An ELISA reader (Thermo-Multiskan FC) was used to measure the HMG-CoA reductase enzyme activity.

Methods

This study was conducted at the Biomedical Laboratory of the Faculty of Medicine and Pharmacy Laboratory of the Faculty of Pharmacy, Universitas Halu Oleo.

Sample collection and preparation

C. frutescensi fruit were collected, then separated between the fruit and the stalks, then washed with running water to separate the fruit from the dirt attached to the sample. The samples were then dried out by being placed in an oven at 40°C. The drying process was carried out until the sample was completely dried and yielded a powder.

Extraction

The maceration method was applied in the extraction process by mixing *C. frutescens* plant powder with pure solvents (pro analysis) in the form of ethanol

P-ISSN: 2406-9388
E-ISSN: 2580-8303

(100%), methanol, and n-hexane at 1 g of dried powder per 10 mL of solvent for 48 h in the dark at room temperature. The extract was filtered through a filter paper until the filtrate was obtained. To obtain a thick extract, the concentration procedure was performed using a Rotary Vacuum Evaporator at 60 °C. To enhance solubility, the samples were diluted in DMSO.

Phytochemical screening

Phyochemical screening was performed as described by Wijaya et al. (2018) and Saripa et al. (2020).

Flavonoidstwo

Two drops of concentrated HCl were used to observe the color changes. The solution was then heated in a water heater for 15 min. The appearance of red, yellow, or orange after heating indicates the presence of flavonoid compounds.

Alkaloids

The residue was dissolved in 5 mL of HCl obtained by evaporating 2 mL of the test extract in a Petri dish. Divide into four tubes. Tube A (blank) was added to HCl. Dragendorff reagent was added with, Mayer reagent was added to tube C, and three drops of Wagner reagent were added to tube D. The white or orange precipitate formed indicated the presence of alkaloids in the test extract.

Terpenoids and sterols

A total of 0.5 mL of chloroform and 0.5 mL of $(CHCO_3)_2O$ were added to the test extract, which was then added to 2 mL of H_2SO_4. A bluish-green color indicates the presence of sterols. If a brown or purple ring forms at the boundary between the two solvents, the terpenoid content is present in the extract.

Phenol

One milliliter of extract (1000 µg/mL) was reacted with two drops of a 1% $FeCl_3$ solution. A strong red, green, or blue color is phenol-positive.

Saponins

The extract was cooled and shaken for 10 s in 10 ml of distilled water in a heated test tube. The foam formed with a height of 1-10 cm for 10 min was added with 2N HCl. The saponin content was considered positive if the foam did not disappear.

Tannins

One milligram was soaked in 96% ethanol. Three drops of a 1% FeCl3 solution were added. If a green or bluish-black color is formed, the tannins are positive.

Measurement of HMG-CoA Reductase Enzyme Activity

Ethanol, methanol, and n-hexane extracts (10 mg) from *C. frutescens* plants were evaporated and dissolved in 5 µL of 100% Dimethyl Sulfoxide (DMSO). The solution was then stirred to dissolve, diluted with 995 µL of deionized water, and stirred again until it dissolved.

Before starting the measurements, the ELISA reader was set at a temperature of 37 °C and wavelength of 340 nm. The work procedure was carried out with a 96 well plate sample measurement program, which was read every 20 s for 10 min. The reagent volumes for the components and samples to be tested are listed in Table 1.

Data analysis

Bioassay data were collected after three repetitions. Percent inhibition (%I) is a measure of the percentage of HMG-CoA reductase enzyme activity inhibited by the sample by estimating the difference between the absorbance value at the last measurement (A0) and the absorbance value at the first measurement (A10) of NADPH molecules in the reaction mixture within 10 min of measurement. Percent inhibition (%I) was estimated using the following formula: %I = [(control absorbance − sample absorbance)/control absorbance] × 100%.

Specific enzyme activity is the ability of the sample to inhibit the activity of the HMG-CoA reductase enzyme in enzyme units per milligram of protein, as determined by the difference between the absorbance value at the last measurement (A30) and the absorbance value at the first measurement (A1) of the NADPH molecules in the reaction sequence with 30 measurements over 10 min using an Elisa Reader (Thermo-Multiskan FC). The specific activity value of the HMG-CoA reductase enzyme was calculated using the following formula: specific activity (unit/mg P) = (ΔA(sample)/min) × volume total/12.44 x volume of enzyme × [enzyme] × light path (0.55 cm). The enzyme concentration was 0.6 mgP/mL.

Table 1. Volume of reagents and samples to be tested

	1 x *Assay Buffer*	**Pravastatin / extract**	**NADPH**	***HMG- CoA***	**HMGR**
Blanko	184 µl	-	4 µl	12 µl	-
Sample	181 µl	1 µl	4 µl	12 µl	2 µl

Abbreviations: NADPH, Nicotinamide adenine dinucleotide phosphate; HMGR, HMG CoA Reductase

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Statistical analyses were performed using the SPSS 25. This analysis aimed to compare the ability of HMG CoA-reductase inhibition among ethanol, methanol, n-hexane extracts, and pravastatin controls. This comparative analysis used a one-way ANOVA hypothesis test. The sample groups were considered distinct if the p-value was less than 0.05. If there were disparities in the sample variation, a post-hoc test was applied to continue the statistical analysis.

RESULTS AND DISCUSSION

Phytochemistry screening

As shown in Table 2, the secondary metabolite compounds in the three extracts of *C. frutescens* were alkaloids, saponins, terpenoids, and flavonoids. The types of secondary metabolites not contained in the three extracts of *C. frutescens* were tannins and steroids. Phenol is a secondary metabolite found only in the ethanol extract of *C. frutescens*.

The ability of *C. frutescens* to reduce HMG-CoA reductase can be attributed to its high flavonoid content, which is known to have the capacity to reduce the enzyme significantly and competitively, similar to the ability of statin drugs to reduce cholesterol levels in the body (Nascimento et al., 2013; Wijaya et al., 2018; Rivera et al., 2019; Bansal, 2021). In a study conducted by Baskaran et al. (2015) on malabar spinach (*Basella alba*) with simvastatin as the positive control, it was found that *B. alba* inhibited HMG-CoA reductase activity, as seen from the % inhibition, which reached 74.1%, which was slightly lower than that of simvastatin (85.1 %). This was related to the secondary metabolite content of *B. alba*, one of the secondary metabolites present in *B. alba* with high levels of luteolin. This is in line with research conducted using samples in the form of *C. frutescens*, which is known to contain very high levels of flavonoids in the form of luteolin (Rivera et al., 2019). Molecular docking research conducted by Nematollahi et al. (2012) showed a very strong interaction between luteolin and the HMG-CoA reductase enzyme, making luteolin highly potential as an HMG-CoA reductase inhibitor.

Inhibition activity of HMG-CoA Reductase

Based on the analysis of research results, it was shown that ethanol, methanol, and n-hexane extracts from *C. frutescens* samples had the capacity to reduce HMG-CoA reductase, which can be seen from the % inhibition value of these three extracts. The mean % inhibition of n-hexane, methanol, and ethanol extracts from C. *frutescens* and pravastatin were 100.11%, 104.70%, 95.74%, and 99.27%, respectively (Figure 1). From the average % inhibition, the sample with the highest % inhibition value was the methanol extract of *C. frutescens*, and the sample with the second highest percentage inhibition was the n-hexane extract of C. frutescens and pravastatin. The sample with the lowest % inhibition value was the *C. frutescens* ethanol extract. The concentration of each extract used in this study was 10 mg in 1000 µL of solvent (0.01%).

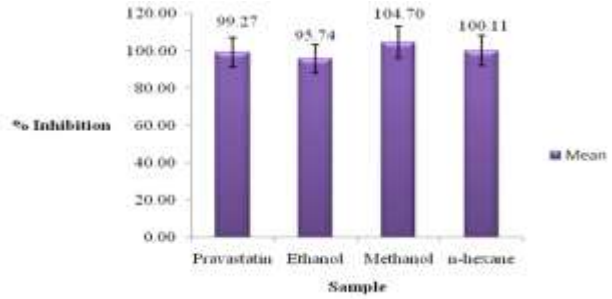

Figure 1. Graph of mean % inhibition values of pravastatin, n-hexane, methanol, and ethanol extracts of cayenne pepper (*C. frutescens*). Pravastatin as a positive control added at 1 µL and n-hexane, methanol, and ethanol extracts as test samples with a concentration of 10 mg in 1000 µL of solvent

Table 2. Phytochemical results of cayenne pepper (*C. frutescens*)

Compound Groups	Ethanol	Methanol	n-Hexane
Flavonoid	+	+	+
Alkaloid			
- Mayer	+	-	+
- Wagner	+	+	+
- *Dragendorff*	-	-	+
Terpenoid	+	+	+
Steroid	-	-	-
Fenol	+	-	-
Saponin	+	+	+
Tanin	-	-	-

Notes: +, detected; -, not detected

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Table 3. Post Hoc test results (multiple comparison) % inhibition value

Dependent variable: Inhibition % value		
Sample (n = 3)		***p-value****
Pravastatin	Ethanol	0.204
	Methanol	0.039
	n-Hexane	0.950
n-Hexane	Pravastatin	0.950
	Ethanol	0.099
	Methanol	0.082
Methanol	Pravastatin	0.039
	Ethanol	0.002
	n-Hexane	0.082
Ethanol	Pravastatin	0.204
	Methanol	0.002
	n-Hexane	0.099

Notes: *Post Hoc One Way ANOVA; significance if $p < 0.05$

This refers to a clinical dose of 10 mg of pravastatin. The inhibitory activity of the n-hexane and methanol extracts was quite high, reaching over 100%, surpassing the inhibitory activity of pravastatin. The crude extract had high inhibitory ability.

In a study conducted by Wijaya et al. (2018) on variations in the concentration of the ethanol extract of bay leaves (*Syzygium polyanthum*), it was found that *S. polyanthum* has the ability to inhibit the activity of HMG-CoA reductase, which can be seen from the % inhibition of bay leaves at different concentrations. One of them was that at a concentration of 600 ppm, the ethanol extract of *S. polyanthum* could inhibit the enzyme with a % inhibition value of 82.76%. This is related to the secondary metabolite content of *S. polyanthum*, where quercetin is one of the secondary metabolites present in S. polyanthum at high levels. This is in line with research carried out using samples in the form of *C. frutescens*, which is known to contain very high levels of flavonoids in the form of quercetin (Nascimento et al., 2013).

Islam et al. (2015) explored the ability of luteolin and quercetin, flavonoid polyphenols, to inhibit HMG-CoA reductase activity. Based on the results obtained, it was found that luteolin and quercetin have a fairly high affinity for the active section of the amino acid residue of the enzyme, which causes the enzyme's catalytic activity to not occur toward the substrate and cosubstrate. This shows that luteolin and quercetin have an excellent ability to inhibit enzyme activity so that they can prevent cholesterol synthesis.

The high activity of *C. frutescens* extract in polar (methanol), nonpolar (n-hexane), and semipolar (ethanol) solvents can be attributed to the high levels of flavonoids in *C. frutescens*. Flavonoids are secondary metabolites that have two different polarities, polar and nonpolar, so that they can dissolve easily in polar, nonpolar, and semipolar solvents (Nascimento et al., 2013; Arifin and Ibrahim, 2018; Rivera et al., 2019). The *C. frutescens* extract in a polar solvent (methanol) had the highest ability with a % inhibition value exceeding that of pravastatin and was statistically significantly different from pravastatin, indicating that the flavonoid content in the *C. frutescens* samples used in this study is thought to be dominated by flavonoids in the form of glycosides, so they are more soluble in polar solvents. Apart from flavonoids, the *C. frutescens* studied is also known to contain a number of secondary metabolite compounds that have the capacity to interact with the active section of HMG-CoA reductase, including phenols, alkaloids, and terpenoids. The results of in silico research show that secondary metabolites in the form of flavonoids, phenols, alkaloids, and terpenoids can interact with the active section of the enzyme, thus inhibiting the catalytic activity of the enzyme against substrates in the form of HMG-CoA and cosubstrates in the form of NADPH (Islam et al., 2015; Hariyanti et al., 2018; Aqeel et al., 2021; Mannino et al., 2021).

The P value between n-hexane extract and ethanol extract ($p > 0.05$) and n-hexane extract and methanol extract ($p > 0.05$) showed that of the three groups of *C. frutescens* extract samples, the HMG enzyme was inhibited by two distinct groups of extracts. The different CoA reductases were ethanol extract and methanol extract ($p < 0.05$), and one group had the same capacity to reduce the enzyme as the other group, namely the n-hexane extract.

Specific activity of HMG-CoA reductase

Based on Figure 2, the enzyme specific activity of the ethanol, methanol, and n-hexane extract samples of *C. frutescens* and pravastatin are shown with the average enzyme specific activity in the following order: 0.5513 mgP; 0.6029 mgP, 0.5765 mgP, and 0.5716 mgP, respectively. Specific activity is the standard of enzyme purity in a series of reactions that contribute to

P-ISSN: 2406-9388
E-ISSN: 2580-8303

the transformation of a particular substance. The specific activity of the enzyme was determined by the number of enzyme units per milligram of protein (units/mg P). Enzymes are proteins and their catalytic activity depends on the integrity of their structure, so the probability that the content of HMG-CoA reductase as an enzyme protein will be high if there is inhibition of the use of NADPH by the inhibitors used in the reaction series. The higher the specific activity of the enzyme, the purer the enzyme contained in the reaction, indicating that the reacted enzyme is not used in the conversion process of a particular compound. Inhibition of enzyme activity by the sample by preventing the reaction between HMG-CoA reductase, HMG-CoA, and NADPH can increase the purity of the enzyme in the reaction. Thus, the higher the specific activity of the enzyme, the higher the ability of the inhibitor to inhibit enzyme activity (Djarkasi et al., 2021).

Data analysis was performed to determine the specific activity of the enzyme. Based on research by Feng et al. (2013) on a mutated uricase enzyme, it is known that measuring the specific activity of the enzyme can help measure the catalytic activity of the enzyme with high sensitivity, even at low activity levels. The basic pathomechanism of hyperuricemia is mutation of the uricase enzyme; therefore, measuring the catalytic activity of the uricase enzyme is essential for determining the progression of the uricase enzyme mutation. The relatively small number of enzymes in cells makes it difficult to determine their presence and concentration. However, the ability to rapidly convert thousands of molecules of a particular substrate into a product makes it easier for each enzyme to detect its presence.

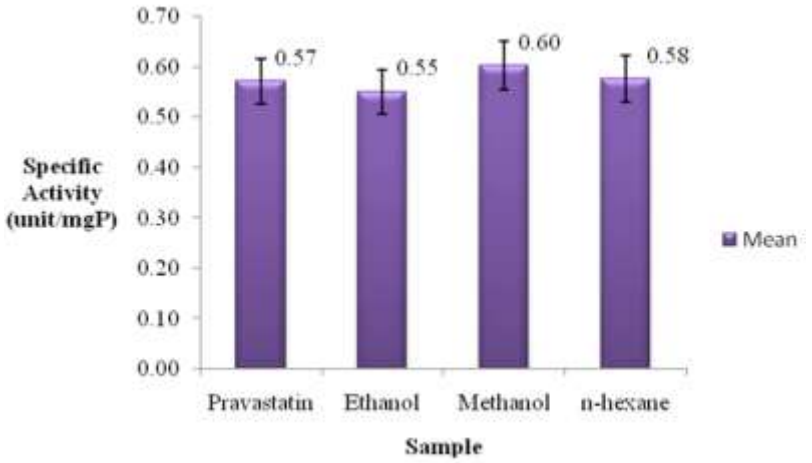

Figure 2. Graph of mean enzyme specific activity of pravastatin, n-hexane, methanol, and ethanol extracts of cayenne pepper (*C. frutescens*)

Table 4. Results of Post Hoc Test (Multiple Comparison) Enzyme Specific Activity

Dependent variable: Enzyme Specific Activity		
Sample (n = 3)		***p-value****
Pravastatin	Ethanol	0.203
	Methanol	0.039
	n-Hexane	0.951
n-Hexane	Pravastatin	0.951
	Ethanol	0.098
	Methanol	0.081
Methanol	Pravastatin	0.039
	Ethanol	0.002
	n-Hexane	0.081
Ethanol	Pravastatin	0.203
	Methanol	0.002
	n-Hexane	0.098

Notes: *Post Hoc One Way ANOVA; significance if $p < 0.05$

P-ISSN: 2406-9388
E-ISSN: 2580-8303

The measurement of enzyme catalytic activity is often used in clinical and research laboratories (Murray et al., 2012). The correlation between specific activity and enzyme catalytic activity, which is directly proportional, shows that exploration of the specific activity of enzymes will greatly assist the development of biotechnology, which is oriented towards progressing clinical aspects in determining the diagnosis or prognosis of diseases related to the metabolism of a particular enzyme.

Significant differences in the specific activity of HMG-CoA reductase from n-hexane, methanol, and ethanol extracts of *C. frutescens* and pravastatin can be identified by looking at the p value from the one-way ANOVA test. The one-way ANOVA test on the sample data revealed that the specific activity of the enzyme was significantly different between the sample groups, as shown in Table 4. Based on data analysis, the average specific activities of the enzyme from n-hexane, methanol, and ethanol extracts of *C. frutescens* and pravastatin, respectively, were as follows: 0.5765 units/mgP, 0.6029 units/mgP, 0.5513 units/mgP, and 0.5716 units/mgP. The sample with the highest specific enzyme activity was the methanol extract of *C. frutescens* with an average specific enzyme activity of 0.6029 units/mgP, followed by the n-hexane extract of *C. frutescens* with an average specific enzyme activity of 0.5765 units/mgP. Both extracts had higher specific enzyme activity than pravastatin with an average specific activity of 0.5716 units/mgP. The average specific enzyme activity of the ethanol extract of *C. frutescens* was lower than that of pravastatin, with an average value of 0.5513 units/mgP. The analysis of specific enzyme activity data showed that the methanol extract and n-hexane extract of *C. frutescens* had the best potential for inhibiting the enzyme.

The results of the phytochemical tests carried out on three groups of *C. frutescens* extract samples showed variations in the content of different secondary metabolite compounds from each extract. *C. frutescens* ethanol extract is a sample that contains the most varied secondary metabolite compounds with secondary metabolite compounds in the form of flavonoids, phenols, alkaloids (positive in the Mayer and Wagner method), terpenoids and saponins. The n-hexane extract of *C. frutescens* is a sample that contains the second most varied secondary metabolite compounds after ethanol with secondary metabolite compounds in the form of flavonoids, alkaloids (positive in the Mayer, Wagner and Dragendorff methods), terpenoids and saponins. The methanol extract of *C. frutescens* contained the smallest variation in secondary metabolite compounds. Flavonoids, alkaloids (positive in the Wagner method), terpenoids, and saponins were some of the secondary metabolites found in the methanol extract of C. frutescens.

Based on the data on the % inhibition value and specific activity of the enzyme obtained, the extract samples with the best potential for inhibiting the enzyme were the methanol extract and n-hexane extract of *C. frutescens*. The sample with the smallest potential for enzyme inhibition was the ethanol extract of *C. frutescens*. This indicates that the high variation of secondary metabolite compounds found in the extract samples in this study does not determine their bioactive capabilities, especially when it comes to enzyme inhibition.

In addition, the characteristics of secondary metabolite compounds which have synergistic and antagonistic properties are known to be one of the factors that determine the potential bioactivity of these secondary metabolite compounds (Kopjar et al., 2016).

In a study conducted by Tavadyan and Minasyan (2019), using the Square Wave Voltammetry (SWV) method on the antioxidant ability of isolated flavonoids, it was found that there was a decrease in the potential bioactivity of flavonoid-derived secondary metabolite compounds in the form of quercetin after adding ascorbic acid (vitamin C). The interaction of a series of chemical groups of the aglycone properties of quercetin as a secondary metabolite derived from flavonoids with vitamin C causes a decrease in the antioxidant activity of quercetin. This shows that the interactions between secondary metabolite compounds and other compounds contained in plants can determine their potential bioactivity.

CONCLUSION

Based on the percentage inhibition value and enzyme-specific activity, the methanol extract of C. frutescens had the best HMG-CoA reductase inhibition capability, with an average inhibition capability of 104.70% and an enzyme-specific activity of 0.60 units/mgP. In addition, there was a substantial difference in HMG-CoA reductase inhibition between the pravastatin and *C. frutescens* extracts.

ACKNOWLEDGMENT

The authors would like to thank the Faculty of Medicine, Halu Oleo University for supporting this research.

AUTHOR CONTRIBUTIONS

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Conceptualization, L.O.M.A., T.; Methodology, L.O.M.A., T.; Software, L.O.M.A., A.E.; Validation, L.O.M.A., A.E.; Formal Analysis, L.O.M.A., A.E.; Investigation, L.O.M.A., T; Resources, L.O.M.A., T.; Data Curration; L.O.M.A., T.; Writing - Original Draft, L.O.M.A.; Writing - Review & Editing, T.; Visualization, L.O.M.A., T.; Supervision, T.; Project Administration, T.; Funding Acquisition, T.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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P-ISSN: 2406-9388
E-ISSN: 2580-8303

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia
Pharmacy and Pharmaceutical Sciences Journal
Published by Faculty of Pharmacy Universitas Airlangga
OPEN ACCESS
E-ISSN 2580-8303
P-ISSN 2406-9388

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia Vol. 11 No. 2 August 2024, 242-252
DOI: 10.20473/jfiki.v11i22024.242-252
Available online at https://e-journal.unair.ac.id/JFIKI/

Review: Indole Alkaloids and Antimalarial Activity in the *Tabernaemontana* Species

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Submitted: 25 June 2024
Revised: 25 July 2024
Accepted: 31 August 2024

Abstract

Background*: Malaria, caused by Plasmodium parasites, is a highly prevalent and lethal illness that shows persistent ability to develop resistance. Antiplasmodial compounds that are indole-based prevent hemozoin formation, exhibiting efficacy against chloroquine-resistant Plasmodium strains. Tabernaemontana is a member of the genus comprised to the Apocynaceae family and has long been known for its efficacy in traditional and herbal tribal medicine. Apocynaceae can be recognized by the existence of indole alkaloids, and Tabernaemontana spp. is widely identifiable for its ability to synthetize a wide variety of indole alkaloids.* ***Objective:*** *This literature review seeks to provide a comprehensive summary of indole alkaloid compounds from Tabernaemontana spp. and the effectiveness of Tabernaemontana spp. as antimalarials.* ***Methods****: Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) protocols were followed to explore the PubMed, Sage Journal, ScienceDirect, and Wiley Library databases.* ***Results:*** *23 publications on the antimalarial activity and indole alkaloids of several species of the genus Tabernaemontana were discovered.* ***Conclusion:*** *Various species of Tabernaemontana contain indole alkaloids, and extracts of the plant or parts of the plant and isolates have weak to strong antimalarial activity.*

Keywords*: antimalarial, indole alkaloid, Tabernaemontana spp., Plasmodium spp.*

How to cite this article:
Hutami, A. T. & Rudyanto, M. & Ekasari, W. (2024). Review: Indole Alkaloids and Antimalarial Activity in the *Tabernaemontana* Species. *Jurnal Farmasi dan Ilmu Kefarmasian Indonesia*, 11(2), 242-252. http://doi.org/10.20473/jfiki.v11i22024.242-252

INTRODUCTION

Tabernaemontana belongs to the family Apocynaceae. The genus has approximately 100 species that can be found in the tropical and subtropical regions of Asia, Africa, Oceania, and the Americas (Silveira et al., 2017). Tabernaemontana species consist of blooming shrubs and small-to medium-sized trees that typically live in savannahs, rocky outcrops, and forest understories (Marinho et al. 2016). The genus can be identified by its white cylindrical flowers, follicular fruit containing seeds enclosed by a yellow to reddish husk, and the occurrence of milky or watery latex secretion, which is typically observed in wounded plants of this genus (Simões et al., 2010).

Plants belonging to the genus Tabernaemontana generally contain a significant amount of alkaloids, which frequently exhibit pharmacological effects (Silveira et al., 2017). The primary types of alkaloids found in species within the Tabernaemontana genus are monoterpene indole and bisindole alkaloids. Additionally, other chemicals include terpenes, lactones, steroids, phenolics, and flavonoids (Van Beek et al., 1984).

Indole has the chemical formula C_8H_7N and shows weak basicity. The substance is composed of a pyrrole ring connected to the benzene nucleus. It has ten π-electrons that orbit around the form. The fundamental properties of indole alkaloids are ascribed to the dispersion of the unshared pair of nitrogen electrons inside the unconstrained motion of the π electronic system. The indole molecule was protonated at the C-3 position, which is more thermodynamically stable. (Omar et al., 2021).

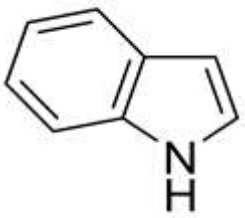

Figure 1. Basic chemical structure of an indole

Indole alkaloids have numerous pharmacological properties. Indole alkaloids have been recorded across multiple important plant families, including Rubiaceae, Apocynaceae, Rubiaceae, Loganiaceae and Nyssaceae, surpassing others in frequency. Indole alkaloids are often identified based on their strong biological effects, including anticancer, anti-inflammatory, antibacterial, antimalarial, antifungal, antidepressant, antiviral, analgesic, hypotensive, anticholinesterase, antileishmanial, antiplatelet, antidiarrheal, spasmolytic, lipid-lowering, antimycobacterial, and antidiabetic properties. (Omar et al., 2021).

Malaria is a deadly parasitic disease that is spread by the bite of the female Anopheles mosquito acting as the vector for humans. It is caused by five different kinds of Plasmodium parasite species, *Plasmodium falciparum (*the most prevalent), *Plasmodium vivax, Plasmodium malariae, Plasmodium ovale,* and *Plasmodium knowlesi* (Milner, 2018). Globally, in 2022, there were an estimated 249 million malaria cases in 85 malaria-endemic countries and areas, an increase of 5 million cases compared to 2021. Globally, the malaria mortality rate was halved from approximately 29 in 200 to 15 in 2015. In 2020, the mortality rate increased again to 15,2, before slightly decresing to 14,3 in 2022. From 2000 to 2019, the number of reported cases in the WHO African Region decreased from 370 to 226 per 1000 people at risk. However, in 2020, it increased to 233.6 per 1000 people at risk. This rise was primarily due to disruptions in service caused by the Coronavirus Disease 2019 (COVID-19) pandemic in 2020 – 2023. According to the World Health Organization (2023), the number of cases per 1000 people at risk has decreased to 229 by 2022 (World Health Organization, 2023).

In Indonesia, malaria cases are increasing from 2020 to 2022, from 254,055 cases in 2020 to 443,530 cases in 2022. The highest number of cases was in Papua Province, which contributed 356,889 positive cases to the national figure. The fatalities are also linear with the increase in positive cases, where in 2022, the number of deaths is 71, which is the highest in 2018 – 2022 (Kemenkes, 2022).

In 2021, four countries in the African region accounted for nearly half of all malaria cases globally: Nigeria (26,6%), the Congo (12,3%), Uganda (5,1%), and Mozambique (4,1%). Four countries also account for over half of malaria deaths globally: Nigeria (31,3%), Congo (12,6%), Tanzania (4,1%), and Niger (3,9%) (Health Organization, 2023).

Currently, researchers are exploring the extensive possibilities of numerous Tabernaemontana species by examining their plant extracts, fractions, chemical constituents, and isolated compounds (Silveira et al., 2017). This review provides detailed information on several species of Tabernaemontana, focusing specifically on their recently published antimalarial activities.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

MATERIALS AND METHODS

This article follows the principles set forth by PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses). This study explored the indole alkaloid compounds identified from the genus Tabernaemontana and their effectiveness in combating malaria parasites.

Search methodology

The publications were gathered by an Internet-based search using selective keywords, such as "Tabernaemontana antimalarial activity" and "Indole alkaloid compounds from Tabernaemontana" across numerous databases, including PubMed, ScienceDirect, MDPI, and Google Scholar. The articles were written in either English or Indonesian. The article search covers the period from 2000 to 2023. The papers used relate to the antimalarial properties of plants belonging to the genus Tabernaemontana and the specific indole alkaloids that have been isolated and identified from them.

Data extraction

The authors collected and assessed the articles using standardized protocols. The selected articles included information on malaria in general as well as the characteristics of Tabernaemontana spp. The antimalarial and/or antiplasmodial activity of the extract from Tabernaemontana spp. is discussed, along with the indole alkaloids identified from the plant species and their antimalarial properties. In addition, the mechanism of action of these indole alkaloids as antimalarial agents was examined.

The criteria for data extraction were as follows: 1) all species belonging to the genus Tabernaemontana spp., 2) the article was published from 2000 until 2023, 3) the extract or fraction of the plants has *in vitro* or *in vivo* antimalarial or antiplasmodial activity, 4) isolated and identified compounds are indole alkaloids, and 5) the article is in English or Indonesian. A total of 23 articles were used in this literature review, as displayed in Table 1.

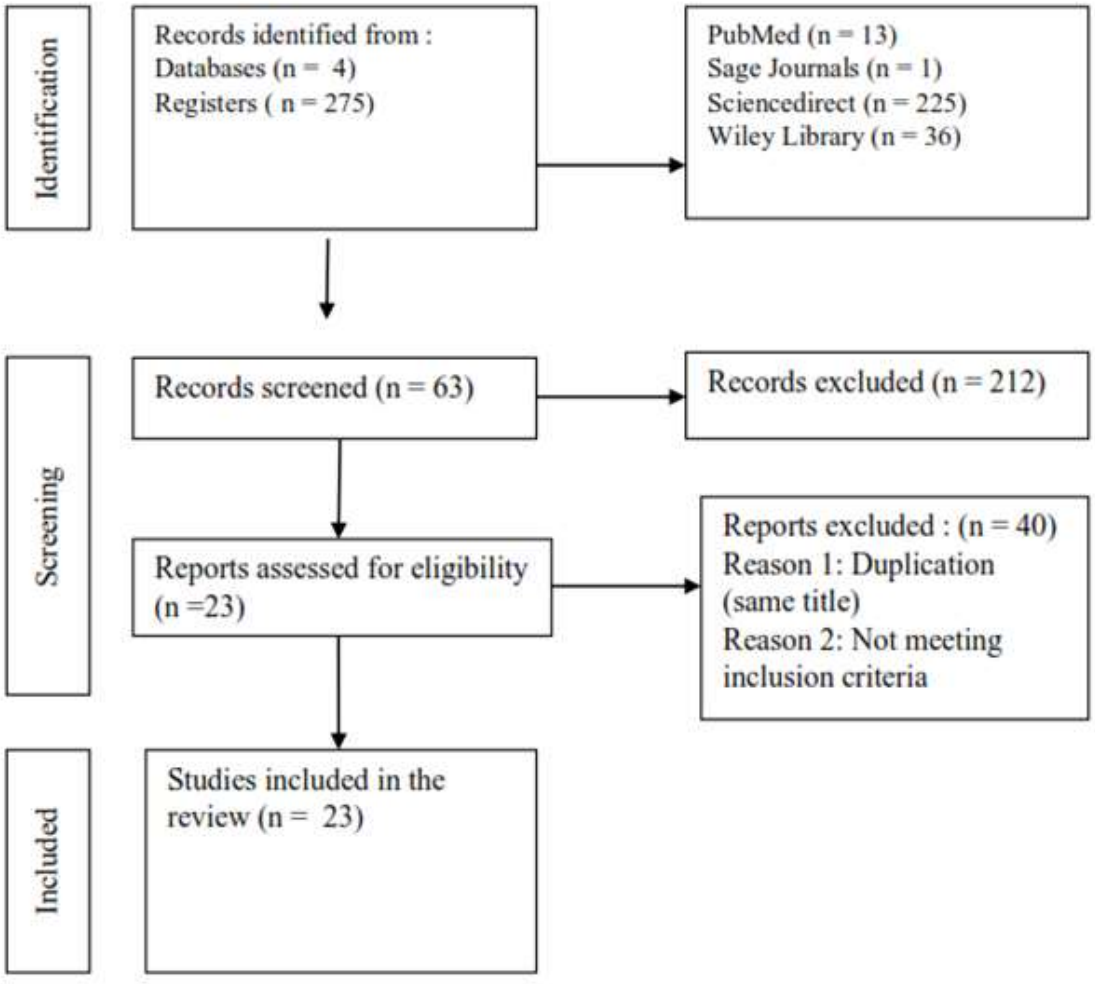

Figure 2. Flow chart of PRISMA guidelines in article collection

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Table 1. Systematic review data table

Source	Indole Alkaloids Isolation and Identification	Antimalarial Activity Evaluation (*in vitro*)
Nge, et.al., 2016	√	
Kam & Sim, 2002	√	
Lim, et.al., 2015	√	
Qu, et.al, 2016	√	
Yuwen, et.al, 2016	√	
Sim, et.al., 2014	√	
H. Zhang, et.al, 2007	√	
Cai, et.al., 2018	√	
Ingkaninan, et.al, 2006	√	
B. J Zhang, et.al., 2015	√	
Hirasawa, et.al, 2019	√	√
Xu et.al., 2019	√	
Pereira, et.al, 2008	√	
Foudjo Melacheu, et.al, 2019	√	
Yu, et.al, 2019	√	
Bitombo, et.al., 2021	√	√
Masuda, et.al, 2000	√	
Bapela, et.al., 2018	√	
Federici et.al, 2000	√	√
Noguchi, et.al, 2016	√	√
Amelia, et.al, 2019	√	√
Ramalhete et.al., 2008		√
Muthaura, et.al, 2015		√

RESULTS AND DISCUSSION

Indole alkaloids antimalarial mechanism of action

The efficacy of the drug against drug-resistant *Plasmodium spp*. remains high even when it possesses a distinct or additional mode of action. It should be emphasized that the presence of the indole nucleus alone does not ensure antimalarial action. Covalently linking the pharmacophore units of hemozoin and PfATP4 inhibitors to an indole backbone using molecular hybridization has the potential to create antiplasmodial medicines that act on both targets simultaneously (Surur et al., 2020).

The most promising approach is to inhibit the detoxification of the plasmodial pathway to become hemozoin. The breakdown of hemoglobin within the parasite's food vacuole results in the release of a significant amount of free haem (ferriprotoporphyron IX). The abundance of free heme is believed to be deadly to the Plasmodium parasite because of its ability to block membranes, cause lipid peroxidation, and induce oxidation of proteins and DNA. *Plasmodium spp*. employs a heme detoxification mechanism known as bio-crystallization, which transforms heme into an insoluble substance called hemozoin. Hemozoin has been proposed to be composed of chains of Fe^{III}-protoporphyrin units, connected to form a polymer. The collection of a substantial amount of free hematin and the complex formed between hematin and antimalarial drugs impairs the parasite's capacity to maintain cationic gradients. This, in conjunction with the harmful effects of free heme, results in parasite death (Kgokong et al., 2005). Certain indole compounds can directly attach to ferriprotoporphyrin IX, thereby interfering with heme polymerization into hemozoin (Surur et al., 2020).

PfATP4 is a P-type ATPase found in *P. falciparum* and serves as a target for the sporoindolone class of antiplasmodial drugs. Sporoindolones disrupt the growth of parasites by disturbing the balance of sodium ions, which is weakened in Plasmodium species with acquired mutations in PfATP4 that confer resistance. PfATP4 has been identified as a critical mechanism for the antimalarial action of new chemotypes, aminopyrazoles, and dihydroisoquinolines, in addition to sporoindolones, which have been used in preclinical studies (Spillman & Kirk, 2015).

By targeting the melatonin receptor, research has revealed the potential for generating therapeutic candidates that target several targets and are based on indole. Melatonin is crucial for the coordination of the cell cycle of malaria parasites. The ubiquitin/proteasome system plays a vital role in regulating genes in Plasmodium, which is essential for maintaining cell cycle and transcriptional activity. This regulation ultimately contributes to the percentage of mature schizonts. Malaria stimulates hepatocyte death by inducing mitochondrial diseases and oxidative stress.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

However, this detrimental effect can be mitigated by administering a large dose of melatonin, as demonstrated by Surur et al. (2020). Bagnaresi et al., 2008 demonstrated that luzindole, an indole-based melatonin antagonist, effectively suppressed trophozoites by disturbing the rhythmicity of the cell cycles of the parasite.

As described above, various modes of action are responsible for their efficacy against chloroquine-resistant strains. Indeed, it is important to note that the indole nucleus alone does not guarantee antimalarial activity, such as alkaloids from *Pandaca*, *Bonafusia*, or *Rauvolfia*, which contain indole nuclei but are not active against Plasmodium parasites (Passemar et al., 2011). Molecular hybridization that covalently links the pharmacophore units of hemozoin inhibitors and PfATP4 inhibitors to an indole structure may lead to dual-acting antimalarial action (Surur et al., 2020).

Indole alkaloids showed synergistic/additive interactions with conventional antimalarial agents, as shown by Bagnaresi et al. (2008), in which mice treated with luzindole (15 mg kg^{-1}) and chloroquine (suboptimal dose at 1.5 mg kg^{-1}) worked synergistically, which reduced the number of intraerythrocytic parasites. Cryptolepine in combination with a 4 mg kg^{-1} dose of artemisinin showed no significant biochemical and histopathological index variations compared to the control group, which ensured an acceptable safety profile (Forkuo et al., 2017).

Toxicity profiles of indole-alkaloid-rich extracts generally show no genotoxicity, cardiotoxicity, or respiratory issues (Surur et al., 2020). However, some compounds such as luzindole and cryptolepine have specific toxic effects, including reduced cardioprotection and cytotoxicity (Gopalan et al., 2011). Cryptolepine showed *in vivo* toxicity in mice and embryonic malformations in zebrafish; however, in *P. berghei*-infected mice, it did not alter the histopathology of the liver, spleen, stomach, or kidney (Forkuo et al., 2017). The observed toxicity of indole alkaloids has partly hindered further preclinical development of indoles. Most of the reported side effects are associated with long-term exposure to indoles; thus, the chronic toxic effect of indole derivatives could be avoided as long as the malarial treatment regimen extends only for a short time (Forkuo et al., 2017).

Indole alkaloids compounds and antimalarial activities of *Tabernaemontana* species

The Tabernaemontana genus possesses an abundance of monoterpene indole alkaloids (MIAs), which are synthesized from tryptophan (an aromatic acid) and secologanin (an iridoid terpene) (Athipornchai, 2018). Various skeletal types have been identified in MIAs, such as seco-tabersonine, bis-vobtusine, and bis-vobsinyl-ibogan indole alkaloids. (Marinho et al., 2016). Heterodimeric bisindole alkaloids are another important family of alkaloids in this plant (Athipornchai, 2018). Table 2 presents a summary of the indole alkaloids identified from the taxa in the genus Tabernaemontana.

Table 2. Summary of indole alkaloids identified from tabernaemontana

No	Species	Plant Part	Reported MIAs	Class Type	Reference
1	*Tabernaemontana corymbosa*	Stem Bark	Conodusine A	Iboga	(Nge et al., 2016)
			Conodusine B	Iboga	(Nge et al., 2016)
			Conodusine C	Iboga	(Nge et al., 2016)
			Conodusine D	Iboga	(Nge et al., 2016)
			Conodusine E	Iboga	(Nge et al., 2016)
			Apocidine A	Aspidosperma	(Nge et al., 2016)
			Apocidine B	Aspidosperma	(Nge et al., 2016)
			Conoduzidine A	Vincamine	(Nge et al., 2016)
			Tabernamidine A	Vobasine-Iboga	(Nge et al., 2016)
			Tabernamidine B	Vobasine – Iboga	(Nge et al., 2016)
			19'(S)-Hydorxytabernamine	Vobasine – Iboga	(Kam & Sim, 2002)
			19'(R)-Hydorxytabernamine	Vobasine – Iboga	(Kam & Sim, 2002)
			16'-Decarbomethoxyvoacamine	Vobasine – Iboga	(Lim et al., 2015)

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No	Species	Plant Part	Reported MIAs	Class Type	Reference
2	*Tabernaemontana litoralis*	Fruits	Isokuammiline	Corynanthe	(Qu et al., 2016)
			18-Hydroxypseudovincadifformine	Iboga	(Qu et al., 2016)
			3,19-Oxidocoronaridine	Iboga	(Qu et al., 2016)
			Strictosidine	Strictosidine	(Qu et al., 2016)
3	*Tabernaemontana divaricata*	Leaves and twigs	Tabervarine A	Iboga	(Yuwen et al., 2019)
			Tabervarine B	Iboga	Yuwen et al., 2019)
			Vobasidine C	Vobasine	(Sim et al., 2014)
			Ervadivaricatine B	Vobasine – Iboga	(H. Zhang et al., 2007)
		Stems	Flabellipparicine	Flabelliformide – Appariicine	(Cai et al., 2018)
			19,20-Dihydrovobparicine	Vobasine-Appariicine	(Cai et al., 2018)
			10'Demethoxy-19,20-Dihydrovobatemsine D	Vobasine-Iboga	(Cai et al., 2018)
			3'-(2-Oxopropyl) Ervahanine A	Sarpagine – Iboga	(Cai et al., 2018)
			19,20-Dihydrotabermine	Vobasine-Iboga	(Ingkaninan et al., 2006)
			Taberdivarines E	Vobasine-Iboga	(B. J. Zhang et al., 2015)
			Hydroxy-3-(2-Oxopropyl) Coronaridine	Iboga	(Ingkaninan et al., 2006)
			Indolenine Deoxytubulosine	Corynanthe bisindole	(Cai et al., 2018)
		Root	Divaricamine A	Vobasine – vobasine – iboga	(Hirasawa et al., 2021)
4	*Tabernaemontana bufalina*	Branches and leaves	(3R,7S,14R,19S,20R)-19-Hydroxypseduovincadiffromine	Iboga	(Xu et al., 2019)
			Voachalotine	Akuammidine	(Pereira et al., 2008)
			12-Methoxyl-Voaphylline	Aspidosperma	(Perera et al., 1984)
			Conophylline	Apsidosperma-Aspidosperma	(Perera et al., 1984)
5	*Tabernaemontana contorta*	Fruits	5,6-Dioxo-11-Methoxy Voacangine	Iboga	(Foudjo Melacheu et al., 2019)
			(-)-Apparicine-21-One	Appariicine	(Foudjo Melacheu et al., 2019)
6	*Tabernaemontana bovina*	Leaves	Tabernabovine A	Corynanthe bisindole	(Yu et al., 2019)
			Tabernabovine B	Aspidosperma	(Yu et al., 2019)
			Tabernabovine C	Iboga	(Yu et al., 2019)
7	*Tabernaemontana penduliflora* K. Schum	Trunk bark	Penduliflorines A		(Bitombo et al., 2021)
			Penduliflorines B		(Bitombo et al., 2021)
			Penduliflorines C		(Bitombo et al., 2021)
			Penduliflorines D		(Bitombo et al., 2021)
			Penduliflorines E		(Bitombo et al., 2021)
			Tabernaemontine		(Bitombo et al., 2021)

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No	Species	Plant Part	Reported MIAs	Class Type	Reference
			10-Hydroxycoronaridine		(Masuda et al., 2000)
			Voacangine		(Masuda et al., 2000)
8	*Tabernaemontana elegans*	Stem bark	Tabernaemontine	Vobasine	(Bapela et al., 2018)
			Dregamine	Vobasine	(Bapela et al., 2018)
9	*Tabernaemontana hystrix*	Stem bark	Voacangine	Vobasine – Iboga	(Federici et al., 2000)
			Coronaridine	Iboga	Federici et al., 2000)
10	*Tabernaemontana dichotoma*	Leaves	16-Hydorxy-16,22-dihydroapparicine	Vobasine – vobasine – Iboga	(Noguchi et al., 2016)
11	*Tabernaemontana macrocarpa*	Bark	16-demethoxycarbonyl voacamine	Vobasine – Iboga	(Amelia et al., 2019)

MIAs: Monoterpene indole alkaloids

Tabernaemontana Species with Antimalarial Activity

***Tabernaemontana penduliflora* K. Schum**

The botanical nomenclature assigned to this plant is *Tabernaemontana penduliflora*. K. Schum, previously named as *Conopharyngia penduliflora* (K. Schum), is a sleek-stemmed bush or little tree indigenous to the woodlands of Cameroon and the southern part of Nigeria (Bitombo et al., 2021). The ethanol extract of *T. penduliflora* exhibits potent antibacterial effects against gram-positive bacteria, as discovered by Van Beek et al. in 1984. *T. penduliflora* root is also used as a traditional treatment for malaria (Titanji et al., 2008).

The trunk bark ethanol of *T. penduliflora* extract was prepared by subjecting the dried plant part to extraction using 90% ethanol under decreased pressure. Subsequently, the extract was combined with a 5% hydrochloric acid solution and subjected to extraction using n-hexane. The remaining portion was treated with NH4OH and subsequently extracted with $CHCl_3$ to yield a crude alkaloid extract. The material was subjected to phytochemical examination using repeated column chromatography (CC) and liquid chromatography (LC), along with high-resolution mass spectrometry (HRMS) (Bitombo et al., 2021).

From the trunk bark of *Tabernaemontana penduliflora*, six new zwitterionic monoterpene indole alkaloids, penduliflorines (A-E), and tabernaemontine, were extracted, along with eight alkaloids that had been identified previously. The *in vitro* activity of penduliflorines A-E and tabernaemontine against 3D7 and Dd2 strains of *Plasmodium falciparum* demonstrated IC_{50} values ranging from 1.85 to 26.69 µg/ml, respectively. Penduliflorine A-B exhibited strong antiplasmodial action against 3D7 and Dd2 strains, with the IC_{50} value of 7.88 and 5.32 µg/ml, respectively. Penduliflorine C and Penduliflorine D-E demonstrated *in vitro* IC_{50} values ranging from 15.71 to 26.69 and 14.41 to 15.87 µg/ml, respectively, against the two strains of *P. falciparum* (Bitombo et al., 2021).

Tabernaemontana elegans

Tabernaemontana elegans (IC_{50} of dichloromethane extract = 26,9 ±3.1 µg/ml), widely utilized for malaria treatment in Mozambique, revealed moderate or no significant activity. It is worth mentioning that plants are commonly used in the treatment of fever, particularly in cases related to malaria (Ramalhete et al., 2008). The absence of *in vitro* antimalarial inactivity in *T. elegans* may be attributed to its potential role as an antipyretic or an enhancer of the immune system, rather than its direct antiparasitic activity. (Ramalhete et al., 2008).

Tabernaemontana hystrix

Tabernaemontana hystrix (formerly *Peschiera fuchsiaefolia*) is indigenous to South America. The use of the stem bark of *T. hystrix* as an antimalarial traditional medicine led Federici et al. (2000) to investigate its basic extract, which showed good *in vitro* activity using the D6 strain of *P. falciparum*, which exhibited an IC_{50} value of 0.495 µg/ml, and W2, which exhibited an IC_{50} value of 0.817 µg/ml. Voacamine, identified from *T. hystrix*, shows the most active antimalarial activity, with IC_{50} values of 0.238 and 0.290 µg/ml against the D2 and W2 strains, respectively (Federici et al., 2000).

Tabernaemontana pachysiphon

The fruit of *Tabernaemontana pachysiphon* has historically been used to prevent miscarriages and cure sores and lesions in Nigeria. (Duru & Mbata, 2010). The plant is believed to exhibit antibacterial properties, and investigation of the leaf extract concluded the existence

P-ISSN: 2406-9388
E-ISSN: 2580-8303

of bioactive phytochemical components, including alkaloids, resins, saponins, flavonoids, polyphenols, and carbohydrates. (Duru & Mbata, 2010). Muthaura et al. (2015) investigated water and methanol extracts from *T. pachysiphon* fruit and leaves and found that the extracts yielded IC_{50} values against D6 and W2, respectively, of 4,8 and 4,4 µg/ml (fruit water extract), 3,9 and 53,7 µg/ml (fruit methanol extract), 25,3 and 70,8 µg/ml (water leaves extract) and 14.7 and 25,4 µg/ml (leaf methanol extract).

Tabernaemontana dichotoma

Tabernaemontana dichotoma exhibits vasorelaxant activity. Zaima et al. (2013) reported the vasorelaxant activity of a methanol extract of the bark of *T. dichotoma* in the rat aorta. Furthermore, a total of eight indole alkaloids were identified, namely 10-methoxyalstonerine, 10-methoxyaffisinine, lochnerine, cathafoline, (-)-alstonerine, 19,20-dehydro-10-methoxyalcarpine, alstonisine, and alstonal.

In 2019, Noguchi and colleagues conducted a study to examine the antimalarial activity of *T. dichotoma* methanol leaf extract. The main constituents of the methanol extract derived from the leaves of *T. dichotoma* are primarily 16-Hidroxy-16,22-dihydroapparacine, which is a 5-nor stemmadenine alkaloid. It exhibits antimalarial properties. The methanol extract of *T. dichotoma* leaf exhibited strong *in vitro* antimalarial activity, with a measured IC_{50} value of 0.59 µg/mL against K1, a chloroquine-resistant strain of *P. falciparum* and 0.35 µg/mL against FCR3 which is a chloroquine-sensitive strain of *P. falciparum*.

Tabernaemontana divaricata

Tabernaemontana divaricata (L) R. Br ex Roem & Schult is a plant native to Asia and Australia, both tropical and subtropical. Currently, it is primarily recognized as a decorative tree found in gardens. However, in Thailand and China, it has long-standing traditional use for alleviating fever and pain (Naidoo et al., 2021). *T. divaricata* is known for its ability to synthesize a diverse range of indole alkaloids. In their study, Hirasawa et al., (2021) discovered a novel trimeric monoterpene alkaloid named divaricamine A. Divaricamine A exhibited strong *in vitro* antimalarial activity against the 3D7 strain of *P. falciparum*, with IC_{50} measured at the value of 1.9 µM.

Tabernaemontana macrocarpa

Tabernaemontana macrocarpa Jack, a tree species found in Borneo, Indonesia, is traditionally used to treat dental diseases such as herpes and dermatitis. This is achieved by utilizing the exudate obtained from the bark of the tree (Ekawati et al., 2023). The stems of *T. macrocarpa* have been analyzed for phytochemicals, including alkaloids, flavonoids, terpenoids, and tannins. Amelia et al., (2021) identified and separated two newly found sarpagine-type indole alkaloids, alongside five previously identified alkaloids (12-methoxy-4-methylvoachalotine, 16-demethoxycarbonylvoacamine, isositrikine, affnisine, affinine). Compound 16-demethoxycarbonylvoacamine exhibited *in vit*ro antimalarial activity against the 3D7 strain of *Plasmodium falciparum*, with an IC_{50} value of 28.8 µM.

Tabernaemontana crassa

Appiah-Opong et al., (2022) examined eight Ghanaian traditional medicinal plants, namely *Cinnamomum zeylanicum*, *Morinda lucida* Benth, *Parkia clappertoniana* Key, *Tabernaemontana crassa* Benth, *Lippia multiflora* Moldenke, *Baphia nitida* Lodd, *Terminalia ivorensis* A.Chev, and *Treculia africana* Decne. Of all eight plants, the root extract of *Tabernaemontana crassa* showed the weakest antiplasmodial activity against the 3D7 strain, which is a strain of *Plasmodium falciparum* that is sensitive to chloroquine. The IC_{50} value for this activity was measured to be 62.33 µg/mL.

CONCLUSION

This literature review evaluated the indole alkaloid compounds found in species of the genus Tabernaemontana and their effectiveness in treating malaria. The antimalarial activity of Tabernaemontana genus members ranged from mild to strong. This antimalarial activity may be attributed to the presence of indole alkaloid molecules and multiple mechanisms. Generally, the toxicity profile of the indole alkaloid-rich extract showed no toxicity but was observable in some compounds.

Although the genus Tabernaemontana contains physiologically active chemical compounds, a significant number of species have not undergone chemical and biological assessments. Additional research is crucial to obtain a deeper understanding of the bioactive chemicals and active pharmacological actions of this genus, particularly in relation to its potential for malaria treatment.

ACKNOWLEDGMENT

We express our gratitude to the Department of Pharmaceutical Science, Faculty of Pharmacy, Universitas Airlangga for their valuable cooperation in facilitating resources and access to the journals and providing resources to support this endeavor.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

AUTHOR CONTRIBUTIONS

Conceptualization, W.E.; Methodology, A.T.H.; Validation, W.E., M.R.; Formal Analysis, A,T.H.; Investigation, A.T.H., W.E.; Resources, W.E., M.R.; Data Curration; W.E., A.T.H.; Writing - Original Draft, A.T.H.; Writing - Review & Editing, A.T.H., W.E., M.R.; Visualization, A.T.H., W.E.; Supervision, W.E., M.R.; Project Administration, A.T.H., W.E., M.R.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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P-ISSN: 2406-9388
E-ISSN: 2580-8303

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia
Pharmacy and Pharmaceutical Sciences Journal
Published by Faculty of Pharmacy Universitas Airlangga
OPEN ACCESS
E-ISSN 2580-8303
P-ISSN 2406-9388

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia Vol. 11 No. 2 August 2024, 253-259
DOI: 10.20473/jfiki.v11i22024.253-259
Available online at https://e-journal.unair.ac.id/JFIKI/

Evaluation of Adverse Drug Reactions (ADRs) in Breast Cancer Patients Who Received Doxorubicin, Cyclophosphamide (AC) and Doxorubicin, Cyclophosphamide, Paclitaxel (AC-T) Chemotherapy at West Nusa Tenggara Provincial Hospital

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Submitted: 20 May 2024
Revised: 27 July 2024
Accepted: 30 August 2024

Abstract

Background*: Chemotherapy is commonly used to treat breast cancer (BC). Chemotherapy may cause ADRs in patients, affecting their physical and psychological wellbeing.* ***Objective:*** *To understand the adverse drug reaction (ADR) profile in patients with breast cancer who received AC-T and AC chemotherapy at the West Nusa Tenggara Provincial Hospital.* ***Methods:*** *This observational study used cross-sectional data collected from medical records and direct interviews with the patients between May and June. Probability categories were measured using the Naranjo algorithm questionnaire, causality categories were measured using a causality flowchart, and the severity level of ADRs was determined using the Common Terminology Criteria for Adverse Events (CTCAE) 5.0.* ***Results:*** *The probability results for the AC-T regimen were as follows: possible (10 %), probable (54.44 %), and definite (35.56 %). whereas The AC regimen showed categories of possible (6.67%), probable (63.33%), and definite (30%). The causality results for the AC-T regimen were categorized as unlikely (1.11%), possible (12.22%), probable (25.56%), or certain (61.11%), whereas those for the AC regimen were categorized as possible (6.67%), probable (43.33%), or certain (50%). The most common ADRs were alopecia and nausea, with the highest probability in the probable category for AC-T (54.44%) and AC (63.33%), respectively.* ***Conclusion:*** *Respondents who received the AC-T regimen experienced more severe ADRs in terms of hematologic disorders (anemia, leukopenia, and thrombocytopenia) and symptoms of nausea, pain, and fever than those who received the AC regimen.*

Keywords*: chemotherapy, breast cancer, adverse drug reactions (ADRs), probability, common terminology criteria for adverse events (CTCAE)*

How to cite this article:
Anjani, B. L. P., Muriani, R. N., Nurbaety, B. & Nopitasari, B. L. (2024). Evaluation of Adverse Drug Reactions (ADRs) in Breast Cancer Patients Who Received Doxorubicin, Cyclophosphamide (AC) and Doxorubicin, Cyclophosphamide, Paclitaxel (AC-T) Chemotherapy at West Nusa Tenggara Provincial Hospital. *Jurnal Farmasi dan Ilmu Kefarmasian Indonesia*, 11(2), 253-259. http://doi.org/10.20473/jfiki.v11i22024.253-259

P-ISSN: 2406-9388
E-ISSN: 2580-8303

INTRODUCTION

Breast cancer (carcinoma mammae) is the abnormal growth of cells in the breast caused by oncogenes, leading to their transformation into cancerous cells (Syamsuddin et al., 2020). According to Global Burden of Cancer (GLOBOCAN) data from the International Agency for Research on Cancer (IARC) in 2018, there was an increase of 18.1 million cases globally, with 9.6 million deaths worldwide, accounting for the highest percentage of deaths at 43.3% (Bray et al., 2018). The Basic Health Research of Indonesia in 2018 showed an increase in the prevalence of breast cancer to 1.79 per 1000 population, up from 1.4 per 1000 2013. Indonesia ranks 23rd in terms of the number of breast cancer cases in Asia. Based on the results of Basic Health Research in West Nusa Tenggara (2018), breast cancer cases increased from 0.6% to 0.85% (Pangribowo, 2019).

Chemotherapy involves treatment with cytostatic drugs that actively target the growing and dividing cells (Piepoli et al., 2016). Advancements in pharmaceutical technology and various anticancer drug discoveries have increased optimism in addressing cancer malignancy. However, chemotherapy treatment has both physical and psychological side effects (Hidayatullah, 2015).

The World Health Organization (WHO) defines ADRs as unfavorable and unintended responses to a drug occurring at doses typically used in humans for the prevention, diagnosis, disease therapy, or modification of physiological functions (Balai Pengawasan Obat dan Makanan Republik Indonesia, 2020). ADRs and differences in hematological profiles before and after chemotherapy in patients with breast cancer breast cancer patients at Yogyakarta City Hospital showed that nausea was the most common manifestation (Basuki et al., 2020). The most commonly received chemotherapy regimens for patients with breast cancer at West Nusa Tenggara Provincial Hospital are AC-T and AC, which potentially lead to different manifestations of ADRs. The objective of this study was to understand the ADR profile in patients with breast cancer receiving AC-T and AC chemotherapy at the West Nusa Tenggara Provincial Hospital.

MATERIALS AND METHODS

Method

This observational study was conducted using cross-sectional data collected from medical records and direct interviews with patients in June 2023. The study population included all patients diagnosed with breast cancer between May and June 2023 at the West Nusa Tenggara Provincial Hospital, who received chemotherapy. Non-probability sampling using a convenience sampling/quota sampling methodology was employed. The inclusion criteria for this study were patients who received combination therapy with AC-T and AC combination therapy. The exclusion criteria were Patients who received radiotherapy or surgery and those who were unwilling to participate were excluded.

Following data collection, a descriptive analysis was performed on general patient profiles by presenting data based on age, occupation, comorbidities, chemotherapy cycles, and breast cancer stages. Probability categories were measured using the Naranjo algorithm questionnaire, causality categories were measured using a causality flowchart, and the severity level of ADRs was determined using the Common Terminology Criteria for Adverse Events (CTCAE) 5.0. This research was approved with Reference Number 00.9/18/0386/RSUDP/2023 and ethically cleared with Approval Number 00.9.1/08/KEP/2023.

RESULTS AND DISCUSSION

The study sample consisted of 120 patients, with 90 patients receiving the AC-T chemotherapy regimen and 30 patients receiving the AC chemotherapy regimen. As shown in Table 1, the breast cancer patients were in the age range of 46-55 years old, with 43 patients (47.78%) received AC-T chemotherapy, and 13 patients (43.33%) received AC chemotherapy. Table 1 indicates that older age at the onset of menopause poses a greater risk of breast cancer than a younger age at menopause. Elevated estrogen levels in women can delay menopause, thus increasing the risk of breast cancer (Wahyuni, 2021).

Table 2 shows that the majority of patients diagnosed with breast cancer were homemakers, with 55 patients (61.11%) receiving AC-T chemotherapy and 19 patients (63.33%) receiving AC chemotherapy. Research indicates that working women have a higher proportion of breast examinations than do non-working women. The primary factor is the lack of self-breast examination (SBE) due to the lack of knowledge and interaction among non-working women compared to their working counterparts (Wongkar et al., 2022).

Tables 3 and 4 show the probability and causality of ADRs observed in each chemotherapy cycle. Respondents who received AC-T chemotherapy were mainly in the probable (54.44%) or certain (61.11%) categories. Respondents to AC chemotherapy regimens were predominantly in the probable (63.33%) or certain (50%) categories. Respondents who received chemotherapy with either AC-T or AC regimens. A higher chemotherapy frequency correlated with a higher

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Naranjo score, indicating an increased ADR category level. This aligns with the theory that as the frequency of chemotherapy increases, more cancer cells undergo damage and death. Similarly, healthy cells in the body also experience damage, and after a few periods, typically one– three weeks, these cells recover but undergo significant damage, leading to a decline in function and overall body resilience. This treatment was continued along with subsequent chemotherapy (Hilli, 2017).

The occurrence of adverse drug reactions varies in each cycle, and is attributed to chemotherapy reactions affecting each patient differently and in diverse ways (Khairani et al., 2019). According to a previous research, ADRs occurring in patients undergoing chemotherapy do not show significant differences in each cycle (Prieto-Callejero et al., 2020). In Table 3 and 4, it can be observed that the most common causality category experienced by patients is the "certain" category (highly associated with drug use), with 55 patients (61.11%) receiving AC-T chemotherapy and 15 patients (50%) in the AC chemotherapy group. The degree of certainty, "certain" and "probable" (likely associated with the drug), indicates a relatively high value for the causality link between the drug and the occurring side effects. These values suggest the sequential occurrence of reactions with chemotherapy administration.

Side effects were aligned with the known profile of the suspected drug. This is assured by the cessation of chemotherapy, which is a three-week interval between administrations. Meanwhile, for the degree of certainty and "possible" (not yet certain association with the drug), as mentioned, other possibilities exist, such as other ailments suffered by the patient or due to other therapies (Sukandar et al., 2014).

Table 1. Respondent characteristics based on age

Age (years)	Number of AC-T Respondent		Number of AC Respondent	
	(n)	(%)	(n)	(%)
17 - 25	0	0	1	3.33%
26 - 35	1	1.11%	1	3.33%
36 - 45	27	30%	9	30%
46 - 55	43	47.78%	13	43.33%
56 - 65	16	17.78%	6	20%
≥65	3	3.33%	0	0
Total	**90**	**100%**	**30**	**100%**

Table 2. Respondents' characteristics based on occupation

Occupation	Number of AC-T Respondents		Number of AC Respondents	
	(n)	(%)	(n)	(%)
Housewife	55	61.11%	19	63.33
Farmer	11	12.22%	5	16.67%
Entrepreneur	11	12.22%	3	10%
Farm laborer	5	5.56%	0	0
Civil servant	5	5.56%	0	0
Teacher	3	3.33%	2	6.67%
Student	0	0	1	3.33%
Total	**90**	**100%**	**30**	**100%**

Table 3. Probability and causality of ADRs based on chemotherapy cycle of respondents who received AC-T

Chemotherapy Cycle	Number of Respondents	Probability			Causality			
		Possible	Probable	Definite	Unlikely	Possible	Probable	Certain
1	21 (23.33%)	8 (38.10%)	13 (61.90%)	0	0	11 (52.38%)	10 (47.62%)	
2	15 (16.67%)	1 (6.67%)	13 (86.67%)	1 (6.67%)	1 (6.67%)	0	8 (53.33%)	6 (40%)
3	18 (20%)	0	9 (50%)	9 (50%)	0	0	3 (16.67%)	15 (83.33%)
4	14 (15.56%)	0	5 (35.71%)	9 (64.29%)	0	0	1 (7.14%)	13 (92.86%)
5	9 (10%)	0	5 (55.56%)	4 (44.44%)	0	0		9 (100%)
6	13	0	4	9	0	0	1	12

P-ISSN: 2406-9388
E-ISSN: 2580-8303

	(14.44%)		(30.77%)	(69.23%)			(7.69%)	(92.31%)
Total	**90 (100%)**	**9 (10%)**	**49 (54.44%)**	**32 (35.56%)**	**1 (1.11%)**	**11 (12.22%)**	**23 (25.56%)**	**55 (61.11%)**

Table 4. Probability and causality of ADRs based on chemotherapy cycle of respondents who received AC

Chemotherapy Cycle	Number of Respondents	Probability				Causality		
		Possible	Probable	Definite	Unlikely	Possible	Probable	Certain
1	3 (10%)	2 (66.67%)	1 (33.33%)	0	0	2 (66.67%)	1 (33.33%)	0
2	1 (3.33%)	0	1 (100%)	0	0	0	1 (100%)	0
3	6 (20%)	0	6 (100%)	0	0	0	4 (66.67%)	2 (33.33%)
4	6 (20%)	0	4 (66.67%)	2 (33.33%)	0	0	2 (33.33%)	4 (66.67%)
5	8 (26.67%)	0	5 (62.50%)	3 (37.50%)	0	0	3 (37.50%)	5 (62.50%)
6	6 (20%)	0	3 (50%)	3 (50%)	0	0	2 (33.33%)	4 (66.67%)
Total	**30 (100%)**	**2 (6.67%)**	**19 (63.33%)**	**9 (30%)**	**0**	**2 (6.67%)**	**13 (43.33%)**	**15 (50%)**

Table 5. Probability and causality based on stage respondent received AC-T

Stage	Number of Respondents	Probability				Causality		
		Possible	Probable	Definite	Unlikely	Possible	Probable	Certain
1	20 (22.22%)	4 (20%)	11 (55%)	5 (25%)	0	4 (20%)	7 (35%)	9 (45%)
2	42 (46.67%)	3 (7.14%)	24 (57.14%)	15 (35.71%)	0	6 (14.29%)	10 (23.81%)	26 (61.90%)
3	27 (30%)	2 (7.41%)	13 (48.15%)	12 (44.44%)	1 (3.70%)	1 (3.70%)	5 (18.52%)	20 (74.07%)
4	1 (1.11%)	0	1 (100%)	0	0	0	1 (100%)	0
Total	**90 (100%)**	**9 (10%)**	**49 (54.44%)**	**32 (35.56%)**	**1 (1.11%)**	**11 (12.22%)**	**23 (25.56%)**	**55 (61.11%)**

Table 6. Probability and Causality Based on Stage Respondent Received AC

Stage	Number of Respondents	Probability				Causality		
		Possible	Probable	Definite	Unlikely	Possible	Probable	Certain
1	4 (13.33%)	1 (25%)	3 (75%)	0	0	1 (25%)	3 (75%)	0
2	18 (60%)	1 (5.56%)	11 (61.11%)	6 (33.33%)	0	1 (5.56%)	7 (38.89%)	10 (55.56%)
3	8 (26.67%)	0	6 (75%)	2 (25%)	0	0	3 (37.50%)	5 (62.50%)
Total	**30 (100%)**	**2 (6.67%)**	**20 (66.67%)**	**8 (26.67%)**	**0**	**2 (6.67%)**	**13 (43.33%)**	**15 (50%)**

Table 7. ADRs Based on CTCAE 5.0

ADRs Experienced	Level CTCAE							
	AC-T				AC			
	Number of Respondents	1	2	3	Number of Respondents	1	2	3
Nausea	**84 (93.33%)**	26 (30.95%)	58 (69.05%)	0	**28 (93.33%)**	6 (78.57%)	22 (21.43%)	0
Vomiting	**38**	38	0	0	**12**	12	0	0

	(42.22%)	(100%)			**(40%)**	(100%)		
Alopecia	**86**	22	64	0	**29**	3	26	0
	(95.56%)	(25.58%)	(74.42%)		**(96.67%)**	(10.34%)	(89.66%)	
Diarrhea	**45**	45	0	0	**13**	13	0	0
	(50%)	(100%)			**(43.33%)**	(100%)		
Constipation	**28**	28	0	0	**7**	7	0	0
	(31.11%)	(100%)			**(23.33)**	(100%)		
Pain	**62**	34	24	4	**22**	11	10	1
	(68.89%)	(54.84%)	(38.71%)	(6.45%)	**(73.33%)**	(50%)	(45.45%)	(4.55%)
Mouth sores	**42**	33	9	0	**13**	9	4	0
	(46.67%)	(78.57%)	(21.43%)		**(43.33%)**	(69.23%)	(30.77%)	
Fever	**32**	31	1	0	**9**	9	0	0
	(35.56%)	(96.88%)	(3.13%)		**(30%)**	(100%)		
Nail discoloration	**12**	12	0	0	**4**	4	0	0
	(13.33%)	(100%)			**(13.33%)**	(100%)		
Anemia	**12**	11	1	0	**3**	3	0	0
	(13.33)	(91.67%)	(8.33%)		**(10%)**	(100%)		
Leukopenia	**11**	7	3	0	**4**	3	1	0
	(12.22%)	(63.64%)	(36.36%)		**(13.33%)**	(75%)	(25%)	
Thrombocytopenia	**3**	2	0	1	**0**	0	0	0
	(3.33%)	(66.67%)		(33.33%)				

The results in Tables 5 and 6 show that the majority of respondents were at stage 2 of breast cancer, both for those receiving AC-T chemotherapy (46.67%) and AC chemotherapy (60%). Chemotherapy is one of the factors that influences success. Patients with lower disease stages have a lower risk of breast cancer recurrence, thus achieving better outcomes with chemotherapy (Wicaksono, 2022). These results indicate that the Naranjo score (which determines the category of ADRs) does not affect cancer staging. This aligns with Belachew et al.'s (2016) research, which indicates a significant influence of age and number of chemotherapy agents on the severity of ADRs, whereas the risk factor of cancer stage does not have a substantial impact on the severity level of ADRs.

CTCAE is a descriptive terminology used to determine the scale of assessment (severity) for reporting adverse events. Grade refers to the severity of adverse events. The CTCAE displays grades 1 through 5, with unique clinical descriptions of the severity of each adverse event. Grade 1, "mild," indicated asymptomatic or mild symptoms, clinical or diagnostic, observation only, and intervention was not indicated. Grade, 2 "moderate," indicated as minimal, and local or noninvasive intervention was indicated. Grade 3 was "severe" or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated a disability. With a grade of 4, life-threatening consequences and urgent interventions were indicated. Grade 5 deaths are related to adverse events (Cancer Institute 2017).

Table 7 shows the patients with breast cancer, including those with nausea, vomiting, alopecia, diarrhea, constipation, pain, mouth sores, fever, nail discoloration, and hematologic disorders (anemia, leukopenia, and thrombocytopenia). According to CTCAE 5.0, the majority of patients experienced ADRs of grades 1 and 2, with the most minor occurrences in grade 3, whereas no patients experienced reactions in grades 4 or 5.

The occurrence of drug reactions based on the Common Terminology Criteria for Adverse Events (CTCAE) (Table 7) revealed that in grade 1 (mild) ADRs, the most commonly experienced was diarrhea, with an increase of <4 stools per day. On average, patients experienced diarrhea approximately 3-5 times per day. These results contrast with those of a study conducted by Van Rossum et al. (2018), who compared toxicity reactions between AC and AC-T chemotherapy regimens, which showed a higher level of anemia and a lower incidence of diarrhea. However, in this study, there was a lower level of anemia and a higher incidence of diarrhea.

The highest grade 2 (moderate) ADRs was associated with alopecia and nausea. Alopecia presented with hair loss of 50% normal for that individual, which was readily apparent to others, whereas nausea was characterized by decreased oral intake without significant weight loss. These results align with those of a study conducted by (Kim et al. 2019), who evaluated the safety of the AC regimen in breast cancer patients, and showed that the most common side effects included nausea, alopecia, general muscle weakness, myalgia, mucositis, anorexia, dyspepsia, and diarrhea.

The most frequently experienced grade 3 ADRs were categorized as severe and limited self-care ADRs. These results align with those of a study by Kang et al. (2021) that evaluated the safety of cyclophosphamide in

anthracycline and taxane-based neoadjuvant chemotherapy in breast cancer patients, indicating that the addition of cyclophosphamide may increase the risk of thrombocytopenia, sensory/motor neuropathy, and nausea/vomiting.

Respondents who received the AC-T chemotherapy regimen experienced more severe grade of ADRs in terms of hematologic disorders, such as anemia (grade 2, 8.33%), leukopenia (36.6%), and thrombocytopenia (grade 3, 33.33%), as well as symptoms of nausea (grade 2, 69.05%), pain (grade 3, 6.45%), and fever (grade 2, 3.13%), compared to respondents who received the AC chemotherapy regimen. However, for ADR grades related to alopecia (grade 2, 89.66%) and mouth sores (grade 2, 30.77%), the respondents who received the AC chemotherapy regimen experienced slightly more severe symptoms than those who received the AC-T regimen.

Chemotherapy-related ADRs affect the quality of life of patients with breast cancer. Quality of life is one of the factors that determine the effectiveness of a chemotherapy regimen. (Oh et al., 2021; Ratna et al., 2021). Hematologic disorder ADRs are one of the most common issues resulting from chemotherapy regimens used in breast cancer treatment. Managing hematologic disorders leads to high treatment costs and increases the economic burden on the patients and their families. The severity of these ADRs is a significant factor in selecting an optimal chemotherapy regimen for patients (Yuniarti et al., 2021). The use of additional medications may also be necessary to manage chemotherapy-induced ADRs. This increases the need for other medications that can pose a risk for drug interactions. Therefore, selecting a chemotherapy regimen with the lowest risk of ADRs is crucial for preventing potential drug interactions (Effendi and Anggun, 2019).

A limitation of this study was that the numbers of respondents who received AC-T and AC chemotherapy regimens were not equal, making comparisons between the two somewhat challenging. This is because the AC-T regimen is more commonly used than the AC regimen at West Nusa Tenggara Provincial Hospital. Future studies are expected to further examine the relationship between ADRs and quality of life, with ADRs potentially serving as determinants of the effectiveness of chemotherapy regimens in breast cancer treatment.

CONCLUSION

Respondents who received the AC-T regimen experienced more severe ADRs in terms of hematologic disorders (anemia, leukopenia, and thrombocytopenia) as well as symptoms of nausea, pain, and fever compared to respondents who received the AC regimen. However, for ADR grades related to symptoms of alopecia and mouth sores, respondents on the AC regimen experienced slightly more severe symptoms than those on the AC-T regimen.

AUTHOR CONTRIBUTIONS

Conceptualization, B.L.P., R.N.M.; Methodology, B.L.P., R.N.M.; Software, B.L.P., R.N.M.; Validation, B.L.P., R.N.M.; Formal Analysis, B.L.P., R.N.M.; Investigation, B.L.P., R.N.M.; Resources, B.L.P., R.N.M.; Data Curration; B.L.P., R.N.M.; Writing - Original Draft, B.L.P., R.N.M.; Writing - Review & Editing, B.N., B.L.N.; Visualization, B.N., B.L.N.; Supervision, B.L.P., R.N.M.; Project Administration, B.L.P., R.N.M.; Funding acquisition, B.L.P., R.N.M.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Jurnal Farmasi dan Ilmu Kefarmasian Indonesia
Pharmacy and Pharmaceutical Sciences Journal
Published by Faculty of Pharmacy Universitas Airlangga
OPEN ACCESS
E-ISSN 2580-8303
P-ISSN 2406-9388

Jurnal Farmasi dan Ilmu Kefarmasian Indonesia Vol. 11 No. 2 August 2024, 260-268
DOI: 10.20473/jfiki.v11i22024.260-268
Available online at https://e-journal.unair.ac.id/JFIKI/

Anti-Ulcer and Antioxidant Activities of *Chrysophyllum albidum* G. Don. Seeds Cotyledons

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Submitted: 30 May 2024
Revised: 27 July 2024
Accepted: 31 August 2024

Abstract

Background*: Gastric ulcers are prevalent gastrointestinal disorders with significant global implications owing to their prevalence and potential complications. Side effects associated with synthetic drugs have led to the search for alternative treatments. Chrysophyllum albidum, a plant traditionally used to manage various diseases, has been investigated for its potential to alleviate ulcerative conditions.* ***Methods:*** *This study assessed the efficacy of extracts from C. albidum seed cotyledons in mitigating ethanol- and diclofenac-induced ulcers in rats. Phytochemical screening was performed using standard methods and antioxidant activities were evaluated using DPPH scavenging and $ABTS^+$-reducing assays.* ***Results:*** *For ethanol-induced gastric ulcers, extracts at doses of 100, 200, and 400 mg/kg produced lesion indices of 7.04 ± 0.44, 5.18 ± 0.38, and 2.53 ± 0.46 mm, respectively, compared to omeprazole's 0.9 ± 1.09 mm. The highest dose showed 87.93% inhibition, which was comparable to that of omeprazole (93.63% inhibition). A similar trend was observed for diclofenac-induced ulcers. Phytochemical analysis revealed the presence of active compounds, such as steroids, flavonoids, polysaccharides, alkaloids, and cardiac glycosides. Antioxidant activity results indicated significant free radical scavenging properties, with an IC_{50} value of 49.24 µg/mL for DPPH and 15.1 µg/mL for $ABTS^+$ at a dose of 400 mg/kg. These findings demonstrate the notable dose-dependent anti-gastritis and anti-ulcer effects of the extract.* ***Conclusion:*** *This study highlights the potential of C. albidum seed cotyledons as a valuable candidate for gastroprotective drug development and supports their traditional use in treating and preventing gastritis and gastric ulcers.*

Keywords*: antioxidant, alternative treatment, DPPH, phytochemicals, ulcer*

How to cite this article:
Owolabi, T. A. & Okubor, P. C. (2024). Anti-Ulcer and Antioxidant Activities of *Chrysophyllum albidum* G. Don. Seeds Cotyledons. *Jurnal Farmasi dan Ilmu Kefarmasian Indonesia*, 11(2), 260-268. http://doi.org/10.20473/jfiki.v11i22024.260-268

P-ISSN: 2406-9388
E-ISSN: 2580-8303

INTRODUCTION

Plants play a significant role in contemporary drug discovery and development, with medicinal plants being historically used to manage and treat various diseases, including ulcers (Shahzad et al., 2023). Gastritis and gastric ulcers are among the most common gastrointestinal disorders, and their prevalence and complications have increased in recent decades, leading to substantial global morbidity and mortality (Sun et al., 2023). Ulcers result from an imbalance between harmful factors, such as acid and pepsin, and the protective mechanisms that maintain mucosal integrity (Périco et al., 2022). Managing gastritis and ulcers involves a combination of medications including proton pump inhibitors, anticholinergics, histamine receptor antagonists, and antibiotics (Arunachalam et al., 2023). Although these drugs are effective, their potential side effects, limited efficacy, and interactions pose significant challenges (Périco et al. 2022). Consequently, there is a growing interest in natural remedies, which are perceived to have fewer side effects and lower costs.

Antioxidants help manage ulcers by counteracting oxidative stress, which is a key factor in the development of gastric and duodenal ulcers. Oxidative damage exacerbates inflammation and mucosal injury, contributing to ulcer formation and hindering healing (Beiranvand et al. 2021). Research suggests that antioxidants, such as vitamin C, vitamin E, flavonoids, polyphenols, and plant-based compounds, can reduce oxidative damage, protect the gastric mucosa by neutralizing free radicals, suppressing inflammatory pathways, and promoting mucosal repair (Beiranvand et al., 2018; Beiranvand et al., 2021). Additionally, they may help lower gastric acid secretion, potentially improve ulcer healing, and reduce the risk of recurrence (Khan et al., 2024). Antioxidants also help combat bacteria such as H. pylori and inhibit pepsinogen production, thereby preventing ulcer formation. Some antioxidants have been shown to boost the levels of prostaglandins and mucus in the gastric mucosa, thereby demonstrating cytoprotective effects. Additionally, several of these compounds can prevent gastric mucosal ulcers triggered by various experimental models and safeguard the gastric lining from various harmful agents (Alharbi et al., 2022).

Chrysophyllum albidum G. Don, a tree from the *Sapotaceae* family, is commonly found in lowland rainforests of East and West Africa (Erukainure et al., 2022). Known as the African Star Apple or Agbalumo and Udara in Nigeria, this fruit is enjoyed as a nutritious snack and is believed to offer health benefits (Imaga et al., 2023; Erukainure et al., 2022). The fruit contains approximately 50 mg/100 g ascorbic acid in its exocarp and pulp (Tsado et al., 2023). Phytochemical analyses have revealed both saturated (palmitic and myristic acids) and unsaturated (linoleic and oleic acids) fatty acids in fruits (Izuakor et al., 2024). Additionally, fruit juice contains significant phenolic compounds such as catechin, chlorogenic acid, caffeic acid, epicatechin, cyanidine-3–O-glycoside, rutin, quercitrin, quercetin, and kaempferol (Ajayi et al., 2024). Ethnomedicinally, C. albidum was used to treat diarrhea, hypertension, malaria, and wounds (Ogunleye et al., 2020). The fruit pulp and peel extracts have demonstrated various pharmacological activities, including anti-nociceptive, anti-inflammatory, hypolipidemic, and antidiabetic effects (Akomolafe et al., 2019; Asagba et al., 2019; Ajayi et al.,2020a; 2020b), and anti-ulcer effects on the bark (Salami et al., 2022). However, the anti-ulcer activity of the seed cotyledons of this plant is yet to be evaluated scientifically; therefore, this study focused on evaluating the anti-ulcer potential of extracts derived from the seed cotyledons of *C. albidum*.

MATERIALS AND METHODS

Materials

C. albidum seeds were sourced from ripe fruits (Figure 1) purchased from local markets in Ewu, Esan, Edo State, Nigeria. The seeds were identified and authenticated at the Pax Herbal Clinic and Research Laboratories, where they were assigned voucher numbers Pax/12/668 and a specimen was deposited. Wistar rats were obtained from the Pax Herbal Clinic and Research Laboratories Animal House and handled according to animal ethics standards. The other materials used were acacia gum, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+}$), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid (Aldrich), potassium persulfate, monosodium phosphate monohydrate, disodium phosphate heptahydrate, methanol, ethanol, and chloroform. All other reagents were of analytical grade and the solvents were redistilled before use.

Tools

The study utilized various apparatus and equipment, including a heating mantle, hot air oven (DHG-9053A), water bath, Soxhlet extractor, grinding machine (DE-DAMAK; GX160, Japan), centrifuge, UV Spectrophotometer (Surgifield; SM-23D, England), and a water circulator.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Method

Extraction

Two hundred grams of the Powdered cotyledons (200 g) were subjected to Soxhlet extraction using methanol as the solvent. Following extraction, the extract was concentrated under reduced pressure using a rotary evaporator, yielding a semi-solid pale-yellow paste, which was then weighed, and the percentage yield of the extract was calculated using the formula.

$$\% \text{ yield} = \frac{\text{Weight of extract}}{\text{Weight of original plant material}} \times 100$$

Phytochemical screening

Phytochemical analysis was performed to detect the presence of various bioactive compounds in the extracts. The tests included alkaloids, flavonoids, saponins, cardiac glycosides, terpenoids, steroids, reducing sugars, and polysaccharides (Owolabi and Salome, 2022). Briefly, 5 g of the extract was dissolved in 5 mL of methanol and diluted with 100 mL of double-distilled water. The resulting solution was used for the following phytochemical tests.

Alkaloid test

To 3 mL of the extract, 3 mL of 1% HCl was added, heated in steam for 30 min, cooled, and centrifuge at 2000-3000 rpm for 10 min. The supernatant was tested with

a) Drangedroff reagent (orange precipitate indicates alkaloids)

b) Mayer's reagent (creamy precipitate indicates alkaloids)

c) Wagner's reagent (reddish-brown precipitate indicates alkaloids)

Flavonoid test

Then, 2 mL of the extract was added to 2 mL of dilute ammonia solution, and then 1 mL of concentrated H2SO4 was added. The yellow coloration, which fades upon standing, confirms the presence of flavonoids.

Saponin test

The extract (0.5 mL) and distilled water (5 mL) were added, and the mixture was shaken vigorously. Persistent frothing indicated the presence of saponins.

Cardiac glycoside test

Two milliliters of the extract, 2 mL of glacial acetic acid, 1 mL of 0.1% FeCl3, and 1 mL of concentrated H2SO4. The green-blue coloration confirms the presence of cardiac glycosides.

Terpenoid test

Add 2 mL of the extract to six drops of Brady's reagent. The yellowish-orange color indicates the presence of terpenoids.

Steroid test

mL of the extract with acetic acid anhydride (0.5 mL) was mixed and cooled on ice, and chloroform (0.5 mL of chloroform and 1 mL) were H2SO4 carefully. A reddish-brown ring at the interface confirms the presence of steroids.

Reducing sugar test

The extract (2 mL) was added to 2 mL of Fehling's solutions A and B, and then heated for 30 min. The red coloration confirms the presence of reducing sugars.

Starch/polysaccharide test

Add 2 mL of the extract to six drops of iodine solution. The blue-black coloration indicates the presence of starch.

Anti-ulcer activity: ethanol and diclofenac-induced gastric ulcer

All animal experimental procedures were conducted in strict adherence to the approved ethical committee on animal handling guidelines of the Research and Ethical Review Committee, Igbinedion University (approval number: IUO/Ethics/054/24), which aligns with the United States National Institute of Health (NIH) Guidelines for the Care and Use of Laboratory Animals in Biomedical Research (NIH, 1985).

Briefly, adult Wistar rats were fasted for 24 h before the experiment although they had free access to water. The rats were randomized and divided into five groups, each containing five rats. Groups 1–3 were treated with *C. albidum* seed cotyledon extract at doses of 100, 200, and 400 mg/kg orally. These doses were chosen based on the previously reported LD_{50} of the seed to be greater than 1000 mg/kg (Onyegbule et al., 2019; Onyegbule et al., 2020). Group 4 received vehicle, while group 5 was administered omeprazole at a dose of 20 mg/kg. After 60 min, each rat was orally administered 1 mL of 96% ethanol or diclofenac via an orogastric cannula. One hour after ethanol/diclofenac administration, the animals were sacrificed under ether anesthesia. The stomachs were then dissected, opened along the greater curvature, rinsed under running water to remove blood clots, fixed in 10% formalin, and examined for lesions using a hand lens. The total number, shape, and coloration of all the lesions in each stomach were observed using a 10X hand lens and recorded as the ulcer index (UI), which was calculated as follows:

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Ulcer index (UI) = UN + US + (UP/10)

UN is the average number of ulcers per animal, UP is the percentage of animals with ulcers, and US is the average severity score, which is shown in table below:

Table 1. Severity scores of ulcer indices

S/N	Observable indices	Scores
1	Normal colored stomach	0
2	Coloration	0.5
3	Spot ulcer	1
4	Hemorrhagic streak	1.5
5	Deep ulcer	2
6	Perforation	3

The percentage of inhibition of ulceration was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{(UI_{control} - UI_{treated})}{UI_{control}} X\ 100$$

Antioxidant activity

The antioxidant potential of the extracts was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) and $ABTS^+$ (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) assays (Munteanu & Apetrei, 2021).

DPPH radical scavenging capacity

DPPH radical scavenging capacity was determined using standard methods (Munteanu & Apetrei, 2021). A 2.4 mL solution (0.1 mM) in ethanol was mixed with 1.6 mL of the extracts at varying concentrations (0-200 µg/mL). The reaction mixture was thoroughly vortexed and incubated in the dark at room temperature for 30 min. Absorbance was measured using a spectrophotometer (Surgifield; SM-23D, England) at 517 nm. The percentage of DPPH radical-scavenging activity was calculated using the following equation:

$$\text{Scavenging activity} = \frac{(A_0-A_1)}{A_0} X\ 100$$

where A_0 is the absorbance of the blank, and A_1 is the absorbance of the sample. The percentage inhibition was plotted against the concentration, and the IC_{50} value was determined from the graph.

Calculation of EC_{50} value

To calculate the EC_{50} value, the plant extract solution in methanol was further diluted and tested using the DPPH assay to determine the concentration required for 50% inhibition. The EC_{50} values were calculated using a graphical method. The EC_{50} value of ascorbic acid (AA) was determined.

$ABTS^+$ assay

The $ABTS^+$ assay was performed using a modified method from Munteanu and Apetrei (2021). A 7 mM $ABTS^+$ stock solution was prepared in water. The $ABTS^+$ radical cation was generated by reacting the stock solution with 2.45 mM potassium persulfate solution. The solution was kept in the dark at room temperature for 12 h prior to use. It was then diluted 50-fold with phosphate buffer (pH 8.04) to achieve an absorbance of 0.7 at 415 nm. Three milliliters of the $ABTS^+$ solution was added to a 1 cm cuvette, followed by the addition of 150, 300, and 600 µL of methanolic plant extract solutions to achieve final concentrations of 50, 100, and 200 ppm, respectively. Trolox was used as a positive control, whereas the $ABTS^+$ solution was used as a negative control. The absorbance was measured at 415 nm. The percentage inhibition was measured using the following formula:

% inhibition = (Ac-As/Ac) x 100

Where;

Ac = Absorbance of control

As = Absorbance of sample

Statistical Analysis

All experiments were conducted in triplicate and repeated at least twice, and the results are expressed as mean ± standard deviation. Statistical analysis was performed using analysis of variance (ANOVA) (SigmaPlot version 15.0). Differences were considered statistically significant at $P < 0.01$ and $P < 0.05$.

RESULTS AND DISCUSSION

Yields of the plant extract

Following exhaustive extraction using continuous hot extraction, 200 g of powdered *C. albidum* seed cotyledons yielded a crude extract of 21.9583 g, which corresponded to 10.98% of the initial plant powder. Extraction and extraction of solvents are the key determinants of the yield of bioactive constituents obtained after each successful extraction procedure. The extraction method used in this study contributed to the high yield obtained through this procedure.

Phytochemical analysis results

Phytochemical screening identified flavonoids, polysaccharides, alkaloids, terpenoids, cardiac glycosides, and steroids in the methanol extract of *C. albidum* seed cotyledons. However, saponins were not detected. Detailed results are presented in Table 2. Some researchers have reported that *C. albidum* contains alkaloids, tannins, phenols, and flavonoids in the stem

P-ISSN: 2406-9388
E-ISSN: 2580-8303

slash and seed cotyledons (Adeboyejo et al., 2019; Imaga et al., 2023; Izuakor et al., 2024), which agrees with the current study, except for the presence of steroids, which has not been previously reported. These variances can be attributed to differences in geographical sources. The presence of these bioactive compounds likely contributes to the observed anti-ulcer and antioxidant activities, as many researchers have linked the therapeutic effects of plants to their phytochemicals (Owolabi & Ayinde, 2022).

Effects of *C. albidum* seed cotyledon extract on ethanol and diclofenac-induced gastric ulcer

Ulcers are a prevalent gastrointestinal disorder characterized by inflamed lesions or erosion of the mucosa and underlying tissues, resulting from a disparity between harmful factors, such as acid, pepsin, and *H. pylori*, and protective factors, such as gastric mucus, bicarbonate ions, and prostaglandins, along with the inherent resistance of mucosal cells (Périco et al., 2022). The incidence of gastric ulcers and gastritis is notably higher in individuals who smoke, use nonsteroidal anti-inflammatory drugs (NSAIDs), or consume alcohol (Aladainan et al., 2021; Xie et al., 2022). Although conventional treatments are effective, both clinical and experimental studies have shown that traditional herbal medicines offer therapeutic benefits in gastric ulcers (Roy et al., 2023).

In the present study, the methanol extract of *C. albidum* seed cotyledons significantly decreased the ulcer indices in both ethanol- and diclofenac-induced ulcer models in a dose-dependent manner. In the ethanol-induced ulcer model, the vehicle control group had an ulcer index of 8.33 ± 0.73, whereas the groups treated at 100, 200, and 400 mg/kg had ulcer indices of 7.04 ± 0.44, 5.18 ± 0.38, and 2.53 ± 0.46, respectively. These results were comparable to those of the standard omeprazole group, which had an ulcer index of 0.9 ± 1.09, with percentage inhibitions of 12.99, 60.72, and 87.93%, respectively, compared to the omeprazole group (93.63%). A similar trend was observed in the diclofenac-induced ulcer model, where the vehicle group produced an ulcer index of 14.98 ± 0.34. The treated groups at 100, 200, and 400 mg/kg showed ulcer indices of 9.92 ± 0.44, 5.93 ± 0.66, and 3.50 ± 0.73, respectively, while the omeprazole group had an ulcer index of 2.49 ± 0.45, as detailed in Table 3. The methanol extract demonstrated significant efficacy in both in vivo ulcer models, suggesting its potential as a therapeutic agent in ulcer management.

Some researchers have reported *C. albidum* as a traditional treatment for ulcers (Imaga et al., 2023). Although Salami et al. (2022) proved this claim, this study is the first to provide an experimental basis for the anti-ulcer activities of seed cotyledons only on the stem bark of the plant.

Antioxidant effect of *C. albidum* seed cotyledon extract

The extract exhibited some level of antioxidant activity, despite the highest concentration (200 µg/mL) yielding the most effective IC_{50} values of: 49.24 ± 0.978 µg/mL DPPH and 15.1 ± 0.07 µg/mL), which are not comparable to the activities of ascorbic acid (17.24 ± 0.425 µg/mL for DPPH, and 7.01 ± 0.2 µg/mL for $ABTS^{+}$). These results indicate robust free radical-scavenging properties. The detailed results are presented in Table 4.

Oxidative stress is believed to initiate and exacerbate digestive system diseases, including stomach ulcers and gastric carcinomas. Ethanol-induced gastric damage is thought to be mediated by free radicals (Périco et al. 2022). Ethanol metabolism generates superoxide anions and hydroperoxyl free radicals. Recent research suggests that antioxidants may offer protection and promote healing in the stomach by boosting the production of gastric mucus glycoproteins and inhibiting prostaglandin production (da Luz et al., 2019). Free radicals play a significant role in ethanol-induced and NSAID-related mucosal damage (Takeuchi 2012). Antioxidants can neutralize ROS, and are expected to aid in the healing and prevention of gastric ulcers. Akanji (2020) and Adetoun et al. (2023) reported that *C. albidum* pulp and stem bark exhibit antioxidant activities, which was also demonstrated for the first time in the seed cotyledons in the current study. In our experiment, we found a significant scavenging potential that suggests that the extracts would have significant antioxidant action and, therefore, significant anti-gastritis and anti-ulcer activity.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Figure 1. *Chrysophyllum albidum* A: tree, B: fruits, C: seeds, D: cotyledons

Table 2. Results of the qualitative phytochemical screening of *C. albidum* seeds cotyledons

Phytoconstituents	**Results**
Cardiac Glycoside	++
Terpenoids	++
Saponin	-
Flavonoid	+
Steroid	+++
Alkaloid	+
Polysaccharide/Starch	++

Key:
+ = Mildly Present ++ = Moderately Present
+++ = Abundantly Present - = Absent
Note: +, ++, +++ represent the extent of either coloration or precipitate produced

Table 3. Effect of the *C. albidum* seed cotyledon extract on ethanol-induced and diclofenac-induced gastric ulceration

	Ethanol-induced		**Diclofenac-induced**	
Sample	Ulcer Index	% Inhibition	Ulcer Index	% Inhibition
Acacia gum (0.2%)	8.33 ± 0.73	-	14.98 ± 0.34	-
C. albidum (100mg/kg)	7.04 ± 0.44	12.99	9.92 ± 0.44**	28.43
C. albidum (200mg/kg)	5.18 ± 0.38*	60.72	5.93 ± 0.66**	53.75
C. albidum (400mg/kg)	2.53 ± 0.46*	87.93	3.50 ± 0.73**	84.19
Omeprazole (20 mg/kg)	0.9 ± 1.09**	93.63	2.49 ± 0.45**	96.45

Values represent the mean ± SD (n=5); *P<0.05, **P<0.01, significant when compared to the control

Table 4. Antioxidant activities *C. albidum* seeds cotyledons extracts

Sample	DPPH IC_{50} (μg/mL)	$ABTS^{+}$ EC_{50} (μg/mL)
AA (25 µg/mL)	17.24 ± 0.425	7.01 ± 0.2
100 µg/mL	62.9 ± 1.02	30.32 ± 0.4
200 µg/mL	51.7 ± 1.57	27.72 ± 0.05
400 µg/mL	49.24 ± 0.978	15.1 ± 0.07

AA is Ascorbic acid
Each value is expressed as Mean ± SD (n = 3), at 100 μg/ml. IC_{50} (μg/ml): the concentration at which 50% is inhibited; EC_{50} (μg/ml): effective concentration at which the absorbance is 0.5.

CONCLUSION

C. albidum seed cotyledons exhibit significant anti-ulcer that can be linked to their antioxidant activities, supporting their traditional use in treating gastrointestinal disorders. Further research is needed to isolate and characterize the active compounds responsible for these effects and to investigate their mechanisms of action.

ACKNOWLEDGMENT

To the entire staff of Prof. Dora Akuyili College of Pharmacy, Igbinedion University, Okada, Edo State, Nigeria.

AUTHOR CONTRIBUTIONS

Conceptualization, O.T.A.; Methodology, O.T.A.; Software, O.P.C.; Validation, O.T.A.; Formal Analysis, O.P.C.; Investigation, O.T.A., O.P.C.; Resources, O.T.A., O.P.C.; Data Curration; O.P.C.; Writing - Original Draft, O.T.A.; Writing - Review & Editing, O.P.C.; Visualization, O.T.A.; Supervision, O.T.A., O.P.C.; Project Administration, O.T.A.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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