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EDITORIAL WORDS

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

Editor in Chief
Borneo J Pharm

Assalamu'alaikum Wr. Wb.

Alhamdulillahirabbil 'alamin. The next edition of **Borneo Journal of Pharmacy** (*Borneo J Pharm*), has been published at November 2024. This edition contains ten articles: Pharmacology-Toxicology, Pharmacognosy-Phytochemistry, Pharmaceutical, Analytical Pharmacy-Medicinal Chemistry, Microbiology Pharmacy, Natural Product Development, and Clinical-Community Pharmacy. This edition includes writings from five countries: India, Indonesia, Saudi Arabia, Sudan, and Taiwan. The authors come from several institutions, including Institut Sains dan Teknologi Nasional, Taipei Medical University, Metro College of Health Sciences and Research, Galgotias University, Yogendra Nath Saxena College of Pharmacy and Research Centre, Shri Gopichand College of Pharmacy, Jadavpur University, Maulana Abul Kalam Azad University of Technology, Universitas Muslim Indonesia, Universitas Jenderal Soedirman, Akademi Farmasi Surabaya, University of Khartoum, National Medicines Quality Control Laboratory, Al Jouf University, Universitas Sari Mulia, Universitas Muhammadiyah Purwokerto, Universitas Muhammadiyah Kalimantan Timur, Universitas Muhammadiyah Palangkaraya, Universitas Wahid Hasyim, Universitas Jenderal Achmad Yani, and Ibrahim Adjie Primary Care.

Editorial boards are fully aware that there are still room for improvement in this edition, hence with all humility willing to accept constructive suggestions and feedback for improvements to the publication for the next editions. The editorial board would like to thank all editors and reviewers, and contributors of the scientific articles who have provided the repetoire in this issue. We hope that all parties, especially the contributors, could re-participate for the publication in the next edition on March 2025.

Wassalamu'alaikum Wr. Wb.

Palangka Raya, November 2024

Editor-in-Chief

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Author Guidelines

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1. Writing is the result of research or review of critical analysis study in the field but not limited to Pharmacology-Toxicology, Pharmacognosy-Phytochemistry, Pharmaceutical, Analytical Pharmacy-Medicinal Chemistry, Natural Product Development, Clinical-Community Pharmacy, Management Pharmacy, and other Pharmacy aspects which have never been published in other scientific publication media.
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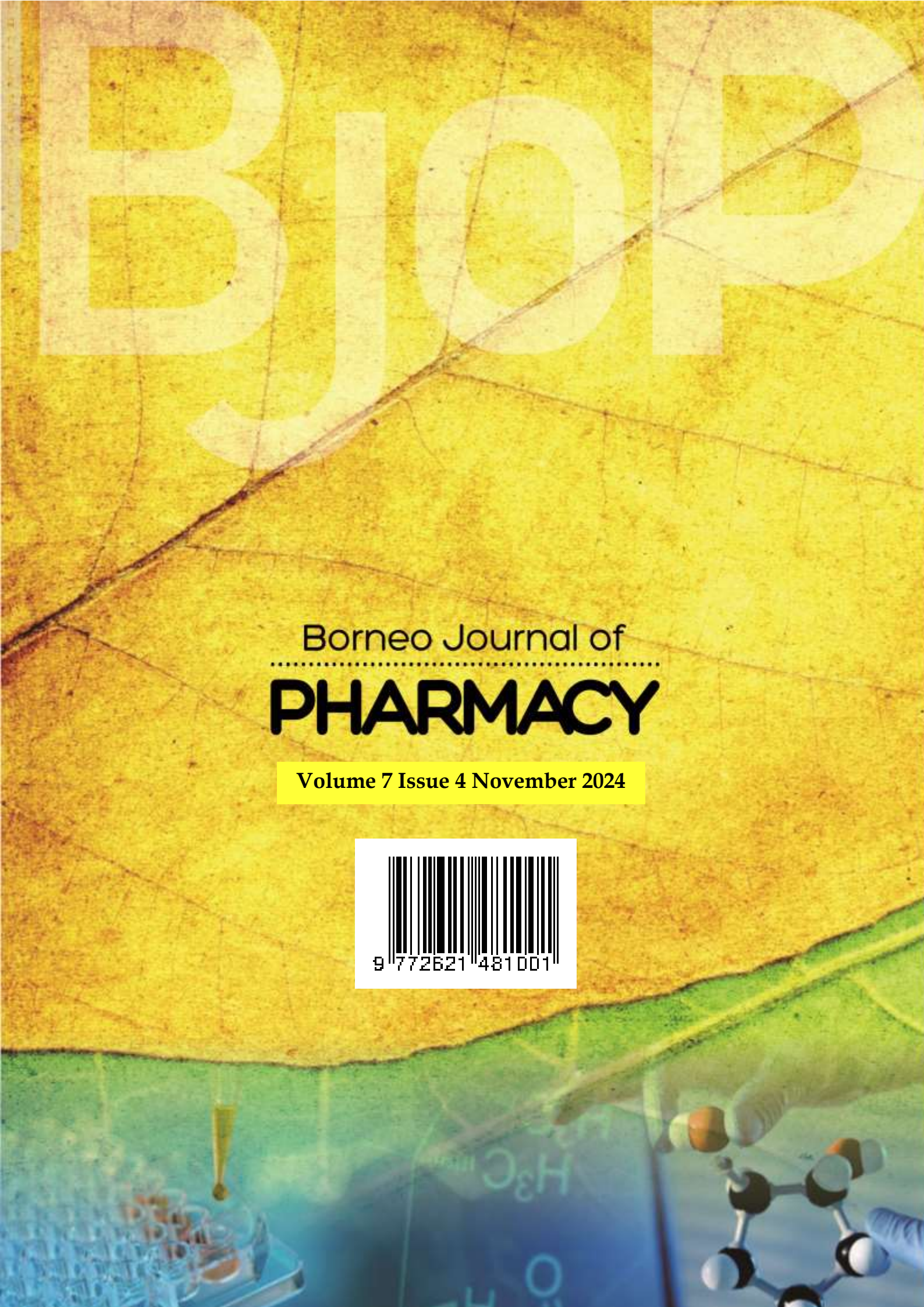
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Research Article

Antidiarrheal Potential of Maja (*Crescentia cujete*) Fruit Extract in Mice

Teodhora [1*]

Rosario Trijuliamos Manalu [1,2]

Richi Andika Saputra [1]

[1] Department of Pharmacy, Institut Sains dan Teknologi Nasional, South Jakarta, Special Capital Region of Jakarta, Indonesia
[2] Program for Cancer Molecular Biology and Drug Discovery, Taipei Medical University, Taipei City, New Taipei City, Taiwan

*email: c.teodhora@istn.ac.id; phone: +6281228481549

Keywords:
Attapulgite
Antidiarrheal
Crescentia cujete
Loperamide

Abstract

Diarrhea, characterized by frequent, loose stools, can be treated with various approaches. This study investigated the antidiarrheal properties of maja (*Cresentia cujete*) fruit extract, which contains secondary metabolites like tannins and flavonoids with astringent properties. The objective was to determine the optimal dose of *C. cujete* fruit extract for treating diarrhea in mice. An *in vivo* experimental design was employed, inducing diarrhea in male mice using *oleum ricini*. The study assessed stools' onset, duration, consistency, frequency, and weight. *Cresentia cujete* fruit extract was administered orally at 125, 250, and 500 mg/kg BW doses. A comparison was made with negative control (CMC-NA) and positive controls (loperamide and attapulgite). Data analysis involved ANOVA followed by the Tukey test. The findings revealed that 500 mg/kg BW of *C. cujete* fruit extract was the most effective dose for treating diarrhea in mice. These results suggest the potential of *C. cujete* fruit extract as a promising natural antidiarrheal agent.

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INTRODUCTION

Diarrhea, a common gastrointestinal ailment characterized by increased stool frequency and altered consistency, remains a significant global health concern. While often underestimated, diarrhea is a leading cause of death, particularly among young children. In fact, it is the third most common cause of death in children under five years of age, surpassed only by pneumonia and tuberculosis[1]. Dehydration, a severe consequence of fluid and electrolyte loss, is the primary cause of mortality associated with diarrhea[2].

Diarrhea remains a significant public health concern, particularly in developing countries like Indonesia. In 2018, diarrheal diseases resulted in a substantial number of deaths, with children under five and adults over five being disproportionately affected. Diarrhea prevalence among Indonesian toddlers increased from 6.7% to 15.2% between 2013 and 2018[3]. Moreover, in 2017, 1725 diarrhea cases were reported across 12 provinces and 17 districts/cities, leading to 34 fatalities. East Java emerged as a hotspot, accounting for the second-highest number of diarrhea cases (151,878) with a prevalence of 7.6%. Surabaya, a major city in East Java, bore a significant burden, reporting approximately half of the total cases in the province[4].

Diarrhea can be treated with either specific or non-specific approaches. Non-specific treatments, such as loperamide, aim to reduce symptoms by suppressing peristalsis[5]. However, this approach is not suitable for all patients, particularly young children. Specific treatments, involving antimicrobial drugs, target the underlying bacterial infection. Yet, indiscriminate use of antibiotics can lead to antibiotic resistance and other adverse effects[6].

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Indonesia, a country renowned for its rich biodiversity, offers a wealth of potential natural remedies. Numerous plants have been traditionally used for various purposes, including medicinal applications. Unfortunately, many plant species are undervalued and face the risk of extinction due to deforestation and habitat loss[7], one of them is maja or *Crescentia cujete*. *Crescentia cujete*, commonly known as the Calabash Tree, is often overlooked due to its unpleasant fruit. The fruit's black, sticky, and malodorous nature has led to the plant being considered poisonous and undesirable. As a result, it is frequently discarded or even destroyed, limiting its potential applications[8]. While traditional uses of *C. cujete* include utilizing its fruit shells for utensils and its pulp as fertilizer or pesticide[9], there is a growing interest in exploring its medicinal properties. Recent studies have investigated the potential of *C. cujete* leaf extract as a treatment for wounds[10], diabetes[11], and cancer[12]. To further unlock the potential of this underutilized plant, additional research is necessary to investigate various aspects of its biology, chemistry, and applications.

Crescentia cujete has long been recognized for its diverse pharmacological properties. Previous studies, such as those conducted by Atmodjo[8], have identified the presence of various secondary metabolites within the plant, which contribute to its potential therapeutic applications. One such potential application is in the realm of antibacterial therapy[13]. Bacterial infections, often contracted through contaminated food or water, can lead to a range of gastrointestinal disorders, including diarrhea[14]. Previous research has demonstrated the therapeutic potential of *C. cujete*, particularly its antioxidant and antibacterial properties, attributed to its rich content of primary and secondary metabolites[15-17]. However, despite extensive studies, the specific antidiarrheal activity of *C. cujete* fruit, particularly at the molecular level, remains underexplored.

This study aimed to investigate the anti-diarrheal potential of *C. cujete* fruit pulp extract prepared using 70% ethanol. To assess its efficacy, an *in vivo* model of castor oil-induced diarrhea in male mice was employed. The parameters evaluated included the onset of diarrhea, fecal consistency, fecal weight, frequency of defecation, and duration of diarrhea. This research provides valuable insights into the potential therapeutic applications of *C. cujete* fruit pulp and encourages further investigation into its underlying mechanisms of action at the molecular level.

MATERIALS AND METHODS

Materials

Analytical balances (Wiggen Hauser), a rotary evaporator (Buchi b-740), and standard laboratory glassware (Pyrex) were employed in this study. The chemicals used were of analytical grade and included: 70% ethanol, 1 N sodium hydroxide, 1% aluminum chloride, 2 N hydrochloric acid, Mayer's reagent, Dragendorff's reagent, 1% ferrous chloride, 1% sodium carboxymethylcellulose, acetate anhydride, sulfuric acid, loperamide hydrochloride (Lodia®), attapulgite (Diatab®), and castor oil. The primary test substance was the fruit flesh of *C. cujete*. Fruits were collected from the campus of the National Institute of Science and Technology, South Jakarta, Indonesia. The correct species identification of *C. cujete* was confirmed by the Indonesian Institute of Science, Bogor, and the Biology Research Center, West Java, Indonesia. This identification was officially recognized with Certificate Number 1083/IPH.1.01/if.07/XI/2020.

Methods

Preparation and extraction of plant materials

Five kilograms of fresh *C. cujete* fruit were thoroughly washed with running water and then peeled, sliced, and dried in an oven at 60°C for 48 hours. The dried fruit was ground into a fine powder using a 40-mesh sieve, yielding approximately 2 kg of dried fruit powder. The powdered fruit was macerated in 70% ethanol at room temperature for three days, protected from light. The resulting extract was filtered and concentrated using a rotary evaporator under reduced pressure to remove the ethanol.

Preliminary phytochemical screening

Preliminary phytochemical screening was conducted to identify the presence of alkaloids, flavonoids, saponins, and tannins in the extract. This screening aimed to establish a pharmacognostic profile for the plant material[18].

Alkaloids: Alkaloids were detected using a standard procedure. One gram of *C. cujete* fruit extract was moistened with 5 mL of 25% ammonia solution and extracted with 20 mL of chloroform. The mixture was heated, filtered, and the filtrate was concentrated to half its original volume. The concentrated extract was acidified with 1 mL of 2 N hydrochloric acid and

partitioned into two layers. The aqueous layer was divided into two portions and tested with Mayer's and Dragendorff's reagent. The formation of white, brown, and red precipitates, respectively, in these tests is indicative of the presence of alkaloids. A positive alkaloid test was confirmed if at least two of these reagents yielded a positive result.

Tannins: One gram of *C. cujete* fruit extract was extracted with 100 mL of hot water. A 5 mL aliquot of the filtrate was mixed with a few drops of 1% ferric chloride solution. A green-purple color indicates the presence of tannins.

Flavonoids: One gram of *C. cujete* fruit extract was filtered into 100 mL of hot water. A 5 mL aliquot of the filtrate was mixed with 1 mL of 5% sodium nitrite solution and 1 mL of 10% aluminum chloride solution. Subsequently, 2 mL of 1 N sodium hydroxide solution was added. A positive test for flavonoids was indicated by the development of a red or orange color.

Saponins: One gram of *C. cujete* fruit extract was extracted and purified using 100 mL of hot water. A 10 mL aliquot of the filtrate was subjected to a foam test to identify saponins. A persistent foam formation of 1-10 cm height indicates the presence of saponins. To confirm this, a drop of 2 N HCl was added to the test tube; the formation of a stable foam (±1 cm) further supports the presence of saponins.

Evaluation of antidiarrheal potential

Ethical approval for this study was obtained from the Medical and Health Research Ethics Committee of Jakarta Veterans University (Letter No. 144/I/2021/KEPK). This approval ensured adherence to ethical guidelines for human and animal research[19]. Male mice (*Mus musculus*) aged 2-3 months and weighing 20-30 g were used for *in vivo* testing. Mice were chosen as the animal model due to their physiological similarity to humans, ease of procurement and care, and rapid regeneration rate. Additionally, male mice were selected to minimize hormonal fluctuations, which can potentially influence experimental outcomes.

Mice were acclimatized to the laboratory environment for one week prior to the experiment. On the day of the experiment, mice were fasted for 30 minutes and then randomly divided into six groups of four mice each, using Federer's formula[20]. In this study, *oleum ricini* (castor oil) was administered orally to induce diarrhea at a dose of 0.8 mL/mouse. Upon ingestion, lipase enzymes in the small intestine hydrolyze castor oil into glycerol and ricinoleic acid. Ricinoleic acid, a potent laxative, irritates the intestinal mucosa and stimulates peristalsis, leading to the characteristic laxative effect. Loperamide and attapulgite were employed as standard antidiarrheal agents for comparison. Thirty minutes post-*oleum ricini* administration, each group received a different treatment: Group I (negative control; 1% CMC-Na), II (loperamide HCl; 7 mg/kg BW), III (attapulgite; 1200 mg/kg BW), and IV to VI (*C. cujete* fruit extract; 125, 250, and 500 mg/kgBW, respectively), as shown in **Table I**.

Table I. Treatment group.

Groups	Treatment - dose administrated (per oral)	Total mice
I	1% CMC-Na 1000 mg	4
II	Loperamide 7 mg/kg BW	4
III	Attapulgite 1200 mg/kg BW	4
IV	*Crescentia cujete* fruit extract 125 mg/kg BW	4
V	*Crescentia cujete* fruit extract 250 mg/kg BW	4
VI	*Crescentia cujete* fruit extract 500 mg/kg BW	4

Mice were monitored for seven hours at 30-minute intervals. Diarrhea was defined as an increase in stool frequency and a change in consistency to a softer or liquid state[21]. Observations included the onset of diarrhea, stool consistency (normal, soft, or watery/mucous), stool weight, stool frequency, and diarrhea duration. The onset of diarrhea was recorded as the time (in minutes) after *oleum ricini* administration when the first instance of diarrhea occurred. Stool weight was measured every 30 minutes. Diarrhea duration was calculated as the difference between the onset of diarrhea and the time when stool consistency returned to normal.

Data analysis

To analyze the diarrhea effect, one-way ANOVA followed by Tukey's HSD post-hoc test was employed using SPSS version 25.0. This statistical analysis allowed for the comparison of mean differences between the treatment groups.

RESULTS AND DISCUSSION

The maceration method was employed for extraction, as it avoids the use of heat, thereby preserving the integrity of heat-sensitive bioactive compounds present in the *C. cujete* fruit. This approach is in line with previous studies[22-24] that have highlighted the benefits of non-thermal extraction methods. Ethanol, a polar solvent, was chosen as the extraction solvent due to its ability to dissolve both polar and non-polar compounds present in *C. cujete* fruit. Additionally, ethanol's inert nature minimizes the risk of unwanted chemical reactions. The use of fungal fermentation, a non-toxic and sustainable method, was optimized to enhance the extraction process and maximize the yield of bioactive compounds[25]. The resulting extraction process yielded a viscous extract weighing 320 g from 545 g of *C. cujete* fruit powder, as detailed in **Table II**.

Table II. Organoleptic analysis and yield of *C. cujete* fruit extract.

Plant identification	Results	Plant	Extract
Color	Blackish green		
Form	Thick extract		
Flavor	Bitter		
Powder weight (g)	545		
Extract weight (g)	320		
Yield (%)	58.7		

To ensure the safety and reliability of the anti-diarrheal testing, the ethanol extract was subjected to a drying process to remove residual solvent. Ethanol traces in the extract could potentially interfere with the study by affecting the central nervous system, leading to ataxia or sedation, and thus skewing the results. Prior to testing, phytochemical screening was conducted to identify the bioactive compounds present in the *C. cujete* fruit extract. Phytochemical screening of *C. cujete* fruit extract revealed the presence of a diverse range of bioactive compounds, including alkaloids, saponins, tannins, and flavonoids. These findings align with previous studies[17,26] that have reported the presence of similar phytoconstituents in other plant species. The positive results for these compounds suggest the potential of *C. cujete* fruit extract for various pharmacological activities, particularly due to the known antioxidant and antimicrobial properties of these phytochemicals.The results of this analysis are summarized in **Table III**.

Table III. Phytochemical screening of *C. cujete* fruit extract.

Phytochemicals		Results	Description
Alkaloids	Mayer's	White precipitate	+
	Dragendorff's	Redbrick precipitate	+
Saponins		Stable foam ± 1 cm	+
Tannins		Greenish black color	+
Flavonoids		Reddish orange color	+

+: presence of phytochemical

The selection of loperamide and attapulgite as comparators was informed by previous studies demonstrating the difference between loperamide and attapulgite in terms of their antidiarrheal mechanism[27]. To comprehensively assess the antidiarrheal effects of *C. cujete* fruit extract, various parameters were monitored, including the onset of diarrhea, stool weight, frequency, duration, and consistency. Administration of *oleum ricini* induced diarrhea in the mice, as evidenced by the onset of liquid fecal excretion. As depicted in **Figure 1**, the successful induction of diarrhea was confirmed by the observation of liquid stool within a specific timeframe following *oleum ricini* administration.

Treatment with 500 mg/kg BW of *C. cujete* fruit extract significantly suppressed the onset of diarrhea, comparable to the effects of 1200 mg/kg BW attapulgite and 4 mg/kg BW loperamide. However, lower doses of the extract (125 and 250 mg/kg BW) exhibited reduced antidiarrheal efficacy. The weight of fecal output was monitored every 30 minutes for 180 minutes post-*oleum ricini* administration (**Figure 2**). The results indicate that the antidiarrheal activity of the extract was dose-dependent. While lower doses (125 and 250 mg/kg BW) exhibited a less pronounced effect on stool weight reduction compared to the standard drugs, the highest dose (500 mg/kg BW) demonstrated comparable antidiarrheal activity to loperamide and attapulgite.

Additionally, the frequency of diarrhea episodes was determined by counting the number of occurrences during the observation period. The results showed that extracts administered at doses of 125 and 250 mg/kg BW exhibited lower

efficacy in suppressing diarrhea compared to the standard drugs, loperamide and attapulgite. However, the 500 mg/kg BW extract demonstrated comparable antidiarrheal activity to both standard drugs in reducing diarrhea frequency. The reduction in diarrhea frequency, as depicted in **Figure 3**, supports the efficacy of the extract at the 500 mg/kg BW dose.

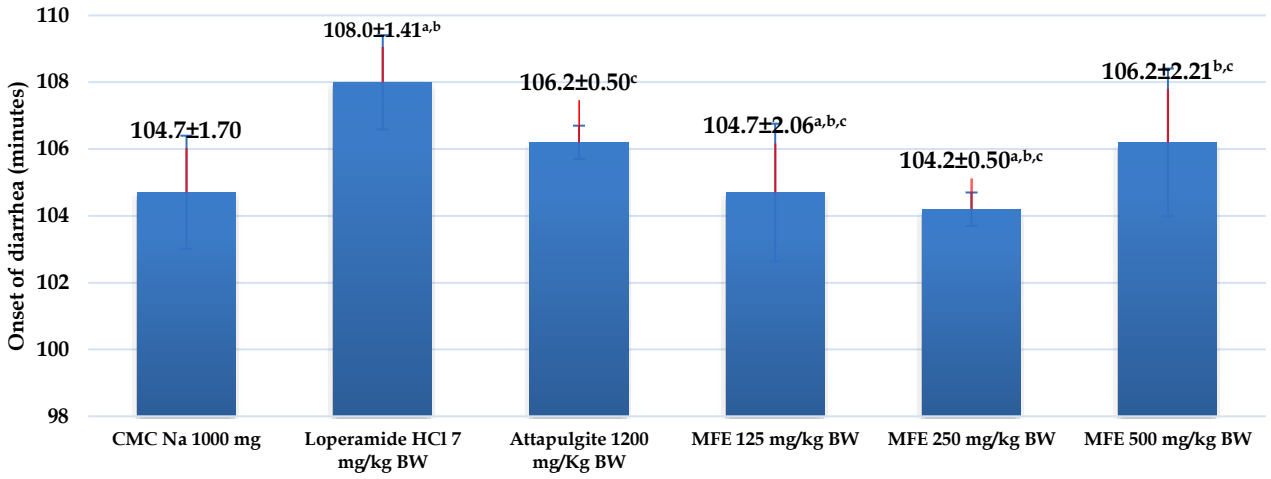

Figure 1. Diarrhea onset of each test group. **a**: There is a significant difference p <0.05; **b,c:** There is no significant difference p >0.05. MFE: *C. cujete* fruit extract.

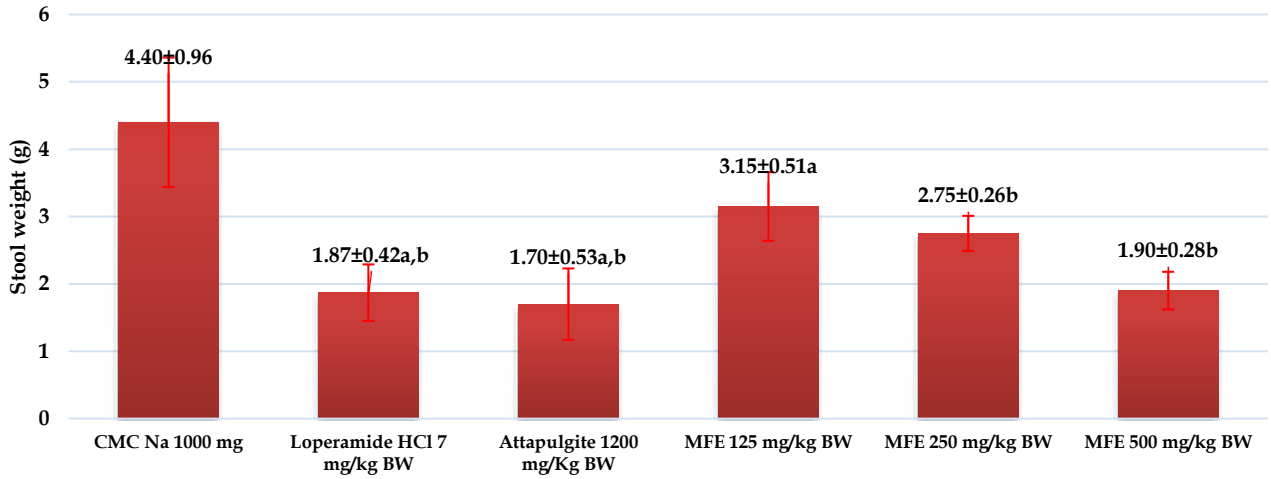

Figure 2. Stool weight of each test group. **a**: There is a significant difference p <0.05; **b,c:** There is no significant difference p >0.05. MFE: *C. cujete* fruit extract.

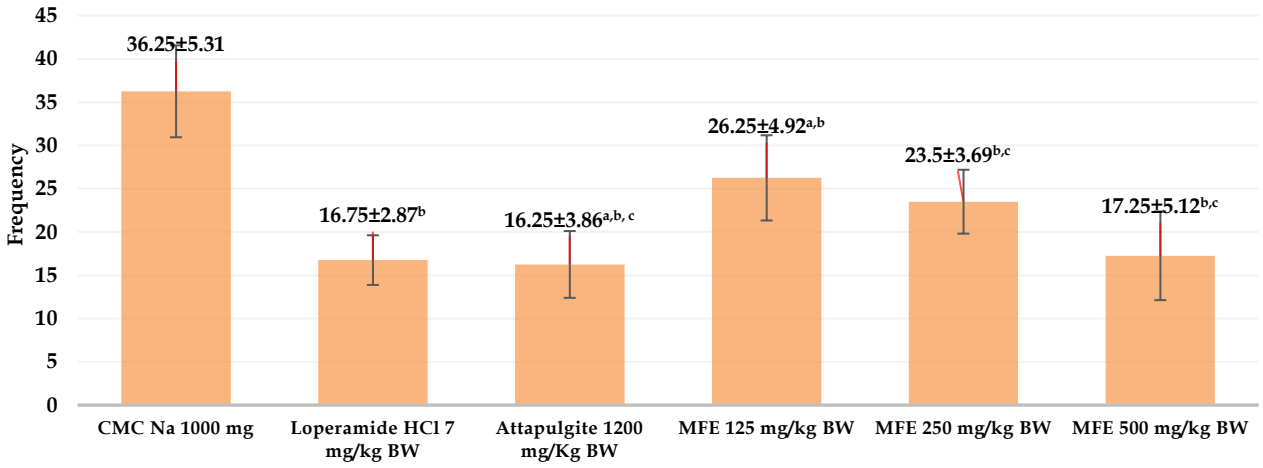

Figure 3. Frequency of diarrhea of each test group. **a**: There is a significant difference p <0.05; **b,c:** There is no significant difference p >0.05. MFE: *C. cujete* fruit extract.

The duration of diarrhea, determined by the time interval between the onset and resolution of liquid stool, was observed across all dose groups (**Figure 4**). Interestingly, the 250 and 500 mg/kg BW dose groups exhibited similar diarrhea durations, comparable to the positive control. In contrast, the 125 mg/kg BW group experienced a notably longer duration of diarrhea. These findings suggest that increasing the dose beyond 250 mg/kg BW may not significantly reduce diarrhea duration but could potentially enhance therapeutic efficacy.

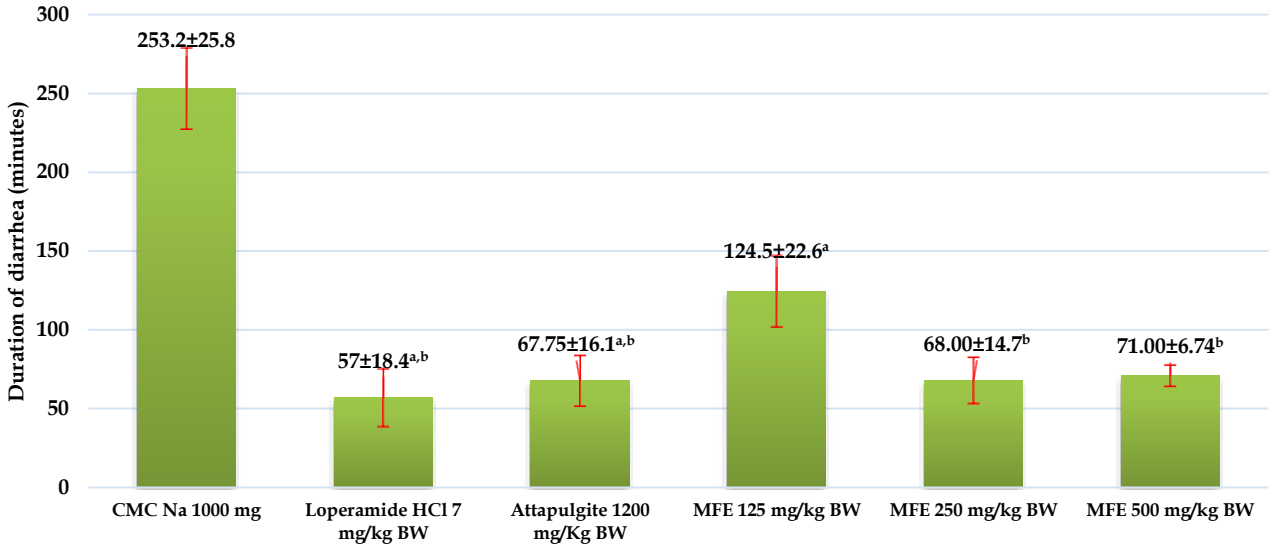

Figure 4. Duration of diarrhea of each test group. **a**: There is a significant difference $p < 0.05$; **b,c**: There is no significant difference $p > 0.05$. MFE: *C. cujete* fruit extract.

Table IV presents the average onset of action for the *C. cujete* fruit extract, attapulgite, and loperamide. Notably, the *C. cujete* fruit extract exhibited comparable anti-diarrheal activity to attapulgite and loperamide at significantly lower doses (500 mg/kg BW compared to 1200 mg/kg BW and 4 mg/kg BW, respectively). These findings suggest that *C. cujete* fruit extract could be a promising natural alternative to conventional anti-diarrheal medications. However, further research is needed to optimize the dosage and formulation of *C. cujete* extract for optimal therapeutic efficacy. Additionally, investigating the underlying mechanisms of action of the active compounds in *C. cujete* fruit extract could provide valuable insights for developing novel anti-diarrheal therapies[28].

Table IV. Phytochemical screening of *C. cujete* fruit extract.

Group	Treatment - dose administrated (per oral)	Onset (minutes±SD)
I	1% CMC-Na 1000 mg	-
II	Loperamide 7 mg/kg BW	124±33.37
III	Attapulgite 1200 mg/kg BW	130±36.34
IV	*Crescentia cujete* fruit extract 125 mg/kg BW	162±97.58
V	*Crescentia cujete* fruit extract 250 mg/kg BW	131±36.85
VI	*Crescentia cujete* fruit extract 500 mg/kg BW	130±36.34

This study's findings corroborate previous research[29,30], which has shown that tannin-rich plants like *C. cujete* possess chelating properties and spasmolytic effects. Tannins interact with proteins in mucus and epithelial cells, forming cross-links that lead to astringency. This mechanism can reduce intestinal motility by inhibiting the release of acetylcholine, a neurotransmitter that regulates smooth muscle contractions[31].

However, further research is necessary to fully elucidate the antidiarrheal properties of *C. cujete*. Future studies should explore different extraction methods, solvents, and pharmaceutical dosage forms. Additionally, comprehensive safety assessments, including acute and chronic toxicity studies, are crucial before clinical trials can be conducted to confirm the safety and efficacy of *C. cujete* as an antidiarrheal agent.

CONCLUSION

The most potent antidiarrheal effect was observed with the 500 mg/kg BW dose of *C. cujete* fruit extract, which was comparable to the standard antidiarrheal medications, loperamide and attapulgite. At this dose, the extract normalized fecal consistency within 210 minutes, indicating significant antidiarrheal activity.

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AUTHORS' CONTRIBUTION

Conceptualization: Teodhora
Data curation: Rosario Trijuliamos Manalu
Formal analysis: Rosario Trijuliamos Manalu
Funding acquisition: -
Investigation: Teodhora
Methodology: Teodhora
Project administration: Teodhora
Resources: -
Software: -
Supervision: Rosario Trijuliamos Manalu
Validation: Rosario Trijuliamos Manalu
Visualization: Teodhora
Writing - original draft: Richi Andika Saputra
Writing - review & editing: Teodhora, Rosario Trijuliamos Manalu

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this study.

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Review Article

Monoclonal Antibodies for Treatment of COVID-19: An Updated Review of Current Evidence

Saurabh Nimesh [1*]
Pratibha Kumari [2]
Rohit Kumar [3]
Gosiya [3]
Md. Quamuddin [1]
Md. Iftekhar Ahmad [4]

[1] Department of Pharmacology, Metro College of Health Sciences and Research, Greater Noida, Uttar Pradesh, India
[2] Department of Pharmacy, Galgotias University, Greater Noida, Uttar Pradesh, India
[3] Department of Pharmacology, Yogendra Nath Saxena College of Pharmacy and Research Centre, Amroha, Uttar Pradesh, India
[4] Department of Pharmaceutics, Shri Gopichand College of Pharmacy, Baghpat, Uttar Pradesh, India

*email: nimeshmiet@gmail.com; phone: +917455923397

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Loperamide

Abstract

The emergence of COVID-19 in December 2019 spurred a global effort to develop effective medical interventions. Therapeutic monoclonal antibodies (mAbs) have emerged as a promising strategy to combat the SARS-CoV-2 virus. Several mAbs targeting the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein have received Emergency Use Authorization (EUA) for treating mild to moderate COVID-19. Additionally, human mAbs and hyperimmune plasma derived from recovered COVID-19 patients have been explored as potential therapeutic options. This review delves into the potential of mAbs for the diagnosis and treatment of COVID-19 infection. We discuss the mechanisms of action of mAbs, as well as their advantages and limitations. Furthermore, we explore the ongoing research and development efforts to optimize mAb-based therapies for COVID-19.

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INTRODUCTION

The COVID-19 pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in Wuhan, China, in December 2019 and rapidly spread globally. This pandemic has resulted in millions of infections and deaths worldwide. SARS-CoV-2 is an enveloped, positive-sense single-stranded RNA virus belonging to the Betacoronavirus genus[1,2]. Its large genome encodes both structural and non-structural proteins, including the spike (S) protein, which plays a crucial role in viral entry into host cells[3].

The S-protein is a prime target for therapeutic intervention, as it is essential for viral attachment and entry. Neutralizing monoclonal antibodies (mAbs) that bind to the S-protein can effectively block viral infection[4]. While significant efforts have been directed towards vaccine development and antiviral therapies, the potential of mAbs as a therapeutic strategy against COVID-19 has gained increasing attention[5]. Monoclonal antibodies can offer several advantages, including rapid onset of action, high specificity, and the potential to target multiple viral epitopes. However, their therapeutic use is often limited by factors such as cost, production complexity, and potential for immunogenicity[6].

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Understanding the complex interplay between the virus and the host immune response is crucial for developing effective therapeutic strategies[7]. In the early stages of COVID-19 infection, antiviral therapies like remdesivir can be beneficial in reducing viral replication. However, as the disease progresses, the host immune response can contribute to severe disease outcomes[8,9]. In such cases, immunomodulatory therapies, such as corticosteroids and interleukin-6 inhibitors, can help mitigate the inflammatory response[10].

The COVID-19 pandemic has underscored the urgent need for innovative therapeutic approaches to combat this highly contagious disease[11]. Monoclonal antibodies have emerged as a promising therapeutic strategy due to their ability to target specific viral proteins and neutralize the virus. Given the ongoing threat of COVID-19 and the emergence of new variants, continued research into the development of novel therapeutic strategies, including mAb-based therapies, is essential[12,13]. This review will explore the development and clinical application of various mAb therapies for COVID-19, providing an overview of their mechanisms of action, efficacy, and safety profiles.

MONOCLONAL ANTIBODIES

Monoclonal antibodies are laboratory-produced antibodies designed to mimic the immune system's ability to fight off harmful antigens. They are typically derived from either convalescent human B-cell lymphocytes or humanized mice. By cloning a single antibody, scientists can produce a large quantity of identical antibodies with high specificity for a particular target antigen[14,15]. Monoclonal antibody therapies have emerged as a promising approach to treat COVID-19. By targeting specific viral proteins, these therapies can help prevent severe illness, reduce viral load, and mitigate symptoms[13,16]. However, it's important to note that mAb therapy is not a substitute for vaccination. Vaccination remains crucial for building herd immunity and preventing the spread of the virus[17]. There are four primary methods for producing mAbs, each with distinct characteristics.

Murine Antibodies

Murine antibodies are derived from mouse proteins and are identified by names ending in "-omab"[18]. For instance, muromonab, the mAb anti-CD3 antibody used as immunosuppressive therapy in kidney, heart, and liver transplant patients[19].

Chimeric Antibodies

Chimeric antibodies are a class of monoclonal antibodies that combine human and mouse antibody sequences. This hybrid approach aims to reduce immunogenicity while maintaining therapeutic efficacy[20]. Chimeric antibodies are named with the suffix "-ximab"[18]. For instance, infliximab is a chimeric antibody approved for the treatment of various inflammatory conditions, including rheumatoid arthritis, inflammatory bowel disease, and ankylosing spondylitis[21].

Humanized Antibodies

Humanized antibodies are a class of therapeutic antibodies that combine the specificity of mouse antibodies with the safety profile of human antibodies. These antibodies are engineered to have a human-like framework, reducing the risk of immunogenicity and improving their therapeutic potential[20]. The names of many humanized antibody drugs end in "-zumab"[18]. For instance, trastuzumab is a well-known humanized monoclonal antibody used to treat certain types of breast, stomach, and gastroesophageal junction cancers[22].

Human Antibodies

Human antibodies, named with the suffix "-umab"[18], are therapeutic proteins derived from human immune cells. These antibodies are engineered to bind specifically to target molecules involved in disease processes[20]. Adalimumab, a well-known example, is a monoclonal antibody that targets tumor necrosis factor-alpha (TNF-α), a key inflammatory cytokine. This targeted approach offers several advantages, including reduced immunogenicity and increased efficacy compared to traditional antibody-based therapies[23].

ANTI-SARS-COV-2 MONOCLONAL ANTIBODIES

The SARS-CoV-2 genome encodes four major structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N). Additionally, several non-structural and accessory proteins are also expressed[24]. The S-protein, composed of S1 and S2 subunits, plays a crucial role in viral entry. The S1 subunit binds to the human angiotensin-converting enzyme 2 (ACE2) receptor, facilitating viral attachment to host cells. Subsequently, the S2 subunit undergoes conformational changes, triggering membrane fusion and viral entry[25-27].

Anti-SARS-CoV-2 mAbs targeting S-protein have demonstrated clinical efficacy in managing SARS-CoV-2 infections. These mAbs have been successfully used for post-exposure prophylaxis in individuals exposed to the virus in residential settings. Additionally, they have been employed in specialist and home care settings during outbreaks[28]. Furthermore, pre-exposure prophylaxis with certain anti-SARS-CoV-2 mAbs has been shown to significantly reduce the risk of infection[29].

The development of mAbs has emerged as a promising strategy for the diagnosis and treatment of COVID-19. Several pharmaceutical companies, including Celltrion, AstraZeneca, and Regeneron, have actively pursued the development of mAbs targeting the SARS-CoV-2 virus. The U.S. Food and Drug Administration (FDA) has granted emergency use authorization (EUA) to several mAb therapies, such as bamlanivimab, a combination therapy of bamlanivimab and etesevimab, casirivimab and imdevimab, and sotrovimab, for the treatment of mild to moderate COVID-19 in high-risk individuals[30]. While mAbs have shown promise in combating COVID-19, the emergence of SARS-CoV-2 variants with mutations that reduce susceptibility to these therapies poses a significant challenge. Additionally, the development of mAb therapies for other viral diseases, such as Ebola virus disease, has demonstrated the potential of this approach in addressing infectious diseases[31].

Bamlanivimab

Bamlanivimab, the mAb specifically designed to block the SARS-CoV-2 spike protein from binding to the human ACE2 receptor, was granted EUA by the FDA on November 9, 2020, for the treatment of mild to moderate COVID-19 in high-risk adult and pediatric patients[32]. Administered as a single 700 mg infusion, bamlanivimab was the first mAb approved for COVID-19 treatment. However, subsequent studies revealed limited efficacy in reducing viral load. Consequently, the FDA revoked the EUA for bamlanivimab monotherapy on April 16, 2021, citing the emergence of SARS-CoV-2 variants resistant to this therapy (**Figure 1**)[33].

Casirivimab and Imdevimab Combination

The R10933-10987-COV-2067 Phase III trial evaluated the efficacy of casirivimab and imdevimab in treating mild to moderate COVID-19 infection[34,35]. Following EUA by the FDA on November 21, 2020, this antibody cocktail, marketed as Regeneron's REGN-COV2, was administered via intravenous infusion or subcutaneous injection. The treatment involves a combination of 600 mg casirivimab and 600 mg imdevimab, targeting non-overlapping epitopes of the SARS-CoV-2 spike protein[36]. However, due to the emergence of the Omicron variant, which exhibits reduced susceptibility to these antibodies, the distribution of casirivimab and imdevimab in the United States has been suspended. Individuals infected with the Omicron variant are unlikely to benefit from this treatment[37].

The clinical studies are evaluating the efficacy of REGN-COV2 in several populations. Firstly, the drug is being tested in adolescents aged 12 and older to assess its ability to alleviate COVID-19 symptoms. Secondly, its effectiveness in reducing viral load in children under 18 years of age is being explored. Additionally, the study is comparing the efficacy of REGN-COV2 to placebo in hospitalized patients with mild to moderate COVID-19, focusing on factors such as survival rates and the need for mechanical ventilation[38,39]. Notably, patients treated with the cocktail did not require hospitalization due to COVID-19 within a 41-day observation period[40].

REGN-COV2 has shown significant promise in combating COVID-19. Clinical trials have demonstrated a 70% reduction in hospitalization and mortality rates among high-risk individuals. Additionally, when administered to individuals exposed to the virus, REGN-COV2 can reduce the risk of symptomatic infection by 80%[13], as shown in **Figure 1**. Notably, individuals with no pre-existing SARS-CoV-2 antibodies experienced the greatest clinical benefit.

Bamlanivimab and Etesevimab Combination

Bamlanivimab and etesevimab, a combination of monoclonal antibodies, target multiple epitopes on the SARS-CoV-2 spike protein. This dual-antibody approach aims to neutralize the virus and prevent its entry into human cells[41]. On February 9, 2021, the FDA granted EUA for this combination therapy to treat mild to moderate COVID-19 in non-hospitalized pediatric patients. By binding to the receptor-binding domain of the spike protein, these antibodies effectively block viral attachment and entry into host cells[30].

Initially, a combination therapy of bamlanivimab (700 mg) and etesevimab (1400 mg) was administered intravenously to individuals in regions with low levels of SARS-CoV-2 mutations. However, official guidelines advised against COVID-19 vaccination within three months of receiving this treatment[42]. A recent phase I clinical trial demonstrated a 70% reduction in COVID-19-related hospitalizations among patients who received bamlanivimab and etesevimab compared to placebo[43]. The distribution of bamlanivimab and etesevimab was temporarily suspended in the United States due to the emergence of the Omicron variant, which exhibits reduced susceptibility to these monoclonal antibodies. As a result, individuals infected with the Omicron variant are unlikely to benefit from this treatment[44]. The product's availability was reinstated as the prevalence of the Gamma and Beta variants declined to less than 5%. The US FDA and the European Medicines Agency (EMA) have recommended the use of monoclonal antibody (mAb) combinations, such as casirivimab and imdevimab (REGN-CoV-2) as well as bamlanivimab and etesevimab, for outpatients at high risk of severe COVID-19 who do not require supplemental oxygen therapy (**Figure 1**)[45].

Sotrovimab

Sotrovimab, the mAb initially discovered in a SARS-CoV survivor in 2003, targets a conserved epitope on the SARS-CoV-2 spike protein's RBD. It received EUA from the FDA in May 2021 for the treatment of mild-to-moderate COVID-19 in high-risk individuals[46]. Administered as a single 500 mg intravenous infusion, sotrovimab has demonstrated a significant reduction in hospitalizations and deaths compared to placebo. In a clinical trial involving 583 participants, only 1% of those treated with sotrovimab required hospitalization or died within 29 days, compared to 7% in the placebo group. This translates to a 6% absolute reduction and an 85% relative reduction in severe outcomes (**Figure 1**). While intravenous administration is the current standard, intramuscular formulations are under clinical investigation[47].

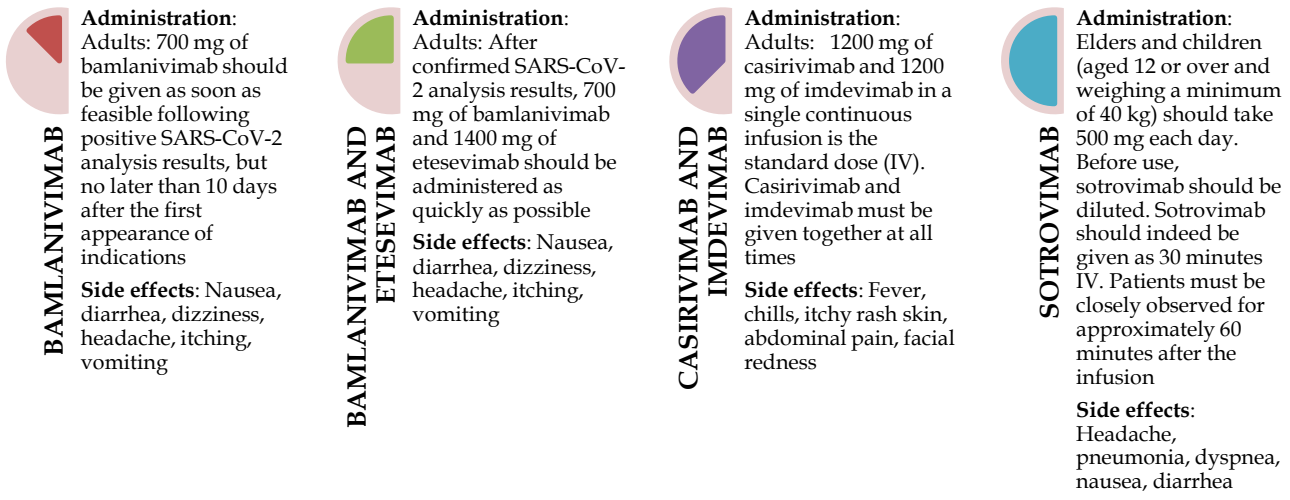

Figure 1. Administration information for mAbs currently approved for COVID-19 and their side effects.

MONOCLONAL ANTIBODIES INFUSION CRITERIA

The FDA EUAs for mAbs targeting SARS-CoV-2 outline specific criteria for individuals at increased risk of severe COVID-19. These criteria were expanded on May 14, 2021, to include individuals diagnosed with other medical conditions who

have mild to moderate COVID-19 and are at high risk of progressing to severe disease. However, the use of mAbs is not recommended for all COVID-19 patients[48,49].

The administration of mAbs is typically considered for individuals with mild to moderate COVID-19 who are at high risk of developing severe disease or hospitalization. This includes patients who have been hospitalized for reasons other than COVID-19 or those who meet specific eligibility criteria for outpatient treatment[50]. Importantly, mAbs are not recommended for patients with severe COVID-19 requiring oxygen therapy. To maximize the effectiveness of mAb treatment, it is crucial to initiate therapy as early as possible after symptom onset and within 10 days of a positive COVID-19 test[51]. Early administration allows mAbs to effectively neutralize the virus and prevent its replication. Beyond this 10-day window, the therapeutic benefit of mAbs diminishes significantly[52].

Patients should be monitored for at least one hour following intravenous or subcutaneous administration of mAb therapy. Additionally, current recommendations advise against receiving COVID-19 vaccination within three months of mAb treatment. This precautionary measure is implemented to prevent potential interference with vaccine-induced immune responses[53]. The Centers for Disease Control and Prevention (CDC) specifically recommends a 90-day interval between the first and second doses of COVID-19 vaccine for individuals who have received mAb therapy[54].

Individuals who are at high risk of severe COVID-19 infection may benefit from therapeutic interventions. This includes those who are unvaccinated or have compromised immune systems due to underlying health conditions or immunosuppressive medications. Additionally, individuals who have frequent close contact with COVID-19 patients may also be considered for therapeutic treatment[55].

High-Risk Conditions

Individuals 12 years and older are considered high-risk for severe COVID-19 if they meet at least one of the following criteria:

1. Age: 65 years or older
2. Obesity: BMI ≥25 kg/m² for adults, BMI ≥85th percentile for children aged 12-17
3. Pregnancy
4. Chronic kidney disease
5. Diabetes
6. Hypertension or cardiovascular disease
7. Chronic lung diseases (e.g., COPD, severe asthma, interstitial lung disease, cystic fibrosis, pulmonary hypertension)
8. Sickle cell disease
9. Neurological disorders (e.g., cerebral palsy)
10. Immunocompromising conditions (e.g., congenital immunodeficiencies, organ transplantation, HIV infection, cancer treatment)
11. Dependency on medical devices (e.g., tracheostomy, gastrostomy, ventilator)
12. Infants under 12 months

It's important to note that the presence of multiple high-risk conditions further increases the risk of severe COVID-19 infection[56].

Mechanism of Action

The SARS-CoV-2 virus relies on its spike protein to invade human cells. This crucial protein binds to the human ACE2 receptor, enabling the virus to enter and replicate. Neutralizing mAbs specifically target this spike protein, effectively blocking viral entry and preventing infection. By hindering the virus's ability to attach to host cells, these mAbs can significantly reduce the severity of COVID-19. The rapid progress in understanding the structure of the SARS-CoV-2 spike protein, aided by prior experience with other human viruses, has accelerated the development of effective mAb-based therapies[4,57].

To initiate infection, SARS-CoV-2 must first bind to the host cell surface receptor, ACE2, through its S-protein. This interaction is facilitated by the transmembrane protease serine 2 (TMPRSS2). Neutralizing mAbs can effectively block this initial step by binding to the S-protein, thereby preventing viral entry into the host cell. While most mAbs target the RBD of the S-protein, which is crucial for ACE2 binding, some mAbs may target other epitopes on the S-protein to achieve

neutralization. The potency of neutralizing antibodies, as demonstrated by previous studies on SARS-CoV and MERS-CoV, is a critical factor in determining their clinical potential[58].

Production

Monoclonal antibodies are produced by B lymphocytes and are essential tools in various biomedical applications. Traditionally, mAbs were generated from murine B cells, limiting their therapeutic potential due to immunogenicity. To overcome this challenge, techniques have been developed to produce mAbs in stable cell lines, including those derived from human B cells[59]. By utilizing human B cells, particularly from convalescent COVID-19 patients, researchers have successfully generated humanized mAbs that exhibit potent neutralizing activity against SARS-CoV-2. These humanized mAbs hold significant promise as therapeutic agents for COVID-19 and other infectious diseases[60].

Traditional production of mAbs in mice involves a multi-step process. First, mice are immunized with a specific antigen, such as the SARS-CoV-2 spike protein. Then, their spleen cells, which produce antibodies against the antigen, are fused with immortalized myeloma cells using a fusing agent like polyethylene glycol. This fusion creates hybridomas, which are cells that can continuously produce the desired mAbs. These hybridomas are then cloned and subcloned to obtain stable cell lines that produce a single type of mAb[61]. Historically, most mAbs produced using this method have been mouse mAbs, and many of these have been used in immunotherapy[62].

To generate recombinant human mAbs, the cDNA sequences encoding the variable regions of the heavy and light chains of interest must be cloned into expression plasmids. These plasmids typically contain the constant regions of human IgG1 heavy chain and Ig kappa light chain, along with a signal sequence (such as the interleukin-2 signal sequence) to facilitate efficient secretion of the recombinant antibody. Following transfection of mammalian cells, such as HEK-293T cells, with the expression plasmids, the recombinant human mAbs are produced and purified using protein A affinity chromatography[63].

Hybridoma technology, introduced in 1975, revolutionized the production of mAbs. By fusing immortalized myeloma cells with antibody-producing B cells, researchers can generate stable cell lines that produce large quantities of specific mAbs. This technique was employed to develop mouse mAbs targeting specific surface epitopes on the SARS-CoV S-protein. Subsequently, through RNA isolation and genetic engineering, these mouse mAbs were humanized or chimerized to reduce immunogenicity in humans[61]. One such humanized mAb, 47D11, demonstrated the ability to neutralize shared epitopes between SARS-CoV and SARS-CoV-2 in Vero cell culture, highlighting its potential as a therapeutic agent against both viruses[64].

Food & Drug Administration Expansion of Authorization of Two mAbs for the Treatment of COVID-19 in Younger Children (including Newborns)

The U.S. FDA has expanded the EUA for bamlanivimab and etesevimab. Previously authorized for pediatric patients aged 12 years and older weighing at least 40 kilograms, the EUA has been extended to include younger pediatric patients, including newborns. This expanded authorization allows for the combined use of bamlanivimab and etesevimab to treat mild-to-moderate COVID-19 in younger pediatric patients who have tested positive for the virus[65]. Additionally, the combination therapy can now be used as a post-exposure prophylaxis to prevent COVID-19 infection in pediatric patients, including newborns, who are at high risk of severe disease or death[66].

COVID-19 Prevention

The COVID-19 pandemic has spurred urgent efforts to develop effective vaccines and therapies. While vaccine development typically takes several years, concerted global efforts have accelerated the timeline for COVID-19 vaccines, with some vaccines becoming available within a year[67]. For individuals who cannot receive vaccination or require immediate protection, monoclonal antibodies (mAbs) offer a promising alternative. By targeting specific viral proteins, mAbs can neutralize the virus and prevent infection or reduce disease severity. Passive administration of mAbs can provide immediate protection, especially for high-risk individuals such as the elderly, immunocompromised, and those with underlying health conditions[68].

However, it's important to note that mAbs are not a long-term solution and should be used judiciously. As vaccine rollout continues, mAbs can play a crucial role in bridging the gap between vaccination and the development of protective

immunity. Additionally, mAbs can be particularly beneficial for individuals who have been exposed to the virus but have not yet developed symptoms[13].

Adverse Effects

One potential side effect of mAb therapy is an allergic reaction. These reactions often occur during or shortly after the infusion and are typically monitored by healthcare providers. Patients who have received anti-SARS-CoV-2 mAbs have experienced a range of hypersensitivity reactions, including anaphylaxis, infusion-related reactions, hives, itching, rashes, diarrhea, dizziness, and pruritis[69]. Additionally, other adverse effects such as fever, chills, nausea, headache, breathing difficulties, hypotension, facial swelling, wheezing, and muscle pain have been reported. Moreover, subcutaneous administration of casirivimab and imdevimab has been associated with injection site reactions, including bruising and redness[70].

EMERGING MONOCLONAL ANTIBODIES

The landscape of mAb therapies for COVID-19 is rapidly evolving. While initial mAbs effectively targeted the original SARS-CoV-2 strain, the emergence of new variants has necessitated the development of next-generation mAbs with enhanced potency and broader spectrum of activity. These novel mAbs are being designed to neutralize multiple SARS-CoV-2 variants, including those with mutations that confer resistance to earlier therapies[71]. Additionally, researchers are exploring mAbs that can modulate the host immune response to COVID-19, potentially reducing disease severity and improving patient outcomes[72].

Next-Generation

The development of mAbs targeting conserved regions of the SARS-CoV-2 virus has emerged as a promising strategy to combat the ongoing pandemic. By focusing on these regions, researchers aim to develop mAbs that are less susceptible to viral mutations and can effectively neutralize a broader range of variants. This approach has the potential to provide long-lasting protection against future outbreaks and variants of concern[73].

Bispecific and Multispecific

A promising avenue in the development of SARS-CoV-2 therapeutics involves the use of innovative antibody-based strategies. Bispecific and multispecific antibodies, which can simultaneously bind to multiple epitopes on the SARS-CoV-2 spike protein, offer a compelling approach to neutralize the virus effectively. By targeting multiple epitopes, these antibodies can reduce the risk of viral escape mutations, which can render conventional monoclonal antibodies ineffective[74].

Long-Acting

Many emerging mAbs are being developed with extended half-lives, offering several advantages. By increasing the duration of their presence in the bloodstream, these mAbs can provide longer-lasting protection against diseases and reduce the frequency of dosing. This is especially beneficial for vulnerable populations, such as the elderly, immunocompromised individuals, and those with chronic conditions, who may require ongoing prophylaxis to prevent infections or disease progression[75].

Enhanced Delivery Mechanisms

To enhance patient convenience and compliance, significant efforts are being directed towards developing innovative delivery mechanisms for mAbs. By formulating mAbs for intramuscular or subcutaneous injection, healthcare providers can administer these therapies outside of hospital settings, reducing the burden on patients and healthcare systems. These advancements have the potential to revolutionize the treatment of various diseases, making mAb therapy more accessible and effective[76].

Combination Therapies

Combination therapy with mAbs and antiviral drugs offers a multifaceted approach to combat viral infections. By targeting different stages of the viral life cycle, such as viral entry, replication, and release, these therapies can disrupt multiple aspects

of viral pathogenesis. This can lead to enhanced antiviral activity and potentially prevent the emergence of drug-resistant viral strains[77].

Broad-Spectrum

Broad-spectrum antibodies offer a promising avenue for combating future coronavirus outbreaks. These antibodies are designed to target conserved regions of the viral spike protein, enabling them to neutralize a wide range of coronaviruses, including SARS-CoV-2. By developing and deploying broad-spectrum antibodies, we can potentially enhance our preparedness for future pandemics and reduce the impact of emerging viral threats[14,78].

Adaptive mAb Platforms

Adaptive mAb platforms represent a promising approach to counter the rapid evolution of viruses like SARS-CoV-2. By utilizing advanced technologies such as phage display and yeast display, these platforms enable the rapid generation of new mAbs with enhanced affinity and specificity for emerging viral variants. This accelerated development process can significantly reduce the time required to develop effective therapeutics and vaccines[79]. Furthermore, adaptive mAb platforms can be tailored to target specific viral epitopes, making them less susceptible to viral escape mutations. As viral pathogens continue to evolve, the development and optimization of adaptive mAb platforms will be crucial for maintaining effective public health strategies[80].

Clinical Trials and Regulatory Pathways

Several mAbs are currently in various stages of clinical trials to evaluate their safety and efficacy against COVID-19. These mAbs target specific viral proteins, neutralizing the virus and preventing infection. Regulatory agencies worldwide are actively working to expedite the approval process for these promising therapies, recognizing the urgent need for effective treatments to combat the ongoing COVID-19 pandemic[14,30].

FUTURE OF MONOCLONAL ANTIBODIES

The potential of mAbs in treating a wide range of diseases is substantial, and ongoing research aims to further optimize their use. While mAbs have proven to be effective therapeutic agents, challenges such as complex manufacturing processes, immunogenicity, and administration routes remain. To address these limitations, researchers are actively exploring innovative strategies to enhance mAb production, improve delivery methods, and reduce adverse immune responses.

A promising strategy involves the development of antibody-drug conjugates (ADCs), which combine the specificity of antibodies with the potency of cytotoxic drugs. ADCs target specific cells, delivering the cytotoxic payload directly to cancer cells while minimizing damage to healthy tissues. This targeted approach offers significant advantages over traditional chemotherapy, which often leads to systemic toxicity. By selectively eliminating cancer cells, ADCs can reduce adverse side effects and improve patient quality of life[81,82].

A recent advancement in mAb therapy involves bispecific antibodies, which possess two distinct antigen-binding sites. This unique characteristic enables them to target multiple antigens simultaneously, often directing cytotoxic T cells to eliminate malignant cells. While these innovative therapies hold immense promise, their clinical application is currently limited to patients who have exhausted standard-of-care treatment options. This limitation arises from rigorous regulatory processes and the need for extensive clinical trials to establish safety and efficacy profiles. Although promising data has emerged from various studies, large-scale, head-to-head comparisons against standard-of-care treatments are still awaited. Nonetheless, the potential of bispecific antibodies to revolutionize cancer therapy is undeniable, potentially rendering more invasive treatments like stem cell transplants obsolete.

CONCLUSION

Monoclonal antibodies have emerged as a promising therapeutic strategy for COVID-19, despite the inherent challenges associated with their development and production. Over the past three decades, mAbs have successfully treated various diseases, demonstrating their efficacy and ease of administration. Their potential application in COVID-19 treatment is

particularly compelling, as they can be derived from recovered patients, providing a rapid and targeted approach. Currently, over 70 mAbs are undergoing clinical trials, promising a swift response to future outbreaks. While vaccines offer long-term protection, mAbs can provide immediate relief and temporary immunity, especially for vulnerable populations like the elderly and immunocompromised individuals. By complementing vaccination strategies, mAbs can offer a comprehensive approach to combatting COVID-19 and future pandemics.

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AUTHORS' CONTRIBUTION

Conceptualization: Saurabh Nimesh, Pratibha Kumari, Md. Iftekhar Ahmad
Data curation: Saurabh Nimesh
Formal analysis: Saurabh Nimesh, Pratibha Kumari, Rohit Kumar, Gosiya, Md. Quamuddin, Md. Iftekhar Ahmad
Funding acquisition: -
Investigation: Saurabh Nimesh, Pratibha Kumari, Rohit Kumar, Gosiya, Md. Quamuddin, Md. Iftekhar Ahmad
Methodology: Saurabh Nimesh
Project administration: -
Resources: Saurabh Nimesh
Software: -
Supervision: Saurabh Nimesh, Pratibha Kumari, Md. Iftekhar Ahmad
Validation: Saurabh Nimesh
Visualization: Saurabh Nimesh
Writing - original draft: Saurabh Nimesh, Rohit Kumar, Gosiya, Md. Quamuddin
Writing - review & editing: Saurabh Nimesh, Md. Iftekhar Ahmad

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this study.

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Mini Review

Post-COVID Mucormycosis: An Emerging Threat in Developing Countries - A Prospective Review

Debpratim Chakraborty [1*]

Sudipa Adhikary [2]

[1] Department of Pharmaceutical Technology, Jadavpur University, Kolkata, West Bengal, India
[2] Department of Pharmaceutical Technology, Maulana Abul Kalam Azad University of Technology, Kolkata, West Bengal, India

*email: debpratim008@gmail.com; phone: +919163138323

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Abstract

COVID-19, with its rapidly mutating strains, poses a significant global health challenge. Recent reports of a surge in mucormycosis cases among COVID-19 patients highlight the urgent need for understanding and addressing this critical complication. This review explores the factors contributing to mucormycosis development in COVID-19 patients and outlines strategies for prevention and management. Several factors, including high glucose levels (diabetes, onset, steroid-induced hyperglycemia), low oxygen levels, elevated iron levels (especially ferritin), metabolic acidosis, and diabetic ketoacidosis, can facilitate the germination of mucor spores. COVID-19 patients with underlying conditions such as diabetes, cancer, or organ transplants are particularly susceptible to mucormycosis due to their immunocompromised state. The growth of the mucor pathogen requires free iron, which is elevated in conditions like diabetic ketoacidosis. This elevated iron level promotes the formation of Cot-H, a crucial component of fungal growth, leading to mucormycosis. Additionally, comorbidities and corticosteroids can suppress the immune system, hindering the body's ability to fight off infections like mucormycosis. Therefore, it is imperative to avoid the indiscriminate use of corticosteroids. Strict control of acute hyperglycemia and comprehensive monitoring of diabetic and immunocompromised COVID-19 patients are essential preventive measures. By addressing these factors, healthcare providers can mitigate the risk of mucormycosis in COVID-19 patients and improve overall outcomes.

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INTRODUCTION

The term "corona" is derived from the Greek word for "crown," aptly describing the appearance of the virus under a microscope: a spherical particle adorned with a crown-like structure of protein spikes, known as peplomers[1]. These peplomers enable the virus to attach to and infect host cells. Throughout history, humanity has faced numerous pandemics, including the Bubonic Plague (1665), the Spanish Flu (1918-1919), the Asian Flu (1957), the Hong Kong Flu (1968), the Swine Flu (2009), and, more recently, the COVID-19 pandemic, declared by the WHO on February 11, 2020[2]. The novel coronavirus responsible for COVID-19 was first identified in Wuhan, Hubei Province, China, in late 2019[3].

Mucormycosis, a rare but potentially fatal fungal infection, was first described by Paltauf in 1885 as phycomycosis[4] and later as mucormycosis by Baker in 1957[5]. This opportunistic infection is caused by a group of fungi, primarily from the order Mucorales, commonly found in the environment, including soil, decaying organic matter, and even the human microbiome[6]. In recent years, there has been a surge in mucormycosis cases, particularly among individuals recovering from COVID-19[7,8]. This mini review aims to explore the factors contributing to the increased incidence of mucormycosis in COVID-19 patients and to discuss preventive measures to mitigate this risk.

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PREVALENCE OF COVID-19

The COVID-19 pandemic has led to a global health crisis, with millions of deaths reported to the WHO[9]. However, the actual number of deaths may be significantly higher, particularly in countries like India, where the pandemic has been exacerbated by the emergence of mucormycosis. While the global prevalence of mucormycosis is relatively low, India has experienced a dramatic surge in cases, with a prevalence rate 80 times higher than developed countries[10,11].

Diabetes mellitus is the most common risk factor for mucormycosis in India, whereas cancer and organ transplantation are leading risk factors in Europe and the United States[12]. However, diabetes remains a significant risk factor globally, contributing to a high mortality rate of 46%[13]. Several factors, including high glucose levels, low oxygen levels, high iron levels, metabolic acidosis, and immunosuppression due to steroid use or underlying conditions like cancer or HIV/AIDS, can predispose individuals to mucormycosis[14]. The most common site of infection is the rhino-maxillary region, particularly in immunocompromised individuals with diabetes or those receiving steroid therapy[15].

MUCORMYCOSIS CASES OF COVID-19 PATIENTS

Recent studies have highlighted a concerning association between COVID-19 infection, uncontrolled diabetes mellitus, and subsequent mucormycosis. For instance, a case report involving a 60-year-old diabetic patient who tested positive for COVID-19 and received treatment with methylprednisolone, oseltamivir, and meropenem eventually developed mucormycosis, as confirmed by nasal biopsy. Similarly, a larger study involving 18 patients with poorly controlled diabetes and a history of steroid use found that 17 (94%) were diagnosed with mucormycosis[16-18]. These findings underscore the importance of vigilant monitoring for mucormycosis in high-risk individuals, particularly those with diabetes and COVID-19 (**Table I**).

Several case reports and studies have highlighted the association between COVID-19 infection and mucormycosis. For instance, a 40-year-old woman and a 54-year-old man with severe COVID-19 infection and corticosteroid treatment developed mucormycosis, confirmed by nasal endoscopy and radiological findings[19]. Similarly, a study involving 25 patients with invasive mucormycosis revealed that 11 of them had concurrent COVID-19 infection, suggesting a potential link between the two diseases, particularly in immunocompromised individuals[20]. A middle-aged diabetic woman with COVID-19 and facial pain, ptosis, and fever was diagnosed with mucormycosis through sinus endoscopy and histopathological examination[21]. A comprehensive review of 101 reported cases of mucormycosis in COVID-19 patients identified diabetes and corticosteroid use as significant risk factors[14].

Table I. Mucormycosis Cases of COVID-19 Patients.

No.	Description	Diabetes	Use of Corticosteroids	COVID-19 Positive	Mucormycosis	Treatment for Mucormycosis	References
1	60-year-old patient	Yes	Yes	Yes	Yes	Amphotericin B	16
2	Study of 18 COVID-19 positive patients	Yes	Yes	Yes	17/18 patients	-	18
3	40-year-old female and 54-year-old male patients	No	Yes	Yes	Yes	Amphotericin B	19
4	Study of 25 patients	No	Yes	11/25 patients	All 25 patients	-	20
5	Middle-aged woman	Yes	Yes	Yes	Yes	Amphotericin B	21
6	Study of 101 patients with COVID-19 and mucormycosis	80/101 patients	77/101 patients	Yes	Yes	Amphotericin B	14
7	3 patients (2 male, 1 female)	Yes	No	No	Yes	Surgery	22
8	Study of 2 male patients aged 60 and 67 years	Yes	No	No	Yes	Amphotericin B	23

CURRENT SITUATION OF MUCORMYCOSIS AND COVID-19

The rapid and ongoing spread of COVID-19, coupled with its high mutation rate, has presented a significant global health challenge. The virus's ability to evolve quickly has hindered efforts to control its transmission and develop effective countermeasures. The SARS-CoV-2 virus has undergone various mutations, leading to the emergence of several variants of concern. Notably, the B.1.617.1 (kappa) and B.1.617.2 (delta) variants were first identified in India[24]. Other significant variants include B.1.1.7 (alpha) from the UK, B.1.35.1 (beta) from Africa, P.1 (gamma) from Brazil, B.1.427/B.1.429 (epsilon) from the USA, P2 (zeta) from Brazil, and B.1.525 (eta) from multiple countries[25]. Given the rapid emergence of COVID-19, the development of a specific drug within a short timeframe has proven challenging. As such, non-pharmaceutical interventions like lockdowns and vaccination have been the primary strategies for preventing the spread of the virus and mitigating its impact. While vaccines cannot directly eliminate the virus, they effectively stimulate the immune system to generate a robust response, providing protection against infection and severe disease[26].

Several vaccines, including BNT162b2/Comirnaty Tozinameran (Pfizer), AZD1222 (AstraZeneca), Covishield (Serum Institute of India), Ad26.Cov2.5 (Janssen), mRNA-1273 (Moderna), SARS-CoV-2 (Vero cell) (Sinopharm/BIBP), and Sputnik V (Gamaleya National Centre), have received regulatory approval, while numerous others are under review or in clinical trials[27]. While vaccination remains a primary strategy to combat COVID-19, antibody-based therapies have emerged as a promising complementary approach. Monoclonal antibodies targeting the SARS-CoV-2 spike protein can neutralize the virus and prevent infection. However, the emergence of viral variants with mutations in the spike protein can reduce the effectiveness of these therapies. To address this challenge, researchers are exploring the development of antibody cocktails that target multiple epitopes on the viral surface, thereby reducing the likelihood of viral escape. This strategy has shown promise in preclinical and clinical studies and is now gaining increasing attention from scientists and clinicians[28,29].

The global health crisis caused by COVID-19 has been further exacerbated by the emergence of secondary infections, such as mucormycosis[30]. In countries like India, a significant number of COVID-19 patients have experienced severe complications due to mucormycosis[6]. This opportunistic fungal infection primarily affects individuals with underlying health conditions, including diabetes, metabolic acidosis, and diabetic ketoacidosis. Patients with elevated blood glucose levels, low oxygen saturation, and high iron levels are particularly susceptible to mucormycosis[31,32].

Iron is an essential element for both mammalian cells and pathogens[33]. In the mammalian host, iron is tightly bound to carrier proteins such as transferrin, ferritin, and lactoferrin to prevent its toxic effects. However, pathogens, including fungi, require free iron for their growth and proliferation. Diabetic ketoacidosis (DKA) can disrupt iron homeostasis by lowering the iron-binding capacity of carrier proteins, leading to increased levels of free iron in the serum. This elevated free iron concentration can provide a favorable environment for fungal growth and infection[34-36].

A compromised immune system, often resulting from long-term steroid use, cancer, organ transplantation, or other underlying health conditions, can increase susceptibility to mucormycosis[37]. While the precise pathophysiology of mucormycosis remains unclear, a proposed mechanism linking COVID-19 to mucormycosis is illustrated in **Figure 1**. The overwhelming demand for oxygen during the COVID-19 pandemic, particularly in India, led to a shortage of medical-grade oxygen cylinders. Consequently, hospitals were forced to use industrial-grade cylinders, which may have been inadequately cleaned and sterilized. This potential contamination with fungal spores could have contributed to the increased incidence of mucormycosis among COVID-19 patients[38].

While the definitive treatment for this condition remains unclear, current literature suggests that amphotericin B and surgical intervention may be potential therapeutic options[39-41]. Additionally, certain homeopathic remedies, such as arsenic album, marksol, gelsenium, nux vomica, sulphur, tuberculinum, and pulsatilla, have been reported to offer potential therapeutic benefits[42,43]. However, further rigorous scientific studies are needed to validate the efficacy and safety of these treatments.

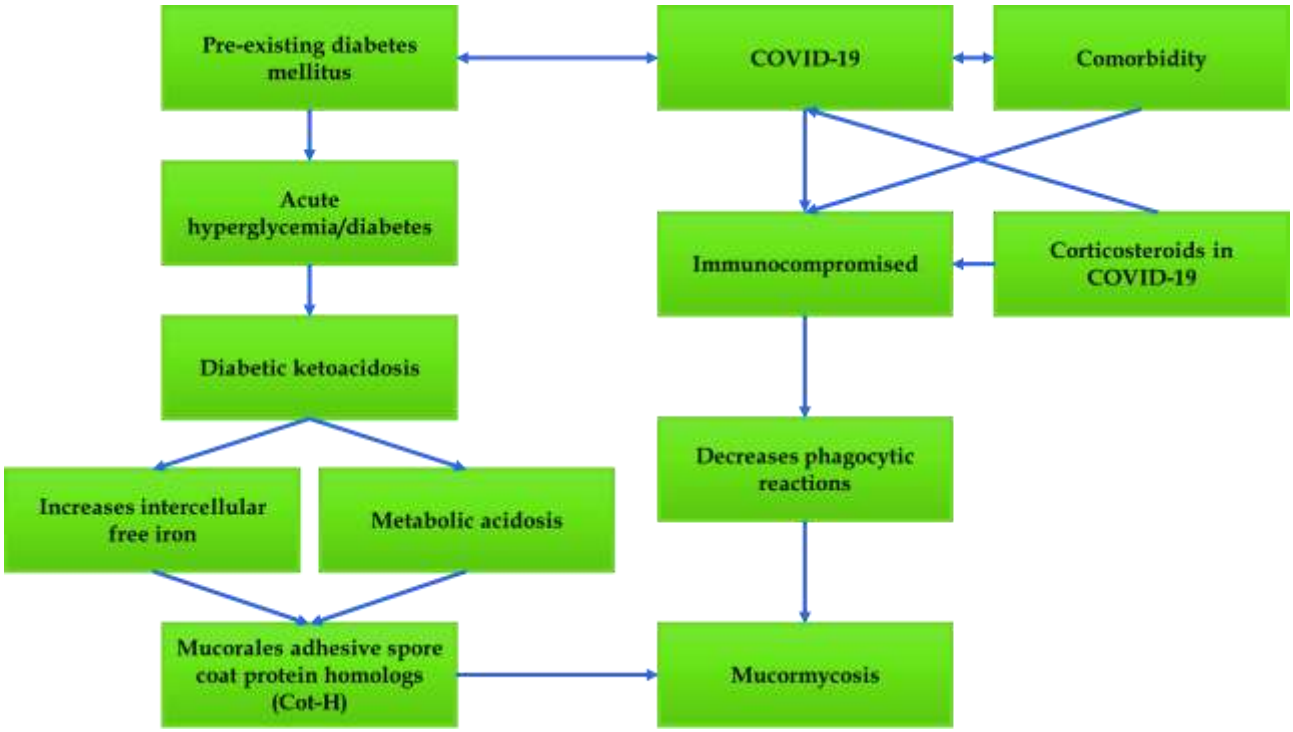

Figure 1. Pathogenesis of mucormycosis in COVID-19 patients.

CONCLUSION

the global COVID-19 pandemic has underscored the importance of comprehensive infection control measures and timely treatment of opportunistic infections like mucormycosis. To mitigate the risk of mucormycosis, it is crucial to minimize the use of corticosteroids, especially in immunocompromised individuals, and to strictly control blood glucose levels in diabetic patients. Early diagnosis and prompt initiation of appropriate antifungal therapy, such as liposomal amphotericin B, posaconazole, or isavuconazole, are essential for improving patient outcomes. Additionally, surgical intervention may be necessary to debride necrotic tissue. Ongoing research is needed to develop novel antifungal agents with improved efficacy and safety profiles, as well as to explore preventive strategies to reduce the incidence of mucormycosis in high-risk populations.

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AUTHORS' CONTRIBUTION

Conceptualization: Debpratim Chakraborty
Data curation: Sudipa Adhikary
Formal analysis: Sudipa Adhikary
Funding acquisition: -
Investigation: Sudipa Adhikary

Methodology: Debpratim Chakraborty
Project administration: -
Resources: Debpratim Chakraborty, Sudipa Adhikary
Software: -
Supervision: Debpratim Chakraborty
Validation: -
Visualization: Debpratim Chakraborty
Writing - original draft: Debpratim Chakraborty, Sudipa Adhikary
Writing - review & editing: Debpratim Chakraborty, Sudipa Adhikary

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this study.

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Research Article

Polyphenol Content and Antioxidant Evaluation of Kawista (*Limonia acidissima*) Leaf Extract

Muammar Fawwaz *

Mamat Pratama

Rais Razak

Yusrianti Andika

Elvina Astika

Feralia

Laboratory of Pharmaceutical Chemistry, Universitas Muslim Indonesia, Makassar, South Sulawesi, Indonesia

*email: muammar.fawwaz@umi.ac.id; phone: +6282125556303

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Abstract

Kawista or *Limonia acidissima*, a plant traditionally used by the Bima community, offers potential health benefits. This study aimed to evaluate the antioxidant potential of ethanol extract from *L. acidissima* leaves by determining total flavonoid content (TFC), total phenolic content (TPC), and antioxidant activity using DPPH, FRAP, and CUPRAC assays. The ethanol extract exhibited significant antioxidant activity, with IC_{50} values of 10.445 and 135.42 µg/mL for DPPH and CUPRAC assays, respectively. TPC and TFC were determined to be 14.63 mgGAE/g extract and 113.9 mgQE/g extract, respectively. These findings suggest that *L. acidissima* leaf extract possesses potent antioxidant properties, which may be attributed to its flavonoid and phenolic content. Further research is warranted to explore this plant extract's underlying mechanisms of action and potential therapeutic applications.

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INTRODUCTION

Antioxidants are vital compounds that act as a defense mechanism against oxidative stress by neutralizing harmful reactive oxygen species (ROS) in the body[1]. These compounds can be classified as endogenous, produced within the body, or exogenous, obtained from external sources. Exogenous antioxidants are abundant in natural sources such as fruits, vegetables, and whole grains[2]. Recognizing the importance of natural antioxidants, this study focuses on the kawista (*Limonia acidissima*) fruit, a plant traditionally used in Bima Regency, Indonesia, to treat various ailments.

Previous studies have demonstrated that *L. acidissima* fruit possesses antioxidant properties, and it is traditionally utilized as a fever reducer, tonic, and remedy for stomach ailments. Furthermore, the fruit skin of *L. acidissima* has also been shown to exhibit antioxidant activity, attributed to the presence of various phytochemicals such as phenolic compounds, flavonoids, steroids, saponins, tannins, and alkaloids[3]. These secondary metabolites possess antioxidant properties due to their ability to donate electrons to neutralize free radicals, effectively stabilizing these reactive species[4]. Beyond its antioxidant potential, *L. acidissima* fruit has shown promise as an anti-diabetic agent, while the leaves have been reported to exhibit hepatoprotective properties[3].

Building upon these empirical observations, this research will investigate the phytochemical profile of *L. acidissima* leaves. Specifically, this study aims to quantify the levels of total flavonoids and phenolics in ethanolic extracts of *L. acidissima* leaves and subsequently evaluate their antioxidant potential. This research will contribute to a better understanding of the phytochemical composition and potential health benefits of *L. acidissima* leaves. Previous studies have employed various methods to evaluate antioxidant potential, including radical scavenging assays[5,6]. This study investigated the antioxidant activity of ethanolic extracts of *L. acidissima* leaves using three complementary assays: 1,1-diphenyl-2-picrylhydrazyl (DPPH)

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radical scavenging, ferric reducing antioxidant power (FRAP), and cupric reducing antioxidant capacity (CUPRAC). All assays were conducted using UV-visible spectrophotometry for quantitative analysis.

MATERIALS AND METHODS

Materials

This study employed analytical instruments including micropipettes (OneMed®), an oven (Memmert®), a pH meter (Jenco®), a centrifuge (OneMed®), a UV-visible spectrophotometer (Aple®), an analytical weighing scale (KERN®), and a vortex mixer (IKA® Vortex Genius 3). All glassware used was of analytical grade (Pyrex®). Analytical-grade chemicals and reagents were procured from commercial sources. Fresh leaves of *L. acidissima* (**Figure 1**), obtained from Bima Regency, Indonesia, were used in this study. The botanical identification of the plant material (specimen No. KW.001.13032024) was confirmed by the Division of Botany, Laboratory of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Muslim Indonesia, Makassar, Indonesia. The reference standards, including quercetin, ascorbic acid, and gallic acid, were obtained from Merck Co. (Darmstadt, Germany). Reagents for antioxidant evaluation, such as DPPH, FRAP, and CUPRAC reagents, were purchased from Sigma-Aldrich (Singapore). Deionized water was produced using a Millipore-Q50 Ultrapure water system (Sartorius). Stock solutions of the reference standards (1000 µg/mL) were prepared by dissolving 10 mg of each standard in 10 mL of ethanol.

Figure 1. *Limonia acidissima* leaves from Bima Regency.

Methods

Extraction

Fresh leaves of *L. acidissima* were meticulously washed with deionized water and subsequently air-dried under ambient conditions. After thorough drying, the leaves were meticulously sorted and then meticulously cut into small pieces using a clean, sterile instrument. The powdered leaf material was obtained by grinding the dried leaf pieces to a fine powder using

a suitable grinder. A total of 100 g of this powdered material was subjected to maceration using 1000 mL of 96% ethanol at room temperature for a duration of three days. The resulting ethanolic extract was meticulously filtered to remove any particulate matter and subsequently concentrated under reduced pressure using a rotary evaporator. The concentrated extract was further dried in a vacuum desiccator to obtain a dry extract. The yield of the extraction process was calculated using **Equation 1**.

$$\%yield = \frac{Total\ weight\ of\ extract\ (g)}{Total\ weight\ of\ sample\ (g)} \text{x} 100\% \quad [1]$$

Qualitative analysis of flavonoids and phenolics

Initially, 5 mg of ethanolic extracts of *L. acidissima* leaves were mixed with 96% ethanol to prepare a stock solution. This mixture was then heated and subsequently filtered. To detect the presence of flavonoids, 1 mL of concentrated HCl, 1 mL of amyl alcohol, and magnesium metal were added to the extract solution. The formation of a yellow color indicated the presence of flavonoids. Furthermore, the presence of phenolic compounds was confirmed by adding 1% $FeCl_3$ to the extract solution. A green-to-black coloration was observed as a positive indicator for phenolics[7].

Determination of total flavonoid content (TFC)

The TFC of the ethanolic extracts of *L. acidissima* leaves was determined using the aluminum chloride colorimetric method, adapted from previously published protocols[8,9]. A standard curve was generated using varying concentrations of quercetin (6, 8, 10, 12, and 14 µg/mL). To each 1 mL quercetin concentration, 1 mL of 2% $AlCl_3$ and 1 mL of 120 mM potassium acetate were added. The mixtures were incubated at room temperature for 30 minutes, followed by absorbance measurement at 449 nm using a UV-Vis spectrophotometer. A linear regression analysis ($y = bx + a$) was performed to determine the best fit for the quercetin standard curve.

Determination of total phenolic content (TPC)

The TPC of the ethanolic extracts of *L. acidissima* leaves was determined using the Folin-Ciocalteu method with slight modifications[10]. A standard curve was prepared using various concentrations of gallic acid (6, 8, 10, 12, and 14 µg/mL). To each 1 mL gallic acid standard, 1 mL Folin-Ciocalteu reagent was added, followed by 1 mL 7% sodium carbonate. After 5 minutes of incubation at room temperature, the absorbance was measured at 752 nm using a UV-Vis spectrophotometer. The absorbance values were plotted against gallic acid concentrations, and a linear regression equation was generated to obtain the standard curve.

Evaluation of antioxidant activity by DPPH assay

The antioxidant activity of the ethanolic extracts of *L. acidissima* leaves was determined using the DPPH radical scavenging assay with slight modifications[6,11]. Quercetin (1000 µg/mL) was used as a positive control and diluted to prepare a concentration series (1, 2, 3, 4, 5, and 6 µg/mL). Briefly, 1 mL of each quercetin concentration or ethanolic extracts of *L. acidissima* leaves (10, 20, 30, 40, and 50 µg/mL) was mixed with 4 mL of DPPH solution (35 µg/mL) in a glass test tube. The mixture was transferred to a disposable polystyrene cuvette and incubated at 37°C for 30 minutes. The absorbance of the reaction mixture was measured at 517 nm using a UV-visible spectrophotometer. A blank sample containing methanol (2 mL) and DPPH solution (1 mL) was prepared similarly. All samples were prepared in triplicate. To minimize photodegradation of the DPPH radical, all samples were protected from light until analysis[6,11].

Evaluation of antioxidant activity by FRAP assay

Antioxidant activity was determined using the FRAP assay, modified from previously published methods[9,12]. Briefly, a standard curve was generated using quercetin at concentrations of 10, 15, 20, 25, and 30 µg/mL. Each quercetin standard (1 mL) was mixed with 1 mL of 0.2 M phosphate buffer (pH 6.6), 1 mL of potassium ferricyanide, and incubated at 50°C for 20 minutes. Subsequently, 1 mL of trichloroacetic acid, 1 mL of deionized water, and 0.5 mL of ferric chloride were added to the mixture. After centrifugation at 3000 rpm for 10 minutes, the absorbance of the supernatant was measured at 744 nm using a UV-visible spectrophotometer. The same procedure was performed in triplicate for the ethanolic extracts of *L. acidissima* leaves at a concentration of 1000 µg/mL. The absorbance values from the quercetin standard curve were used to construct a linear regression equation.

Evaluation of antioxidant capacity by CUPRAC assay

The antioxidant capacity of the ethanolic extracts of *L. acidissima* leaves was determined using the CUPRAC assay with slight modifications[13]. Ascorbic acid served as the positive control. A stock solution of 1000 µg/mL ascorbic acid was prepared by dissolving 2.5 mg of ascorbic acid in 1% oxalic acid. Serial dilutions were then made to obtain concentrations of 100, 150, 200, 300, and 350 µg/mL. To each concentration of ascorbic acid and the ethanolic extracts of *L. acidissima* leaves (50, 100, 150, 200, 300, and 400 µg/mL), 1 mL of neocuproine reagent, 1 mL of ammonium acetate buffer (pH 7), and 1 mL of ethanol were added. The mixtures were incubated for 30 minutes at room temperature. Subsequently, the absorbance of each sample was measured at 450 nm using a UV-Visible spectrophotometer. In the CUPRAC assay, antioxidant compounds reduce Cu^{2+} to Cu^{+}, which then forms a chromophore with neocuproine, resulting in the formation of a yellow-colored complex.

Data analysis

The TFC of the ethanolic extracts of *L. acidissima* leaves was determined by plotting its absorbance on the quercetin standard curve and expressing the result as mg of quercetin equivalents (mgQE) per g of extract (mgQE/g) using **Equation 2**. This procedure was repeated three times for each ethanolic extracts of *L. acidissima* leaves sample to ensure reproducibility. The TPC of the ethanolic extracts of *L. acidissima* leaves was determined by measuring the absorbance of the ethanolic extracts of *L. acidissima* leaves samples in triplicate and interpolating the values onto the gallic acid standard curve. The results were expressed as mg of gallic acid equivalents (mgGAE) per g of extract (mgGAE/g) using **Equation 3**[9,10].

$$Total\ flavonoid\ content\ (mgQE/g) = \frac{Sample\ volume\ (L) \times Initial\ concentration\ (mg/L)}{Extract\ weight\ (g)} \quad [2]$$

$$Total\ phenolic\ content\ (mgGAE/g) = \frac{Sample\ volume\ (L) \times Initial\ concentration\ (mg/L)}{Extract\ weight\ (g)} \quad [3]$$

The percentage of DPPH radical scavenging activity was calculated using the following **Equation 4**, in which ACO is the absorbance of the control (DPPH solution alone) at t=0 and AAT is the absorbance of the sample at t=30 minutes. The antioxidant activity of ethanolic extracts of *L. acidissima* leaves was expressed as mgQE/g using the following **Equation 5**. The antioxidant activity of the ethanolic extracts of *L. acidissima* leaves was expressed as the EC_{50} value, which represents the concentration of the extract required to inhibit the reduction of Cu(II) by 50%. The EC_{50} values were determined from the calibration curve generated using the ascorbic acid standards[14] and calculated using **Equation 6**.

$$\%inhibition = \frac{(A_{CO} - A_{AT})}{A_{CO}} \text{x} 100\% \quad [4]$$

$$Antioxidant\ capacity\ (mgQE/g) = \frac{Sample\ volume\ (L) \times Initial\ concentration\ (mg/L)}{Extract\ weight\ (g)} \quad [5]$$

$$\%capacity = (1 - T)\ \text{x}\ 100\% \quad [6]$$

RESULTS AND DISCUSSION

Extraction of *L. acidissima* leaves was performed using the maceration method with 96% ethanol. A yield of 11.83% (w/w) of ethanolic extract of *L. acidissima* leaves was obtained from 200 g of dried leaf powder. Qualitative phytochemical screening revealed the presence of flavonoids, evidenced by the formation of a yellow color upon reaction with $AlCl_3$. Furthermore, the presence of phenolic compounds was confirmed by the green color reaction with $FeCl_3$. Quantitative analysis of TFC and TPC was conducted by measuring the absorbance of the ethanolic extract of *L. acidissima* leaves and comparing the values to standard curves generated from quercetin and gallic acid, respectively. The absorbance data and the corresponding TFC and TPC levels are summarized in **Table I**.

The DPPH radical scavenging assay is a well-established and widely utilized method for evaluating the antioxidant capacity of various compounds, including flavonoids, ascorbic acid, peptides, and phenolic compounds[5]. This method leverages the stable free radical nature of DPPH, offering several advantages such as simplicity, cost-effectiveness, and rapid analysis compared to other antioxidant assays. In its stable form, DPPH exhibits a characteristic deep purple color. However, upon

interaction with an antioxidant compound, DPPH undergoes reduction, resulting in a color change from deep purple to yellow[15]. This color change signifies the antioxidant's ability to donate either a hydrogen atom or an electron to the DPPH radical[16].

Spectrophotometry is a well-established technique for assessing color changes and evaluating antioxidant activity[17]. In this study, the DPPH radical scavenging activity was measured at 515 nm, its maximum absorbance wavelength. The IC_{50} value, representing the concentration of the extract required to inhibit 50% of DPPH radicals, was determined to quantify antioxidant potency. A lower IC_{50} value generally indicates higher antioxidant activity[18]. Based on established criteria, antioxidant activity can be categorized as very strong (IC_{50} <50 µg/mL), strong (50-100 µg/mL), moderate (100-250 µg/mL), and weak (250-500 µg/mL)[19]. The ethanolic extract of *L. acidissima* leaves exhibited potent antioxidant activity with an IC_{50} value of 10.445 µg/mL, classifying it as a very strong antioxidant. However, quercetin, a potent antioxidant flavonoid, demonstrated slightly higher antioxidant activity with an IC_{50} of 6.207 µg/mL, as shown in **Table II**. This observation is expected, considering quercetin is a pure compound with well-documented antioxidant properties. A previous study reported that *L. acidissima* fruit possesses antioxidant activity, exhibiting an IC_{50} value of 41.35 g/mL in a DPPH radical scavenging assay[20]. These findings suggest that the antioxidant potential of ethanolic extract of *L. acidissima* leaves surpasses that of *L. acidissima* fruit, indicating a promising avenue for further exploration of its potential as a natural antioxidant.

Table I. TFC and TPC level of ethanolic extract of *L. acidissima* leaves.

Analysis	Absorbance (449 nm)	Linearity regression	Initial concentration (mg/L)	TFC (mgQE/g)/ TPC (mgGAE/g)	Average (mgQE/g)/ (mgGAE/g)
TFC	0.326	$y = 0.0132x + 0.148$ (r = 0.990)	35.909	118.4	113.9
	0.333		36.439	115.6	
	0.306		34.393	107.0	
TPC	0.225	$y = 0.067x - 0.2621$ (r = 0.998)	7.270	14.25	14.63
	0.264		7.852	14.72	
	0.266		7.882	15.01	

Table II. Antioxidant activity of ethanolic extract of *L. acidissima* leaves by DPPH assay.

Analysis	Concentrations (µg/mL)	Absorbance (516 nm)	Inhibition (%)	IC_{50} (µg/mL)
Quercetin	1	0.668	26.187	6.207
	3	0.578	36.132	
	4	0.553	38.895	
	5	0.52	42.541	
	6	0.497	45.082	
Ethanolic extract of *L. acidissima* leaves	10	0.807	1.328	10.445
	20	0.79	3.207	
	30	0.671	16.356	
	40	0.589	25.417	
	50	0.452	40.555	

The antioxidant activity of ethanolic extract of *L. acidissima* leaves was evaluated using the FRAP assay. This assay measures the ability of the extract to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). The results, presented in **Table III**, demonstrate that ethanolic extract of *L. acidissima* leaves possesses significant antioxidant capacity, with an average value of 473.41 mgQE/g. This value is comparable to the antioxidant capacity of quercetin itself, as evidenced by the linear regression analysis of quercetin ($y = 0.0088x + 0.1204$, r = 0.997). Free radicals, characterized by unpaired electrons, are highly reactive species that can readily interact with cellular macromolecules such as lipids, proteins, and DNA, leading to oxidative damage. These reactive species are generated during normal cellular metabolism and can also be produced in response to external factors like air pollution and ultraviolet radiation[21,22]. The observed antioxidant activity of ethanolic extract of *L. acidissima* leaves can be attributed to the presence of polyphenols, which have been well-documented to possess strong antioxidant properties[23,24]. This finding suggests that ethanolic extract of *L. acidissima* leaves may have potential as a natural source of antioxidants for various applications.

The CUPRAC assay was employed to evaluate the antioxidant capacity of ethanolic extract of *L. acidissima* leaves by assessing its ability to reduce Cu^{2+} to Cu^{+} through free radical scavenging[25]. This method offers several advantages, including high selectivity, cost-effectiveness, and stability compared to other colorimetric assays. Notably, the CUPRAC assay is not susceptible to interference from environmental factors such as air, moisture, or sunlight[26]. Ascorbic acid served as a positive control in this study. The EC_{50} value for ethanolic extract of *L. acidissima* leaves was determined to be 135.42

µg/mL, which was significantly higher than that of ascorbic acid, indicating lower antioxidant activity. The detailed antioxidant activity of ethanolic extract of *L. acidissima* leaves and ascorbic acid is presented in **Table IV**.

Table III. Antioxidant activity of ethanolic extract of *L. acidissima* leaves by FRAP assay.

Analysis	Concentrations (µg/mL)	Absorbance (744 nm)	Antioxidant capacity (mgQE/g)	Average antioxidant capacity (mgQE/g)
Quercetin	10	0.214	-	-
	20	0.25	-	
	30	0.289	-	
	40	0.346	-	
	50	0.387	-	
Ethanolic extract of *L. acidissima*	1000	0.561	500.68	473.41
leaves	1000	0.564	504.09	
	1000	0.486	415.45	

Table IV. Antioxidant activity of ethanolic extract of *L. acidissima* leaves by CUPRAC assay.

Analysis	Concentrations (µg/mL)	Absorbance (516 nm)	Inhibition (%)	IC_{50} (µg/mL)
Ascorbic acid	350	0.594	73.023	20.43
	300	0.485	65.236	
	200	0.317	48.95	
	150	0.26	33.319	
	100	0.205	23.264	
Ethanolic extract of *L. acidissima* leaves	50	0.318	23.264	135.42
	100	0.298	46.912	
	150	0.42	59.913	
	200	0.541	69.661	
	300	0.663	77.091	
	400	0.799	83.251	

Limited data exists on the antioxidant activity of ethanolic extract of *L. acidissima* leaves. Therefore, this study compared the antioxidant activity of *L. acidissima* leaf extracts with that of *L. acidissima* fruit. Previous research has demonstrated antioxidant potential in *L. acidissima* fruit, with an IC_{50} value of 30.28 µg/mL determined using the ABTS method[20]. These findings suggest that *L. acidissima* fruit exhibits higher antioxidant activity compared to the leaf extracts evaluated in the present study.

CONCLUSION

The current study demonstrated that the ethanolic extract of *L. acidissima* leaves exhibits potent antioxidant activity, as evidenced by its strong scavenging ability against DPPH radicals. This antioxidant activity was comparable to that of quercetin, a well-known flavonoid with potent antioxidant properties. Further supporting these findings, the CUPRAC and FRAP assays also revealed significant antioxidant capacity of ethanolic extract of *L. acidissima* leaves. These results collectively suggest that ethanolic extract of *L. acidissima* leaves holds considerable promise as a potential source of natural antioxidants. However, further *in vivo* studies are crucial to validate these findings and assess the potential therapeutic applications of ethanolic extract of *L. acidissima* leaves.

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AUTHORS' CONTRIBUTION

Conceptualization: Muammar Fawwaz, Mamat Pratama, Rais Razak

Data curation: Yusrianti Andika, Elvina Astika, Feralia
Formal analysis: Muammar Fawwaz, Mamat Pratama, Rais Razak, Yusrianti Andika, Elvina Astika, Feralia
Funding acquisition: Muammar Fawwaz
Investigation: Yusrianti Andika, Elvina Astika, Feralia
Methodology: Muammar Fawwaz, Mamat Pratama, Rais Razak
Project administration: Muammar Fawwaz
Resources: Muammar Fawwaz, Mamat Pratama, Rais Razak
Software: -
Supervision: Muammar Fawwaz, Mamat Pratama, Rais Razak
Validation: Muammar Fawwaz, Mamat Pratama, Rais Razak
Visualization: -
Writing - original draft: Muammar Fawwaz, Yusrianti Andika, Elvina Astika, Feralia
Writing - review & editing: Muammar Fawwaz, Mamat Pratama, Rais Razak

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this study.

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Research Article

Formulation and Evaluation of Soursop (*Annona muricata*) Leaf Extract Nanoemulgel Against *Propionibacterium acnes*

Nabila Ikramina [1]

Rehana [1]

Rahmad Aji Prasetya [2]

Dhadhang Wahyu Kurniawan [1,3*]

[1] Department of Pharmacy, Universitas Jenderal Soedirman, Purwokerto, Central Java, Indonesia
[2] Department of Clinical Pharmacy, Akademi Farmasi Surabaya, Surabaya, East Java, Indonesia
[3] Graduate School, Universitas Jenderal Soedirman, Purwokerto, Central Java, Indonesia

*email: dhadhang.kurniawan@unsoed.ac.id; phone: +628112541628

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Abstract

Annona muricata (soursop) leaves are rich in antimicrobial compounds such as flavonoids, alkaloids, tannins, saponins, and phenols. This study aimed to develop a nanoemulgel formulation incorporating *A. muricata* leaf ethanol extract to enhance its efficacy against *Propionibacterium acnes*, a bacterium associated with acne vulgaris. Four nanoemulgel formulations containing varying concentrations of the extract (0%, 0.5%, 0.7%, and 1%) were prepared and evaluated for their physical properties (organoleptic, homogeneity, pH, spreadability, and viscosity) and stability through freeze-thaw cycles. The formulation with the highest extract concentration (Formula III) was selected for further characterization (particle size, morphology, and zeta potential) and antimicrobial testing against *P. acnes*. All formulations met the established physical property and stability criteria. Formula III exhibited a particle size of 20.5 nm and a zeta potential of 9.8 mV, indicating a stable nanoemulsion with well-dispersed particles. Antimicrobial testing revealed that Formula III demonstrated a strong inhibitory effect against *P. acnes*, with an average inhibition zone of 19.00 mm. These findings suggest that *A. muricata* leaf extract-loaded nanoemulgel has the potential to be a promising topical formulation for acne treatment. Further research is warranted to optimize the formulation and evaluate its efficacy in clinical settings.

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INTRODUCTION

Acne vulgaris is a common skin condition characterized by the inflammation of pilosebaceous units. *Propionibacterium acnes* plays a crucial role in the pathogenesis of acne by exacerbating inflammation within the hair follicles[1]. Conventional acne treatments often rely on topical formulations containing synthetic antibacterial agents, such as benzoyl peroxide, retinoids, and antibiotics. However, these agents can induce adverse side effects, including erythema, skin peeling, dryness, and burning sensations[2]. Consequently, there is a growing interest in exploring alternative therapeutic approaches utilizing natural ingredients. Natural remedies offer the potential for safer and more tolerable long-term acne management due to their generally lower risk of side effects[3].

Soursop (*Annona muricata*) has been traditionally recognized for its potential in treating acne. Previous studies have demonstrated that *A. muricata* leaves possess valuable pharmacological properties, including anti-inflammatory, antioxidant, and antibacterial activities[4]. Phytochemical analysis of *A. muricata* leaf ethanol extracts has revealed the presence of bioactive compounds such as flavonoids, saponins, tannins, and alkaloids, which are known to exhibit antibacterial properties[5-7]. Furthermore, the presence of phenols in these extracts contributes to their antioxidant activity[8]. *In vitro* studies

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have confirmed the antibacterial efficacy of *A. muricata* leaf ethanol extract against *P. acnes*, a key bacterium implicated in acne vulgaris, with significant zones of inhibition observed at 1% and 5% concentrations[9].

However, direct topical application of crude extracts can be challenging due to their often-greasy texture and potential for skin irritation. To overcome these limitations, formulating the extract into a nanoemulgel offers several advantages[10]. Nanoemulgels are characterized by their non-greasy nature, ease of spreading, water-solubility, enhanced stability, and transparent appearance, making them more cosmetically appealing and potentially more effective than conventional ointments and creams for topical delivery[11].

Nanoemulgels, colloidal systems comprising nanoemulsions incorporated within a gel matrix, offer a promising approach for transdermal drug delivery. Characterized by small droplet sizes typically ranging from 20 to 200 nm, nanoemulgels exhibit enhanced skin permeation and improved drug absorption compared to conventional emulsions[12,13]. However, a common limitation of nanoemulsions is their low viscosity, which can hinder their spreadability and stability[14]. This study aimed to address this challenge by formulating nanoemulgels incorporating *A. muricata* leaf extract. Four formulations were prepared, containing 0%, 0.5%, 0.7%, and 1% (w/w) of *A. muricata* leaf extract, respectively. These formulations were then characterized and evaluated for their antibacterial activity against *P. acnes*, a bacterium implicated in acne vulgaris.

MATERIALS AND METHODS

Materials

This study utilized *A. muricata* leaf powder obtained from CV. Lansida Group, Yogyakarta. Other materials employed included 70% ethanol (CV. C'Jaya Q-Mia, Purwokerto), isopropyl myristate (CV. C'Jaya Q-Mia, Purwokerto), surfactants such as Tween 80 and propylene glycol (both from PT. Brataco, Purwokerto), preservatives like methylparaben and propylparaben (both from PT. Brataco, Purwokerto), and a gelling agent, carbopol (CV. C'Jaya Q-Mia, Purwokerto), along with triethanolamine (PT. Brataco, Purwokerto). Paper discs (± 6 mm in diameter) were used for antimicrobial susceptibility testing. The microbiological media used were Mueller Hinton agar (MHA) (Oxoid®) and McFarland standard solution obtained from the Microbiology Laboratory, Faculty of Medicine, Universitas Jenderal Soedirman, Purwokerto, Indonesia. The study also utilized a clinical isolate of *P. acnes* obtained from the collection of the Microbiology Laboratory, Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, Indonesia.

Methods

Extraction

One kilogram of dried *A. muricata* leaves was subjected to maceration with 7 L of 70% ethanol (1 : 7 w/v ratio) for 72 hours at room temperature. The mixture was filtered after each 24-hour period, and the residue was re-macerated with fresh 70% ethanol for an additional 24 hours. The combined filtrates were then concentrated using a rotary evaporator under reduced pressure at 40°C to obtain a thick ethanolic extract of *A. muricata* leaves.

Nanoemulgel formulation and preparation

The formulation of *A. muricata* ethanol extract nanoemulgel is outlined in **Table I**. The oil phase was prepared by dissolving isopropyl myristate in Tween 80. Methylparaben and propylparaben were then incorporated into the oil phase and mixed thoroughly. The aqueous phase was prepared by combining propylene glycol with distilled water. Subsequently, the oil and water phases were mixed using a magnetic stirrer at 500 rpm and 50°C for 15 minutes to form a stable nanoemulsion. *Annona muricata* ethanol extract was then added to the nanoemulsion at varying concentrations and the mixture was stirred at 500 rpm and 50°C until homogenous. The resulting nanoemulsion was allowed to stand undisturbed for 24 hours to ensure clarity and the absence of bubbles.

Separately, 25 g of Carbopol 940 was dispersed in distilled water and neutralized with triethanolamine to achieve the desired pH range (as listed in **Table I**). The Carbopol 940 solution was then allowed to swell for 24 hours. Finally, the *A. muricata* ethanol extract-loaded nanoemulsion was incorporated into the swollen Carbopol 940 gel base using a magnetic stirrer at 500 rpm. Distilled water was added to a final volume of 100 mL, and the mixture was stirred until a homogenous gel was obtained.

Table I. Nanoemulsion formula composition.

Materials	Concentration (%)			
	F0	FI	FII	FIII
Annona muricata leaf ethanol extract	0	0.5	0.75	1
Isopropyl myristate	5	5	5	5
Tween 80	22.5	22.5	22.5	22.5
Propylene glycol	22.5	22.5	22.5	22.5
Methylparaben	0.2	0.2	0.2	0.2
Propylparaben	0.01	0.01	0.01	0.01
Triethanolamine	1.5	1.5	1.5	1.5
Carbopol 940	25	25	25	25
Distilled water	ad 100	ad 100	ad 100	ad 100

Physical properties examination

Physical characterization of the nanoemulgel encompassed a comprehensive evaluation of its properties, including organoleptic assessment (color, shape, and odor), homogeneity, pH, viscosity, spreadability, and stability through freeze-thaw cycles. These physical properties were assessed at predetermined time points: day 0, 7, 14, 21, and 28, to monitor any changes over time.

Organoleptic and homogeneity tests: Organoleptic evaluation was conducted to assess the physical characteristics of the nanoemulgel, including color, odor, and shape[15]. Homogeneity testing was performed to ensure the uniform dispersion of the formulation. Homogeneity was determined by visually inspecting a small sample of the nanoemulgel applied to a glass slide. The absence of visible particles, both coarse and fine, under gentle pressure indicated a homogeneous formulation[16,17].

pH test: The pH of the nanoemulgel was determined to ensure its compatibility with the skin. Skin pH typically ranges between 4 and 6[18]. To measure the pH, 2 g of the prepared nanoemulgel were accurately weighed and dispersed in 20 mL of distilled water in a beaker glass. The pH of the resulting solution was then measured using a calibrated pH meter[17].

Viscosity test: The viscosity of the nanoemulgel was determined using a rheometer. A specific volume of nanoemulgel was placed within the measuring chamber, and the viscosity was measured at 30 rpm using spindle 63. This method provides valuable information about the flow properties of the nanoemulgel, which is crucial for its subsequent application and stability[19].

Spreadability test: The spreadability of the nanoemulgel was evaluated to assess its ease of application and distribution on the skin. A quantity of 0.5 g of nanoemulgel was placed on a clean glass slide. A second glass slide was then placed on top and a weight of 150 g was applied for 1 minute. The diameter of the spread nanoemulgel was measured after the weight was removed. This method simulates the pressure exerted during topical application of the formulation to the skin[2].

Freeze and thaw test: The freeze-thaw cycling test was conducted to evaluate the stability of the nanoemulgel during storage and transportation. The nanoemulgel was subjected to six freeze-thaw cycles, each consisting of 48 hours of refrigeration at 4°C followed by 48 hours of incubation at 40°C in an oven. Visual observations were made after each cycle to assess any changes in organoleptic properties such as phase separation, color changes, or viscosity alterations[2].

Nanoemulgel characterization

Based on the results of the physical properties tests, a single formulation of nanoemulgel was selected for further characterization. This selected formulation underwent a comprehensive characterization process to assess its physicochemical properties. Characterization studies included evaluation of morphology, particle size distribution, and zeta potential.

Morphology: Transmission electron microscopy (TEM) was employed to visualize the morphology of the nanoemulgel. This technique enabled the evaluation of crucial parameters such as the sphericity and uniformity of the nanoemulgel particles. By examining TEM images, we aimed to gain insights into the physical characteristics of the formulated nanoemulgel, which are crucial for its stability, drug delivery efficiency, and overall performance[20].

Particle size and zeta potential: Particle size and zeta potential were determined using a particle size analyzer (PSA) to assess the physical stability of the nanoemulgel. This technique provided valuable information on the average particle size and size distribution, crucial parameters for evaluating the stability and potential for *in vivo* performance of the nanoemulgel. Additionally, zeta potential measurements were conducted to assess the surface charge of the nanoemulgel, which significantly influences its colloidal stability and interactions with biological systems[21].

Antibacterial activity test

Mueller Hinton agar media was prepared according to the manufacturer's instructions and poured into Petri dishes. After solidification, the plates were inoculated with a standardized suspension of *P. acnes*. Briefly, a sterile cotton swab was dipped into the bacterial suspension and then evenly swabbed onto the surface of the MHA. The plates were incubated at 37°C for 24 hours to allow bacterial growth.

Sterile paper discs were impregnated with 50 µL of each test solution: 1% clindamycin gel (positive control), Formula 0 (negative control), selected formula, and 1% extract solution prepared in 10% DMSO. The impregnated discs were then placed onto the surface of the inoculated MHA plates. The plates were subsequently incubated at 37°C for 24 hours. The presence of antibacterial activity was determined by measuring the diameter of the zones of inhibition around the discs using vernier calipers. All experiments were conducted in triplicate, with each experiment repeated three times for reproducibility.

Data analysis

Data analysis was performed using GraphPad Prism version 8.0.1 for Windows. All data are presented as mean ± standard deviation (SD) from three independent experiments. Data normality was assessed using the Kolmogorov-Smirnov test. Statistical significance was determined using one-way ANOVA followed by appropriate post-hoc tests for multiple comparisons, such as Tukey's HSD test. For comparisons between two groups, an unpaired t-test was employed. Statistical significance was considered at $p < 0.05$.

RESULTS AND DISCUSSION

Physical properties examination

Organoleptic and homogeneity tests

All *A. muricata* leaf extract nanoemulgel formulations exhibited excellent physical stability throughout the 28-day observation period, with no discernible changes in shape, odor, or color homogeneity. This observation is crucial, as it indicates that the developed formulations meet the essential physical stability criteria. A characteristic *A. muricata* leaf odor was perceptible in all formulations (**Table II**). Furthermore, visual examination revealed that all formulations were free from any coarse or fine particles, indicating a smooth and homogenous texture. These findings suggest that the developed nanoemulgel formulations possess desirable physical properties, making them suitable for potential topical application.

pH test

The pH of the *A. muricata* leaf extract nanoemulgel formulations was determined to be within a narrow range of 4.70 to 5.06. Normality of the data was assessed using the Kolmogorov-Smirnov test, which revealed a normal distribution (p-value 0.019). Subsequent analysis of variance (ANOVA) indicated no statistically significant differences in pH among the various formulations (p-value 0.566) (**Table III**).

Viscosity test

Viscosity measurements were conducted over 28 days of storage, revealing a range of 543.33 to 928.00 cP for the nanoemulgel preparations. **Table IV** summarizes these findings, indicating that Formula 0 and III exhibited greater viscosity stability compared to Formula I and II. Statistical analysis using the Kolmogorov-Smirnov test confirmed that the viscosity data were normally distributed (p-value 0.00). Furthermore, a One-way ANOVA test demonstrated significant differences in viscosity among the four formulations (p-value 0.000; **Table IV**). These findings suggest that the specific formulation and composition of the nanoemulgel significantly influence its viscosity and stability over time.

Table II. The results of nanoemulgel organoleptic observations.

Day - parameters		Formula 0	Formula I	Formula II	Formula III	Information
0	Shape	+	++	++	++	Shape :
	Color	+	++	++	+++	+ : slightly thick
	Odor	K	K	K	K	++ : thick
7	Shape	+	++	++	++	+++ : very thick
	Color	+	++	++	+++	
	Odor	K	K	K	K	Color :
14	Shape	+	++	++	++	+ : colorless
	Color	+	++	++	+++	++ : brown
	Odor	K	K	K	K	+++: dark brown
21	Shape	+	++	++	++	
	Color	+	++	++	+++	Odor :
	Odor	K	K	K	K	K: *A. muricata* leaf ethanol
28	Shape	+	++	++	++	extract
	Color	+	++	++	+++	
	Odor	K	K	K	K	

Table III. The results of nanoemulgel pH observations.

Day	pH of nanoemulgel (Average ± SD)			
	Formula 0	Formula I	Formula II	Formula III
0	5.01 ± 0.00	5.06 ± 0.06	5.00 ± 0.00	5.00 ± 0.00
7	5.00 ± 0.00	5.00 ± 0.00	5.00 ± 0.00	4.97 ± 0.06
14	5.00 ± 0.00	4.97 ± 0.06	4.93 ± 0.06	4.90 ± 0.00
21	4.87 ± 0.06	4.80 ± 0.10	4.87 ± 0.06	4.73 ± 0.06
28	4.87 ± 0.05	4.77 ± 0.05	4.77 ± 0.06	4.70 ± 0.00

Table IV. The results of nanoemulgel viscosity observations.

Day	Viscosity of nanoemulgel (cP; average ± SD)			
	Formula 0	Formula I	Formula II	Formula III
0	543.33 ± 2.89	560.67 ± 1.15	566.67 ± 2.31	559.33 ± 1.15
7	546.67 ± 2.31	588.00 ± 0.00	894.67 ± 2.31	568.67 ± 1.15
14	550.00 ± 0.00	604.67 ± 1.15	928.00 ± 0.00	576.00 ± 0.00
21	550.67 ± 2.31	622.67 ± 2.31	928.00 ± 0.00	577.33 ± 2.31
28	546.67 ± 2.31	568.00 ± 8.00	913.33 ± 2.31	577.33 ± 2.31

Spreadability test

The spreadability of the nanoemulgel formulations was evaluated over 28 days of storage and found to range from 6.7 to 8.15 cm. Statistical analysis using the Kolmogorov-Smirnov test revealed that the data were normally distributed (p-value 0.02). Furthermore, the One-way ANOVA test indicated no significant difference in spreadability among the different formulations (p-value 0.551) during the storage period (**Table V**). This suggests that the formulations exhibited consistent spreadability characteristics throughout the storage period, indicating their potential for effective topical application.

Table V. The results of nanoemulgel spreadability observations.

Day	Spreadability of nanoemulgel (cm; average ± SD)			
	Formula 0	Formula I	Formula II	Formula III
0	8.15 ± 0.49	7.60 ± 0.57	7.33 ± 0.67	7.73 ± 0.67
7	8.00 ± 0.57	7.95 ± 0.64	6.70 ± 0.57	7.60 ± 0.57
14	7.60 ± 0.57	7.45 ± 0.64	6.98 ± 0.88	7.65 ± 0.64
21	7.50 ± 0.71	7.20 ± 1.13	7.25 ± 0.92	7.48 ± 0.95
28	7.75 ± 0.49	7.30 ± 1.27	7.30 ± 1.13	7.73 ± 0.74

Freeze and thaw test

Stability studies revealed that all formulations maintained their physical integrity throughout the testing period. No significant changes were observed in color, odor, or shape, and no phase separation occurred. These observations suggest that all materials were compatible and that the formulations were stable under various storage conditions, including low temperatures, room temperature, and elevated temperatures.

Nanoemulgel characterization

Based on a comprehensive evaluation of organoleptic properties (shape, odor, color), homogeneity, pH, viscosity, spreadability, and freeze-thaw stability, Formula III was selected for further characterization. This selection was based on its

superior overall performance across these parameters. Subsequent characterization studies included an assessment of the formulation's morphology, particle size distribution, and zeta potential.

Morphology

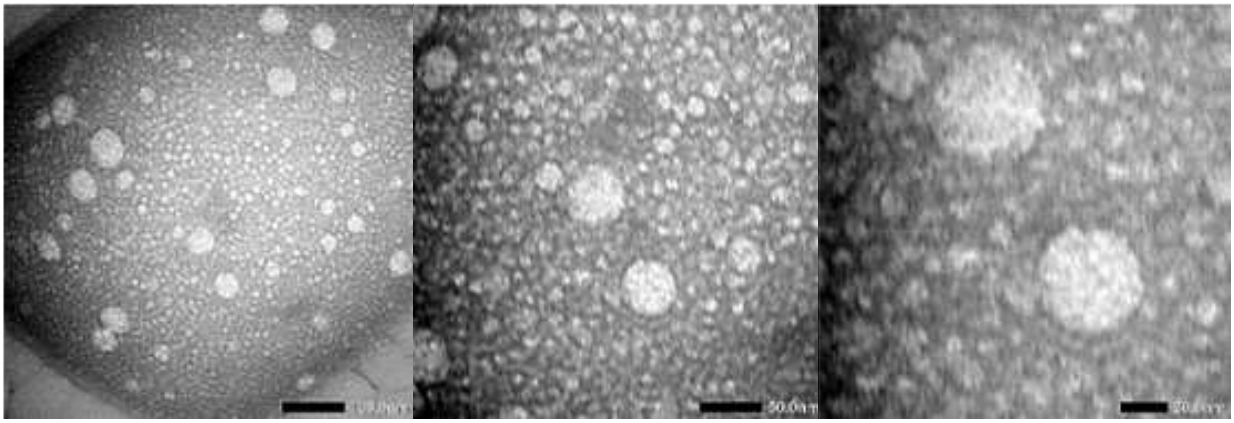
Transmission electron microscopy analysis revealed that the selected nanoemulgel exhibited a uniform dispersion of spherical globules. Notably, no signs of coalescence were observed (**Figure 1**).

Particle size and particle size distribution

The characterization of the developed nanoemulsion revealed a mean particle size of 20.5 nm with a polydispersity index (PDI) of 0.446 (**Figure 2**). This particle size falls within the desirable range for nanoemulsions, typically considered to be between 20 and 200 nm[12]. A narrow particle size distribution, as indicated by the relatively low PDI value, is crucial for achieving optimal stability and enhancing the bioavailability of developed nanoemulsion.

Zeta potential

Zeta potential measurements were conducted to assess the electrostatic stability of the developed nanoemulsion. Zeta potential reflects the surface charge of particles and plays a crucial role in determining the stability of colloidal systems. A high absolute value of zeta potential, typically above (+/-) 30 mV, is generally considered indicative of good colloidal stability, minimizing the risk of particle aggregation[22,23]. In this study, the zeta potential of the nanoemulsion was determined to be -9.8 mV, suggesting moderate electrostatic stability (**Figure 3**). While this value may not fall within the generally accepted range for high stability, the observed stability of the nanoemulsion (as evident in morphology and particle size analysis) indicates that other stabilization mechanisms, such as steric stabilization, may be contributing to the overall stability of the system.



a **b** **c**

Figure 1. Observation of nanoemulgel with TEM at (**a**) 20x, (**b**) 50x, and (**c**) 100x magnification.

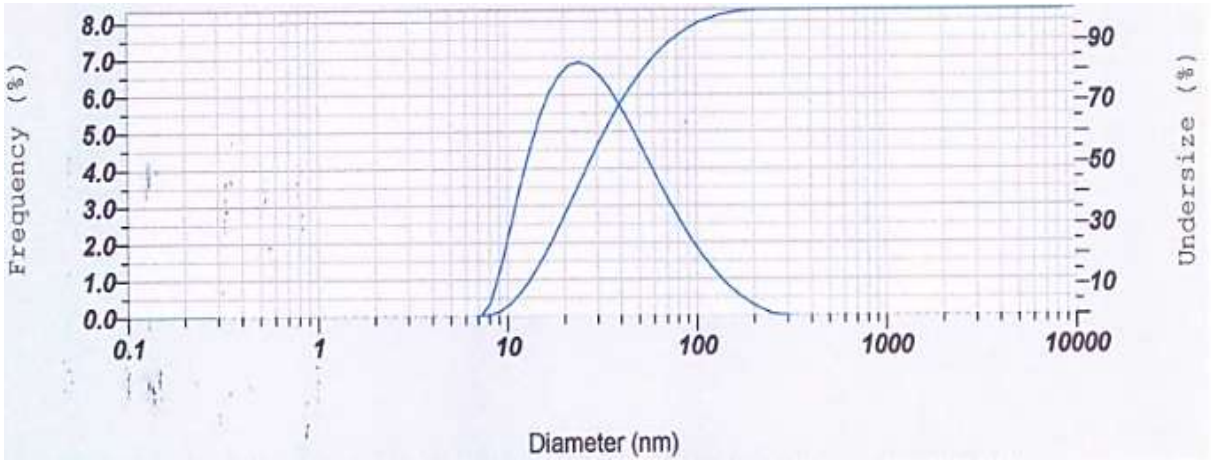


Figure 2. Nanoemulgel particle size measurement.

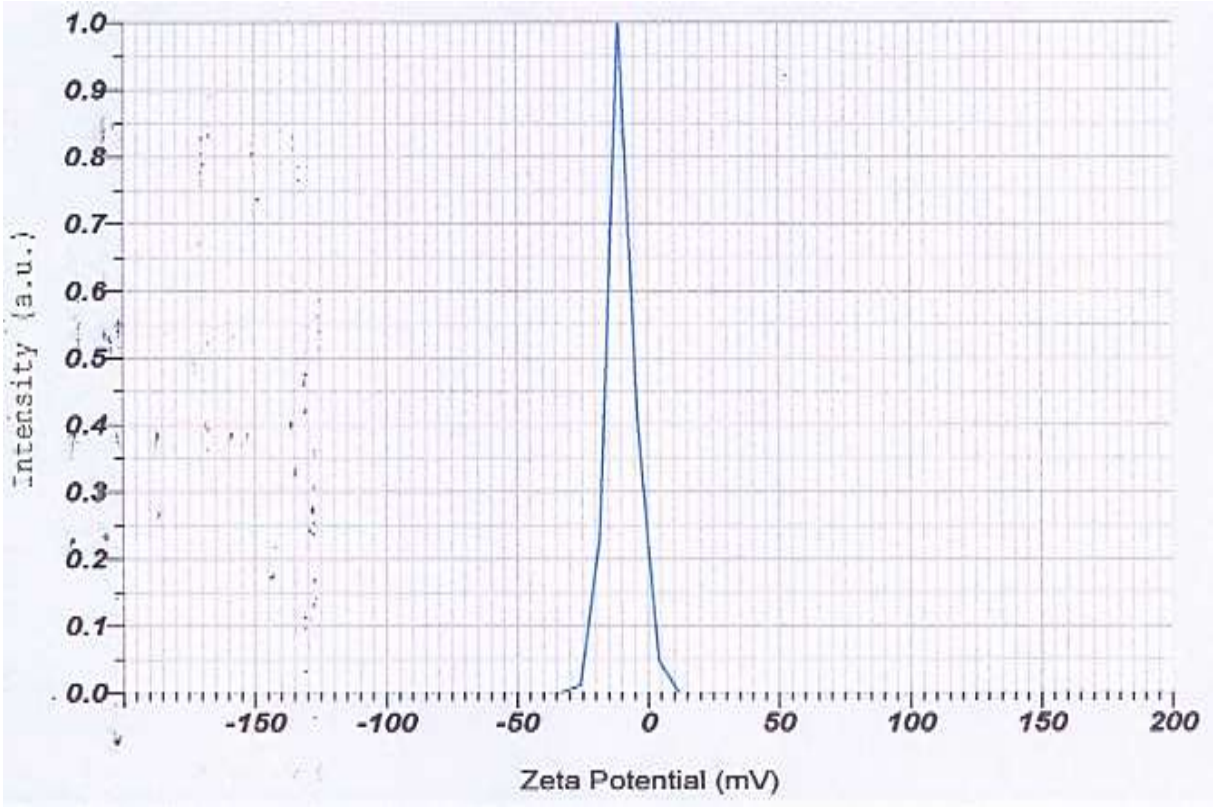
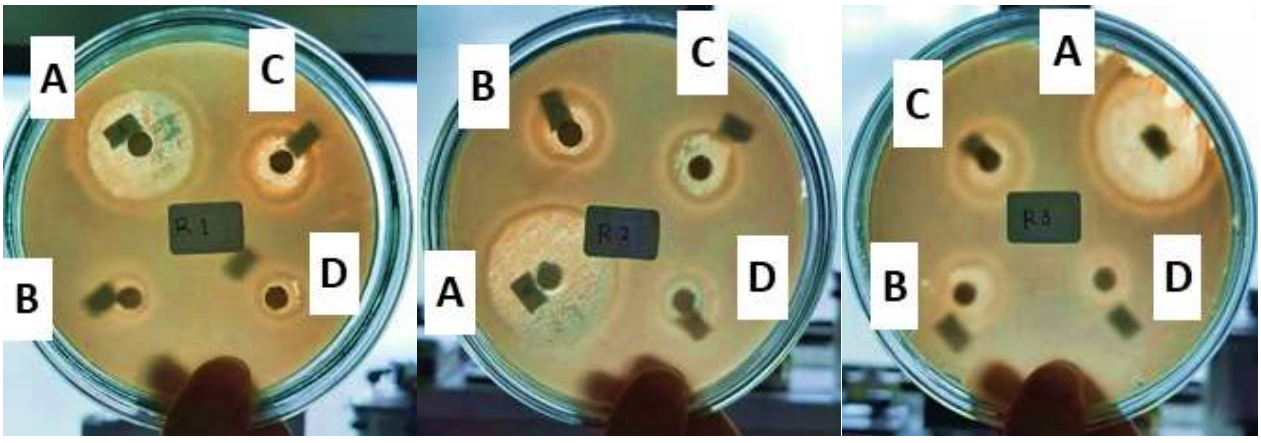


Figure 3. Nanoemulgel zeta potential.

Antibacterial activity test

The antibacterial activity of the selected formulation (Formula III) against *P. acnes* was evaluated and compared to a clindamycin gel (positive control), a negative control (base formulation without active ingredients), and a 1% *A. muricata* leaf ethanol extract solution in 10% DMSO (**Figure 4**). The results of the zone of inhibition tests, depicted in **Figure 5**, demonstrated that Formula III exhibited significant antibacterial activity against *P. acnes* with an average inhibition zone diameter of 19.00 ± 2.65 mm. This value was significantly higher ($p < 0.05$) than that observed for the negative control and the 1% extract solution. However, the antibacterial activity of Formula III was significantly lower ($p < 0.01$) than that of the clindamycin gel, which exhibited an average inhibition zone diameter of 29.33 ± 2.31 mm.



a **b** **c**

Figure 4. Antibacterial test result using disc diffusion method against *P. acnes* in triplicate (**a**: replication 1 or R1; **b**: R2; and **c**: R3). Clindamycin was used as a positive control (A) and Formula 0 as a negative control (B). Nanoemulgel formula III was selected as a sample test (C), while 1% *A. muricata* leaf ethanol extract solution in 10% DMSO was used to examine the influence of other components in the working formula (D).

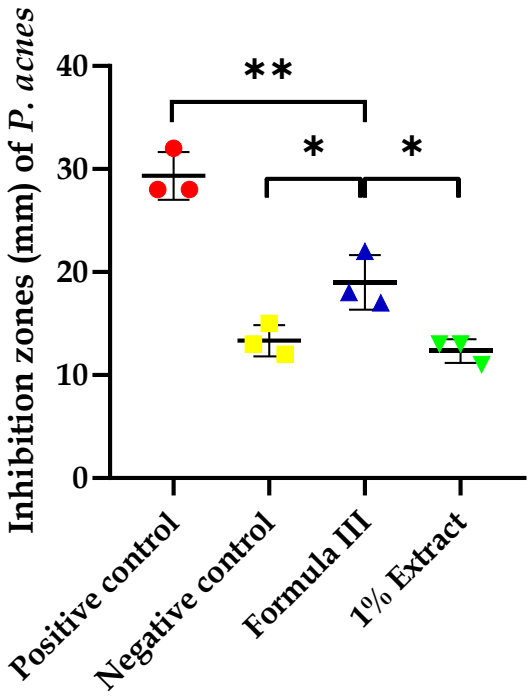


Figure 5. Inhibition zones as results of antibacterial test using disc diffusion method against *P. acnes* in triplicate. Clindamycin was used as a positive control and Formula 0 as a negative control. Data are presented as means ± SD (mm). An unpaired t-test was used for two-group comparisons. *p <0.05, **p <0.01.

Previous studies have demonstrated the potential of *A. muricata* leaf extracts in treating acne due to their inherent antibacterial and antioxidant properties[5-8]. However, direct topical application of crude leaf extracts can be challenging due to poor skin penetration and potential for irritation. To overcome these limitations, this study investigated the development of a nanoemulgel formulation to enhance the delivery and efficacy of *A. muricata* leaf extract. The combination of nanoemulsion and gel was chosen to leverage the advantages of both systems. Nanoemulsions offer enhanced skin penetration and improved drug delivery, while the gel matrix provides stability, viscosity, and improved spreadability.

All developed formulations exhibited acceptable spreadability, with no significant differences observed between them. However, significant variations in viscosity were observed among the formulations (**Tables III** and **IV**), likely influenced by the specific formulation components and their concentrations. All formulations demonstrated excellent physical stability, maintaining homogeneity and stability throughout 28 days of storage and freeze-thaw cycling. Minor color variations were observed among the formulations, which can be attributed to the varying concentrations of the plant extract (**Table I**).

The pH of all nanoemulgel formulations fell within the acceptable range for topical application (pH 4-6)[17]. This slight decrease in pH compared to the extract itself (pH 5.96-6.08)[24] is likely attributed to the use of Carbopol 940, an acidic gelling agent. To adjust the pH, the addition of TEA can be considered.

The zeta potential of the nanoemulgel was measured at -9.8 mV, indicating good stability. This negative surface charge effectively prevented particle aggregation, as confirmed by the physical stability evaluation over 28 days of storage. The successful formation of the nanoemulsion can be attributed to the high concentrations of surfactants and co-surfactants employed, which significantly reduced interfacial tension and facilitated the formation and stabilization of nanodroplets[25].

Formula III was selected for further characterization based on its superior physical stability, particularly its viscosity. As depicted in **Figures 1** and **2**, the optimized nanoemulgel exhibited a spherical morphology with an average particle size of 20.5 nm, a characteristic that is favorable for enhanced skin penetration. While a dedicated permeation study was not conducted in this investigation, the nanoemulgel demonstrated significant antibacterial activity against *P. acnes* in the effectivity testing. This activity can be attributed to the synergistic effects of several factors. Firstly, *A. muricata* leaf ethanol extract, rich in flavonoids, alkaloids, tannins, saponins, and phenols, possesses intrinsic antibacterial properties. These phytochemicals exert their antimicrobial effects through various mechanisms, including disruption of bacterial cell membranes (flavonoids), inhibition of enzyme production and cell wall synthesis (tannins), and interference with vital

cellular processes such as DNA replication (alkaloids)[5,6,8,26]. Secondly, the inclusion of methylparaben and propylparaben in the formulation further enhances its antimicrobial activity[27]. Consequently, Formula III exhibited larger inhibition zones compared to the extract solution alone (**Figure 4**). As reported by Sheskey *et al*.[28], the presence of parabens can significantly enhance the antimicrobial efficacy of formulations within the pH range of 4-8, which is relevant for topical applications. While the negative control, lacking active ingredients from the plant extract, still exhibited some antimicrobial activity due to the presence of the parabens, the positive control (clindamycin gel) demonstrated its expected inhibitory effect by interfering with bacterial protein synthesis through binding to the 50S ribosomal subunit[29].

CONCLUSION

In this study, the concentration of ethanolic extract of *A. muricata* leaves influences physical properties, including organoleptic, pH, viscosity, and spreadability. However, it did not affect freeze and thaw as well as the homogeneity of nanoemulgel. The selected nanoemulgel formula, Formula III with 1% extract content, was effective against *P. acnes* with an inhibition zone of 19.00±2.65 mm.

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AUTHORS' CONTRIBUTION

Conceptualization: Nabila Ikramına, Rehana, Dhadhang Wahyu Kurnıawan
Data curation: Nabila Ikramına, Rehana, Rahmad Aji Prasetya, Dhadhang Wahyu Kurnıawan
Formal analysis: Nabila Ikramına, Rehana, Rahmad Aji Prasetya, Dhadhang Wahyu Kurnıawan
Funding acquisition: -
Investigation: Nabila Ikramına, Rehana, Dhadhang Wahyu Kurnıawan
Methodology: Nabila Ikramına, Rehana, Dhadhang Wahyu Kurnıawan
Project administration: Nabila Ikramına, Rehana
Resources: Nabila Ikramına, Rehana, Dhadhang Wahyu Kurnıawan
Software: -
Supervision: Dhadhang Wahyu Kurnıawan
Validation: Rahmad Aji Prasetya, Dhadhang Wahyu Kurnıawan
Visualization: Rahmad Aji Prasetya, Dhadhang Wahyu Kurnıawan
Writing - original draft: Nabila Ikramına, Rehana
Writing - review & editing: Rahmad Aji Prasetya, Dhadhang Wahyu Kurnıawan

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this study.

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Research Article

Stability Evaluation on Diminazene Diaceturate and Phenazone in Bulk and Combined Formulations using Validated Chromatographic Method

Amna Mubarak [1,2]

Shaza Wagiealla Shantier [1*]

Magdi Awadalla Mohamed [3]

Elrasheed Ahmed Gadkariem [1]

[1] Department of Pharmaceutical Chemistry, University of Khartoum, Khartoum, Sudan
[2] National Medicines Quality Control Laboratory, Khartoum, Sudan
[3] Department of Pharmaceutical Chemistry, Al Jouf University, Sakakah, Al-Jouf, Saudi Arabia

*email: sshantier@yahoo.com; phone: +249912637509

Keywords:
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HPLC
Phenazone
Stability

Abstract

The combined therapy of diminazene aceturate (DMZ) and phenazone (PHENZ) is widely used in veterinary medicine to combat trypanosomiasis and babesiosis. This study presents a novel, validated HPLC method for accurately quantifying DMZ and PHENZ in various pharmaceutical formulations, including bulk powders, sachets, vials, and injectables. The chromatographic separation was achieved using a C18 column (150 x 4.6 mm, 5 µm particle size) with a mobile phase composed of phosphate buffer (pH 3.0) and methanol (70:30 v/v) at a flow rate of 1 mL/minute. UV detection was set at 250 nm. The method demonstrated linearity over a concentration range of 20-100 µg/mL for DMZ and 25-125 µg/mL for PHENZ, with correlation coefficients exceeding 0.999. Forced degradation studies were conducted under various stress conditions to assess the method's stability-indicating power. DMZ exhibited first-order degradation under acidic pH conditions. While slight degradation (2.4-3.1%) was observed under alkaline, UV, and indoor room light conditions, PHENZ remained stable. The validated HPLC method effectively quantified DMZ and PHENZ in the presence of their degradation products and impurities, demonstrating its suitability for quality control and stability studies of these combined drug formulations.

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INTRODUCTION

Sudan, a vast and diverse nation, possesses a rich abundance of livestock, serving as a vital source of food and a cornerstone of the national economy[1]. Ensuring the health and well-being of these animals is paramount, necessitating the implementation of efficient veterinary services and robust drug quality control systems. Validated analytical methods with high accuracy and precision are crucial for guaranteeing the efficacy and safety of veterinary medications[2].

Diminazene diaceturate (DMZ) and phenazone (PHENZ) (**Figure 1**) are commonly employed in combination as an anthelmintic treatment for livestock in Sudan and other developing countries. This combination therapy is primarily effective against trypanosomiasis and babesiosis, with DMZ acting as the primary trypanocide and babesiacide. Diminazene diaceturate, an aromatic diamidine derivative with acidic properties, exhibits limited stability in aqueous solutions, typically degrading within 2-3 days[3]. Phenazone, a pyrazolone derivative with basic properties, serves as a stabilizer for DMZ in aqueous formulations, enhancing its shelf-life and efficacy[4].

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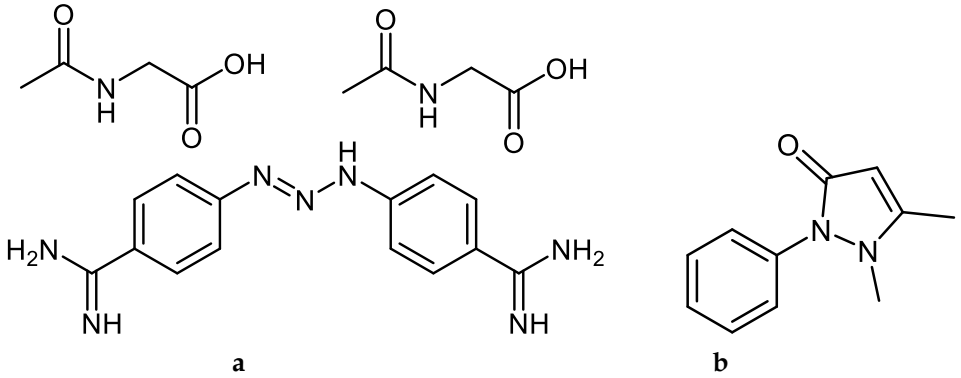

a **b**

Figure 1. Two-dimensional chemical structures of (**a**) DMZ and (**b**) PHENZ.

Despite the widespread use of this combination therapy, a notable gap exists in the availability of standardized quality control methods. While some HPLC methods and a derivative spectrophotometric method have been reported for their analysis[3-5], these methods primarily focus on the determination of drug concentrations in biological fluids or tissues[6-16]. A robust and reliable method for the quality control of DMZ and PHENZ formulations in veterinary products is crucial to ensure their efficacy and safety in animal health.

Several analytical methods have been reported for its quantification in pharmaceutical formulations, including HPLC coupled with LC/MS for the identification of related substances. Additionally, studies have investigated the degradation of DMZ in acidic aqueous solutions[17,18]. The selection of an appropriate mobile phase pH is crucial for successful HPLC analysis, particularly for basic or acidic drugs like DMZ. Proper pH optimization is essential to ensure efficient chromatographic separation, minimizing peak tailing and achieving optimal retention times. This is crucial to avoid interactions between the analyte and the silanol groups present on the silica-based stationary phase, which can lead to peak broadening and decreased resolution[19].

This study aimed to develop, optimize, and validate a simple, robust, and stability-indicating HPLC method for the simultaneous determination of DMZ and PHENZ in their various pharmaceutical formulations marketed in Sudan, including sachets, vials, and ready-to-use solutions. The method was designed to be applicable under both normal storage conditions and under accelerated stress conditions, including exposure to heat, light, acid, and alkali[20]. The optimization process focused on identifying the most suitable chromatographic conditions for achieving optimal resolution and sensitivity for DMZ and PHENZ. This included selecting a commonly available C18 column, utilizing an isocratic mobile phase consisting of a phosphate buffer (pH 3.0) and methanol (70:30 v/v) at a flow rate of 1.0 mL/minute, and employing a detection wavelength of 254 nm, a wavelength suitable for the detection of most organic compounds. The method's validity was rigorously assessed according to The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines. This comprehensive evaluation encompassed several critical parameters, including linearity, range, limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision[21]. By establishing this optimized and validated HPLC method, this study aimed to provide a valuable analytical tool for quality control, ensuring the efficacy and safety of veterinary medicines containing DMZ and PHENZ in Sudan. This will facilitate post-market surveillance by regulatory authorities, ultimately contributing to the improvement of animal health and welfare.

MATERIALS AND METHODS

Materials

Chromatographic analyses were performed using a Shimadzu liquid chromatograph equipped with a UV-visible detector, an isocratic and low-pressure gradient pump, and PC control software (Shimadzu, Japan). An electronic balance (Shimadzu, Japan) was used for accurate weighing of standards and samples. Samples were sonicated in a Power Sonic 405 ultrasonic bath (Branson, USA). pH measurements were conducted using a Jenway 3150 pH meter (Cole-Parmer, UK). Analytical grade potassium dihydrogen phosphate, methanol (HPLC grade), sodium dihydrogen phosphate, and buffer solutions (pH 4, 7, and 10) were obtained from Scharlau (Spain). Reference standards of DMZ (100.67% purity) and PHENZ (99.90%

purity) were procured from Laprovet Laboratories (France). More than ten samples of commercially available combined DMZ and PHENZ formulations were collected. These included sachets, vials (containing 70 and 87.3 mg/mL of DMZ and PHENZ, respectively), and ready-to-use solutions (containing 70 and 375 mg/mL of DMZ and PHENZ, respectively). Samples were sourced from local markets and kindly provided by local company representatives.

Methods

Preparation of standard stock mixture solutions of DMZ and PHENZ

To prepare the stock solution (Solution A), 0.02 g of DMZ and 0.025 g of PHENZ were accurately weighed and dissolved in distilled water. The solution was then transferred to a 100 mL volumetric flask and diluted to the mark with distilled water, resulting in a final concentration of 200 µg/mL for DMZ and 250 µg/mL for PHENZ. Subsequently, 30 mL of Solution A was transferred to a 100 mL volumetric flask and diluted to the mark with distilled water, yielding Solution C with final concentrations of 60 µg/mL for DMZ and 75 µg/mL for PHENZ.

Preparation of sample stock mixture solutions of DMZ and PHENZ

Drug granules or powder equivalents of 0.06 g DMZ and 0.075 g PHENZ were accurately weighed and dissolved in distilled water. The solutions were then transferred quantitatively to 100 mL volumetric flasks and diluted to volume with distilled water, resulting in Solution B (DMZ concentration: 600 µg/mL; PHENZ concentration: 750 µg/mL). Subsequently, 10 mL of Solution B was transferred to another 100 mL volumetric flask and diluted to volume with distilled water, yielding Solution D (DMZ concentration: 60 µg/mL; PHENZ concentration: 75 µg/mL). For analysis of the ready-to-use injection, a suitable volume was diluted with distilled water to obtain a working solution (Solution E) with a final concentration of 60 µg/mL DMZ and 75 µg/mL PHENZ, matching the concentration of Solution D.

Standard curve of DMZ and PHENZ combination

Calibration curves for DMZ and PHENZ were generated using a five-point standard curve method. Varying volumes of standard solutions were diluted with distilled water to obtain a series of concentrations within the ranges of 20-100 µg/mL for DMZ and 25-125 µg/mL for PHENZ. Each concentration was analyzed in triplicate, and the peak areas were recorded. Calibration curves were constructed by plotting the peak area against the corresponding concentration for each analyte. Regression analysis was performed on the mean peak areas using Microsoft Excel to determine the linear regression equations for each calibration curve.

Data analysis

Method validation

Method validation was conducted rigorously in accordance with the guidelines outlined by the ICH. Key parameters assessed included linearity, range, LOD, LOQ, accuracy, and precision, ensuring the reliability and robustness of the analytical method[22].

Forced degradation under acidic conditions: To investigate the stability of the compounds, 25 mL aliquots of solutions C and D were separately treated with 1 mL of 0.1 M HCl. Neutralization was then performed at zero time for one aliquot, and the remaining aliquots were neutralized at 10-minute intervals. Following neutralization, each solution was injected into the chromatographic system. The area under the peak for each compound was determined. Subsequently, peak purity and content percentage were calculated for each time point.

Forced degradation under alkaline conditions: Forced degradation under alkaline conditions was conducted using a procedure analogous to that employed for acidic conditions, with the substitution of HCl with NaOH as the stressor.

Heating condition: A 25 mL aliquot of both Solution C and Solution D was separately heated in a boiling water bath for a duration of two hours. Following the heating period, each solution was allowed to cool to room temperature before being injected into the chromatographic column. The peak area corresponding to each analyte was subsequently determined. Finally, peak purity and percent content were calculated for each sample.

Light condition: To investigate the effect of light exposure, two 50 mL aliquots of solution C were prepared. One aliquot (Tube I) was exposed to a UV lamp (254 nm) for 48 hours, while the other aliquot (Tube II) was exposed to direct light for the same duration. Subsequently, each solution was subjected to chromatographic analysis, and the corresponding peak areas were determined. This procedure was repeated using 50 mL aliquots of solution D.

pH profile: A volume of 3 mL of the solution containing DMZ (60 µg/mL) and PHENZ (75 µg/mL) was transferred into a 10 mL volumetric flask to assess the pH stability of the formulations. The solution was then diluted to the mark with buffer solutions ranging from pH 1.0 to 14.0. Each solution was subsequently injected into the chromatographic system, and the corresponding peak area was determined. This procedure was repeated thirteen times to obtain robust data.

The powder of each sachet and vial separately was accurately dissolved in 15 mL of water for injection. The pH was then measured using a pH meter at 0, 3, 6, 9, 24, and 48 hours. The procedure was repeated using pond water and water boiled in a kettle instead of water for injection.

RESULTS AND DISCUSSION

Stability studies are crucial for evaluating the quality and safety of pharmaceutical formulations. These studies provide valuable information regarding the stability of active ingredients, which directly affects the efficacy of the drug product. Furthermore, they help identify the formation of any toxic degradation products that may arise during storage, posing potential safety risks to patients. By determining shelf-life and calculating the half-life of the active ingredient, stability studies provide crucial insights into optimal storage conditions for maintaining drug efficacy and safety[23]. In the present study, a stability-indicating HPLC method was developed for the analysis of DMZ and PHENZ. This method employed a simple binary mobile phase consisting of phosphate buffer (pH 3.0) and methanol (70:30 v/v) on a C18 column (150 x 4.6 mm, 5 µm) at a flow rate of 1 mL/minute. UV detection at 250 nm was found to provide the highest peak intensities for the analyte. The optimized chromatographic method demonstrated successful separation of the combined drugs, DMZ and PHENZ, with retention times of 3.1 and 7.1 minutes, respectively (**Figure 2**). This efficient separation was crucial for accurate quantification.

During the initial stages of method development, a significant challenge was encountered. Filtration of the solutions containing DMZ and PHENZ through filter paper resulted in the adsorption of DMZ onto the filter paper, leading to a noticeable yellow coloration and a significant reduction in the peak area. This issue was successfully addressed by employing a microsyringe filter for solution filtration. Consequently, the use of a microsyringe filter was deemed essential for accurate and reliable analysis of DMZ and PHENZ. Furthermore, the study highlighted the significant impact of filtration on the chromatographic analysis. As depicted in **Figure 3**, filtration effectively minimized potential interference from particulate matter, resulting in cleaner chromatographic peaks, particularly for DMZ.

Subsequently, the optimized HPLC conditions were determined through a systematic investigation of various parameters, including the selection of a suitable column, mobile phase composition and pH, diluent, and detection wavelength[24]. The primary objective of this optimization process was to achieve optimal separation of DMZ and PHENZ peaks from each other and from any potential degradants, while ensuring robust and reliable system suitability parameters. By carefully adjusting these variables, we aimed to enhance the accuracy and precision of the HPLC method for the quantitative analysis of DMZ and PHENZ in pharmaceutical formulations.

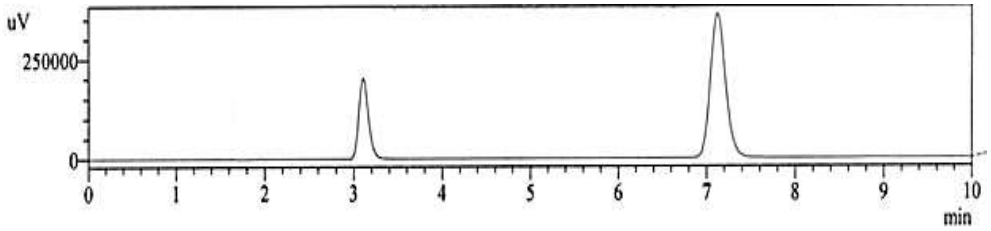

Figure 2. Typical chromatograms for DMZ (**left**, 3.1 minutes) and PHENZ (**right**, 7.1 minutes).

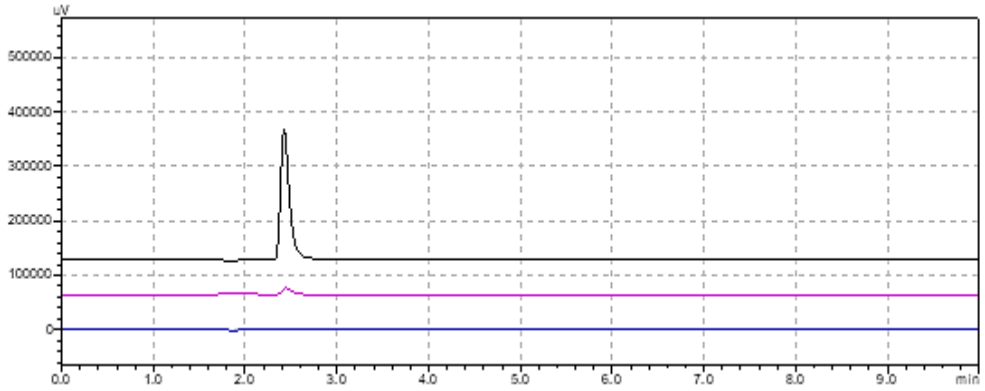

Figure 3. Effect of filtration on DMZ (**black**: microsyringe; **pink**: filter paper; **blue**: blank).

The results of the method validation studies, including system suitability parameters and assay results, are presented in **Tables I**, **II**, and **III**. The developed method demonstrated excellent analytical performance[25], exhibiting linearity with acceptable precision (relative standard deviation <2%) within the ICH guidelines[17,18]. Further validation through assessment of system suitability parameters, including theoretical plates, peak symmetry factor, chromatographic resolution, and capacity factor, confirmed the method's suitability for quantitative analysis. Subsequently, the validated method was applied to analyze commercially available DMZ-PHENZ combination formulations registered in Sudan. Notably, 42% of the analyzed samples exhibited drug content outside the acceptable limits set by the National Medicine and Poisons Board (NMPB). Intriguingly, a wide range of pH values (3.4 to 10.9) was observed in the aqueous solutions of different brands, which may contribute to the observed discrepancies in drug content. However, further investigations are warranted to elucidate the underlying reasons for this significant variation in pH among the analyzed formulations and its potential impact on drug stability and efficacy. These data demonstrate the accuracy, precision, and robustness of the developed analytical method.

Table I. Regression data and validation results of the method developed (n=3).

Parameter	DMZ	PHENZ
Range (µg/mL)	20-100	25-125
Slope	2.52	5.85
Intercept	-13.11	0.77
Correlation coefficient (r)	0.999	1.000
LOD (µg/mL)	1.40	0.50
LOQ (µg/mL)	4.31	1.62
Repeatability (%± RSD)	102.4±0.45	97.7±0.36
Intermediate precision (%±RSD)	102.8±0.55	98.3±0.86
Recovery (%±RSD)	102.1±0.59	99.1±0.76

Table II. System suitability parameters.

Parameter	DMZ	PHENZ
Asymmetry factor	1.03	1.25
Number of theoretical plates	2919.25	8090.68
Resolution	18.4	

Table III. DMZ and PHENZ test results using the developed method (n=3).

Formulation	DMZ	PHENZ
Sachets	102.8±0.6	98.0±0.2
Vials	108.7±0.5	94.7±0.9
Ready-to-use solutions	85.0±0.1	98.8±0.1

Subsequently, the validated method was employed to assess the stability of the drug solution under various stress conditions. The stability of DMZ was investigated under various stress conditions, including pH variations (pH 1.0-14.0), acid hydrolysis, base hydrolysis, light exposure, and heat stress. The results revealed significant degradation of DMZ in the acidic pH range (pH 1.0-5.0), with more than 95% degradation observed at pH 1.0-3.0. Degradation also increased with increasing pH values in the alkaline range (pH 11.0-14.0), with more than 60% degradation observed at pH 13.0 and 14.0.

Notably, the formation of a precipitate was observed at higher pH values (pH 11.0-14.0). Acid hydrolysis studies using different concentrations of HCl demonstrated that a 10-minute incubation time provided a measurable degradation rate with a good correlation coefficient[26].

Chromatographic analysis revealed the formation of degradation products in the acidic and alkaline conditions. The impact of these stress conditions on drug degradation was evaluated, and the results are graphically depicted in **Figures 4** and **5**. **Figure 4** illustrates the presence of extra peaks in the chromatograms of DMZ at pH 1-4, indicating degradation. **Figure 5** depicts a typical chromatogram of degraded DMZ after treatment with 0.1 M HCl for 30 minutes, showing a decrease in the peak area of the parent compound and the emergence of a new peak at a retention time of 2.5 minutes, corresponding to a more polar degradation product. This observation aligns with the known susceptibility of DMZ to degradation due to the presence of a triazene bridge in its structure. Previous studies have confirmed that 4-aminobenzamidine and 4-hydroxybenzamidine are the major degradation products of DMZ[13]. Kinetic analysis revealed that the degradation of DMZ followed first-order kinetics, as evidenced by the linear relationship between the logarithm of the percentage of drug remaining and the time interval (**Figure 6**). Notably, while alkali, light, and heat exposure resulted in minimal degradation (2.4-3.1%), acidic conditions led to a significant loss of DMZ, with up to 98% degradation observed within one hour. Stability studies conducted using the validated method demonstrated that PHENZ exhibited greater stability compared to DMZ under various stress conditions, including exposure to acid, base, light, and heat (**Table IV**). This finding aligns with the observations of Miao *et al.*[16], who reported that PHENZ degradation requires exposure to highly reactive conditions, such as ozone treatment.

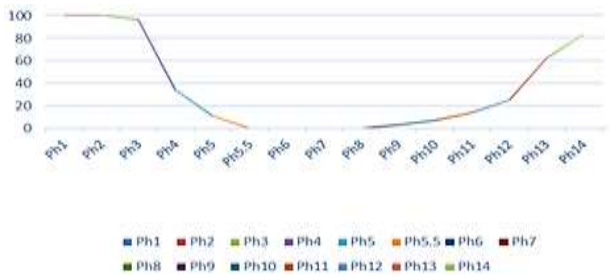

Figure 4. pH profile of DMZ degradation.

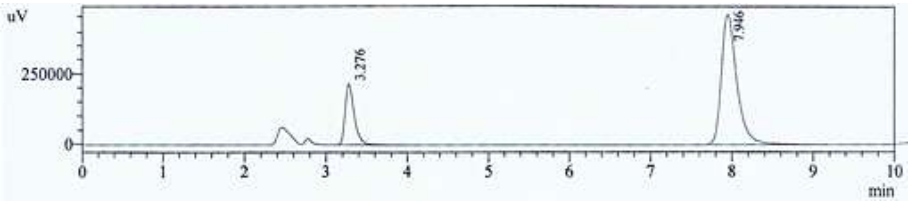

Figure 5. Typical chromatogram for 0.1 M HCl effect on drug degradation after 30 minutes.

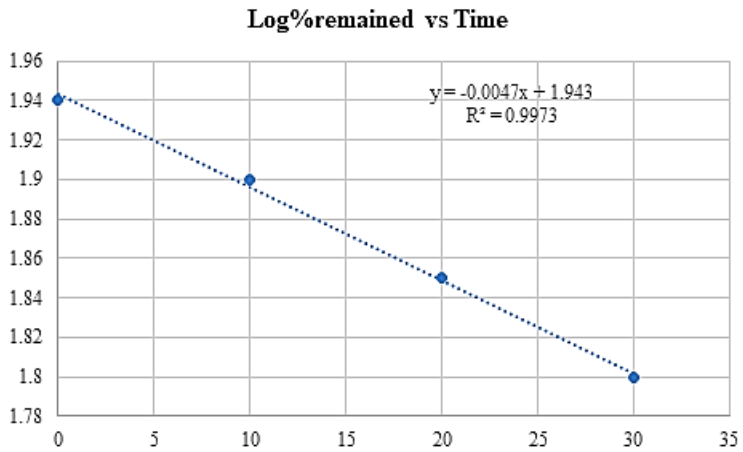

Figure 6. Reaction kinetics of acid influence on DMZ degradation.

Table IV. Summary results for the effect of stress conditions on drug stability.

Condition	Drug	Mean Peak Area (x10³)	Recovery (%)	Degradation (%)
Heat	DMZ	181.229	97.3	2.7
	PHENZ	61728.201	99.9	0.1
UV	DMZ	181.788	97.6	2.4
	PHENZ	61173.019	99.1	0.9
Acidic	DMZ	42.839	23	77
	PHENZ	61234.747	99.2	0.8
Alkaline	DMZ	180.484	96.9	3.1
	PHENZ	61296.476	99.3	0.7

While Abualhasan *et al*.[14] developed a stability-indicating HPLC method for analysis utilizing a C18 column and a mobile phase comprising phosphate buffer (pH 5.2), sodium hexane sulfonate, methanol, and acetonitrile, this method presents several practical limitations that hinder its routine application in quality control. The complex mobile phase composition, involving an ion-pairing reagent like sodium hexane sulfonate, necessitates specialized handling and increases the risk of analytical errors. Sodium hexane sulfonate can irreversibly bind to the C18 column, requiring extensive and potentially ineffective cleaning procedures. Even after thorough flushing, trace amounts of the ion-pairing reagent can remain on the column, altering its selectivity and impacting the reproducibility of subsequent analyses. This not only increases the risk of inaccurate results but also significantly increases the operational costs due to the need for frequent column replacements and specialized cleaning solvents[27]. Moreover, ion pairing needs more time for the mobile phase to equilibrate with the column and any changes in temperature or mobile phase organic can disrupt the equilibrium. Thus, it is taken as the last choice for routine laboratory use if there are no other alternatives[28].

The observed stability of DMZ in the acidic environment (0.1 N HCl) can be attributed to the pH of the mobile phase used in the HPLC analysis. The mobile phase buffer (pH 5.2) likely buffered the acidic environment, preventing significant pH changes in the DMZ solution and thus minimizing degradation. This contrasts with the observed degradation of DMZ in aqueous solution, highlighting the importance of pH control in maintaining drug stability[3,4,29]. This study introduces several novel aspects. Firstly, it presents a tailored optimization of HPLC conditions specifically for the stability assessment of DMZ and PHENZ. Secondly, it emphasizes the importance of understanding the conditions under which DMZ can be safely formulated and the potential impact of inappropriate reconstitution methods on drug stability.

Our observations during sample collection revealed that end-users often deviate from the recommended reconstitution instructions for DMZ and PHENZ powders. The use of raw or potable water, which may contain various impurities such as electrolytes, organic matter, and microorganisms, can significantly impact the physical and chemical properties of the reconstituted drug solution[4]. To investigate this, the drug powder was reconstituted with kettle-boiled water and pond water, and the resultant solutions were compared with those prepared using water for injection. Notably, changes in physical appearance and pH were observed in the solutions prepared with kettle-boiled water and pond water, highlighting the critical role of water quality in maintaining drug stability and efficacy.

CONCLUSION

A robust and accurate HPLC method was developed and validated according to ICH guidelines for the simultaneous determination of DMZ and PHENZ in their bulk forms and combined dosage forms. The method demonstrated excellent linearity, precision, and accuracy, making it suitable for stability studies and routine quality control of pharmaceutical preparations containing these drugs. This validated HPLC method is recommended for the reliable and consistent analysis of DMZ and PHENZ in both bulk and formulated products.

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AUTHORS' CONTRIBUTION

Conceptualization: Amna Mubarak, Shaza Wagiealla Shantier
Data curation: Amna Mubarak
Formal analysis: Amna Mubarak
Funding acquisition: -
Investigation: Amna Mubarak, Shaza Wagiealla Shantier, Magdi Awadalla Mohamed, Elrasheed Ahmed Gadkariem
Methodology: Shaza Wagiealla Shantier, Magdi Awadalla Mohamed, Elrasheed Ahmed Gadkariem
Project administration: Shaza Wagiealla Shantier
Resources: Shaza Wagiealla Shantier, Magdi Awadalla Mohamed, Elrasheed Ahmed Gadkariem
Software: -
Supervision: Shaza Wagiealla Shantier, Magdi Awadalla Mohamed, Elrasheed Ahmed Gadkariem
Validation: Shaza Wagiealla Shantier, Magdi Awadalla Mohamed, Elrasheed Ahmed Gadkariem
Visualization: Amna Mubarak, Shaza Wagiealla Shantier
Writing - original draft: Amna Mubarak, Shaza Wagiealla Shantier
Writing - review & editing: Shaza Wagiealla Shantier, Magdi Awadalla Mohamed, Elrasheed Ahmed Gadkariem

DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this study.

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Research Article

Isolation and Identification of Marine Bacteria in Raja Ampat Islands West Papua Producing Antibacterial Against *Salmonella typhi* and *Staphylococcus aureus*

Dede Mahdiyah *

Muhammad Rifqi Dharmawan

Noval

Department of Pharmacy, Universitas Sari Mulia, Banjarmasin, South Kalimantan, Indonesia

*email: mahdiyahmukti@gmail.com; phone: +6282148508393

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Minimum inhibitory concentration
Salmonella typhi
Secondary metabolites
Staphylococcus aureus

Abstract

Indonesia's vast archipelago harbors a wealth of natural resources, including marine bacteria with potential antibacterial properties. Given the increasing prevalence of antibiotic resistance, particularly against *Salmonella typhi* and *Staphylococcus aureus*, there is a pressing need to explore alternative antimicrobial agents. This study aimed to isolate and characterize marine bacteria with antibacterial activity and evaluate their efficacy against *S. typhi* and *S. aureus*. Isolation of marine bacteria was conducted using the spread plate method. Antibacterial activity screening of the secondary metabolites was performed using the well diffusion method. Minimum inhibitory concentration (MIC) was determined using the dilution method, while minimum bactericidal concentration (MBC) was determined using the spread plate method. Seven bacterial isolates were obtained, all identified as Gram-negative bacilli. The secondary metabolites of these marine bacteria demonstrated antibacterial activity against both S. *typhi* and *S. aureus*, with inhibition zones of 8.50 mm and 8.46 mm, respectively. The MIC for both bacteria was determined to be 1500 µg/mL. Statistical analysis revealed a significant difference in antibacterial activity between the isolates (Kruskal-Wallis Test, p-value = 0.007) and between *S. typhi* and *S. aureus* (Mann-Whitney Test, p-value = 0.025). While the secondary metabolites exhibited antibacterial activity against both bacteria, they did not demonstrate bactericidal activity as measured by the MBC test.

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INTRODUCTION

Indonesia, an archipelago nation, possesses a vast maritime domain, its sea area exceeding its landmass by a factor of 2.5. This expansive marine environment presents immense potential, offering a wealth of natural resources and crucial environmental services that can significantly contribute to economic growth at local, regional, and national levels[1]. Among the myriad of Indonesian seas, the Raja Ampat Islands in Papua stand out as a region of exceptional marine biodiversity and ecological significance.

The Raja Ampat Islands, a renowned archipelago situated in the western part of Papua Island, Indonesia, comprise four major island groups: Waigeo, Misool, Salawati, and Batanta. This region, renowned for its breathtaking underwater scenery, has captivated divers and researchers alike[2]. Importantly, the Raja Ampat Islands lie within the Coral Triangle, recognized globally as a center of marine biodiversity. This exceptional biodiversity extends to the microbial realm, with the region

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harboring a rich diversity of marine bacteria. Given the potential for these bacteria to produce novel bioactive compounds, including secondary metabolites, exploring the microbial diversity of the Raja Ampat Islands presents a significant opportunity for scientific discovery and potential applications in various fields, such as medicine and biotechnology[3].

Marine environments offer a unique and diverse source of novel bioactive compounds, particularly from bacterial secondary metabolites. These metabolites often exhibit distinctive structures and potent bioactivities, driven by the complex and challenging conditions of their marine habitats[4]. Previous studies have demonstrated the potential of marine bacteria, such as those associated with *Japis* sp., to produce bioactive compounds, including protease inhibitors[5]. Notably, these protease inhibitors have shown significant antibacterial activity, offering a promising avenue for the development of novel antibiotics. The increasing prevalence of antibiotic resistance poses a significant global health threat. According to the Centers for Disease Control and Prevention (CDC), antibiotic resistance contributes to approximately 2 million infections and 23,000 deaths annually in the United States alone, with *Salmonella typhi* and *Staphylococcus aureus* being major concerns[6]. The development of new antibacterial agents, such as those derived from marine bacterial secondary metabolites, is crucial to combat this growing public health challenge[7]. This research aims to investigate the potential of marine bacterial isolates from the coast of Waigeo Regency, Raja Ampat Islands, West Papua, Indonesia, to produce antibacterial compounds with activity against *S. typhi* and *S. aureus*.

MATERIALS AND METHODS

Materials

This study utilized several laboratory materials, including Petri dishes, micropipettes (Accumax pro fix), pycnometer (Pyrex), magnetic stirrer (Oem), Erlenmeyer flasks (Pyrex and Herma), analytical balance (Shimadzu Corporation), autoclave, incubator, hot plate (Thermo Scientific), laminar air flow (LAF) cabinet, and test tubes (Pyrex). Biological materials used included marine bacteria isolated from the Raja Ampat Islands, pure cultures of *S. typhi* ATCC 13311 and *S. aureus* ATCC 25923, nutrient agar (NA), nutrient broth (NB), sterile cotton, 10% NaCl solution, distilled water, and Gentian Violet dye.

Methods

Bacterial isolation

Water samples were collected from coastal areas within Waigeo Regency, Raja Ampat Islands, West Papua, Indonesia. Serial dilutions of each water sample were prepared, ranging from 10^{-1} to 10^{-5}. Subsequently, 20 mL of each diluted sample was aseptically transferred onto NA plates. The plates were then spread evenly using a sterile glass rod and incubated at 37°C for 24-48 hours. Following incubation, bacterial colonies were observed and differentiated based on their morphological characteristics, including colony color, shape, elevation, and edge[8].

Bacterial morphological identification

Bacterial morphological identification aimed to characterize the physical appearance of the isolated bacterial colonies. This involved a visual examination of colony characteristics, including color, shape, elevation, and edge morphology. The observed characteristics were then recorded in a table to facilitate further analysis. Bacterial colony morphology, particularly its shape, provides valuable initial clues for bacterial identification[9].

Antibacterial activity screening

Antibacterial activity of the marine bacterial supernatants was evaluated using the well diffusion method. Briefly, 20 µL of bacterial suspensions of *S. typhi* and *S. aureus*, standardized to 0.5 McFarland turbidity, were inoculated onto Mueller-Hinton agar (MHA) plates and spread evenly using a sterile cotton swab. Three wells, each with a diameter of 6 mm, were then created in the agar using a sterile cork borer. Subsequently, 50 µL of each marine bacterial supernatant was carefully added to the respective wells. Control wells received 50 µL of sterile distilled water. The plates were then incubated at 37°C for 18-24 hours. After incubation, the plates were examined for the presence of zones of inhibition around the wells, indicating antibacterial activity. The diameters of the inhibition zones were measured using a caliper and recorded.

Determination of minimum inhibitory concentration

To determine the minimum inhibitory concentration (MIC) of the marine bacterial supernatant against pathogenic bacteria, serial two-fold dilutions were prepared in NB. The supernatant was added to sterile test tubes containing NB at concentrations of 500 µL, 700 µL, 1000 µL, and 1500 µL. Subsequently, each test tube was inoculated with 100 µL of standardized suspensions of *S. typhi* and *S. aureus*, adjusted to 0.5 McFarland turbidity standards. The inoculated tubes were then incubated at 37°C for 24 hours. The MIC was determined as the lowest concentration of the marine bacterial supernatant that exhibited no visible turbidity, indicating the absence of bacterial growth.

Determination of minimum bactericidal concentration

To determine the minimum bactericidal concentration (MBC), 20 µL of each bacterial culture from the MIC broth dilutions exhibiting no visible growth were transferred to sterile NA plates. The bacterial suspensions were then spread evenly over the agar surface using sterile L-shaped glass rods. The inoculated plates were incubated at 37°C for 24 hours. After incubation, the plates were visually inspected for the presence of bacterial colonies. The MBC was defined as the lowest concentration of the test substance that resulted in no visible bacterial growth after subculturing on solid media.

Data analysis

Data analysis in this study employed a descriptive approach. To determine significant differences among multiple groups, the One-Way ANOVA was utilized, a parametric test suitable for comparing means across three or more groups. The prerequisites for employing ANOVA are normality and homogeneity of variances within the groups. Normality of data distribution was assessed using the Shapiro-Wilk test, while homogeneity of variances was evaluated using Levene's test. If the data violated the assumptions of normality or homogeneity, the non-parametric Kruskal-Wallis test was employed as an alternative. Subsequently, if significant differences were detected by the Kruskal-Wallis test, pairwise comparisons were performed using the non-parametric Mann-Whitney U test to identify specific groups that differed significantly from each other.

RESULTS AND DISCUSSION

Bacterial isolation

Bacterial isolation from seawater samples collected from the Raja Ampat Islands, West Papua, yielded seven distinct bacterial isolates (MB 1 to MB 7; **Figure 1**). The density of bacterial colonies observed on the agar plates decreased progressively with increasing dilution factors. At the 10^{-1} dilution, a high density of bacterial colonies was observed, indicating a significant bacterial load in the original seawater sample. This trend is expected, as higher dilutions result in a significant reduction in the number of viable bacterial cells present in the sample[10]. These findings demonstrate the presence of a diverse bacterial community within the seawater of the Raja Ampat Islands.

Bacterial morphological identification

Morphological characterization of the marine bacterial isolates revealed a predominance of rod-shaped (*bacillus*) morphology. Seven isolates exhibited this characteristic, with variations in colony color observed (**Table I**). Isolate 1, 4, and 5 displayed a cream color, while isolates 2, 3, and 6 were clear white, and isolate 7 exhibited a milky white coloration. All isolates shared common morphological features, including a flat elevation and flat edges. These findings are consistent with previous studies conducted by Marzuki *et al*.[11], which reported that marine bacterial isolates from Melawai Beach, Balikpapan, predominantly exhibited rod-shaped morphology and displayed a range of colors, including cream, milky white, and clear white. Furthermore, Sariadji[12] as well as Sabdaningsih *et al*.[13] also reported the presence of flat edges in their respective studies on marine bacterial isolates.

Antibacterial activity screening

Antibacterial activity screening was conducted using the disc diffusion method. Isolate 7, designated as MB 7, isolated from marine bacteria in the Raja Ampat Islands, West Papua, exhibited antibacterial activity against the test organism (**Table II**). The average diameter of the inhibition zones produced by MB 7 against *S. typhi* and *S. aureus* were 8.5 and 8.46 mm, respectively, which falls within the category of moderate inhibition according to the classification system defined by

Prijatmoko *et al*[14]. This classification system categorizes inhibition zones as weak (diameter ≤5 mm), moderate (5-10 mm), strong (10-20 mm), and very strong (≥20 mm)[15]. It is noteworthy that marine bacteria in symbiosis with other organisms, such as sponges, have been reported to produce a significantly higher diversity of secondary metabolites compared to free-living marine bacteria[16]. Since MB 7 is a free-living marine bacterium, the observed moderate antibacterial activity aligns with this expectation. According to Kusuma *et al*.[17], bacteria with high antibacterial activity often exhibit specific morphological characteristics, such as rod shape, milky white color, and flat edges. Interestingly, MB 7 displays these characteristics, further supporting its observed antibacterial potential.

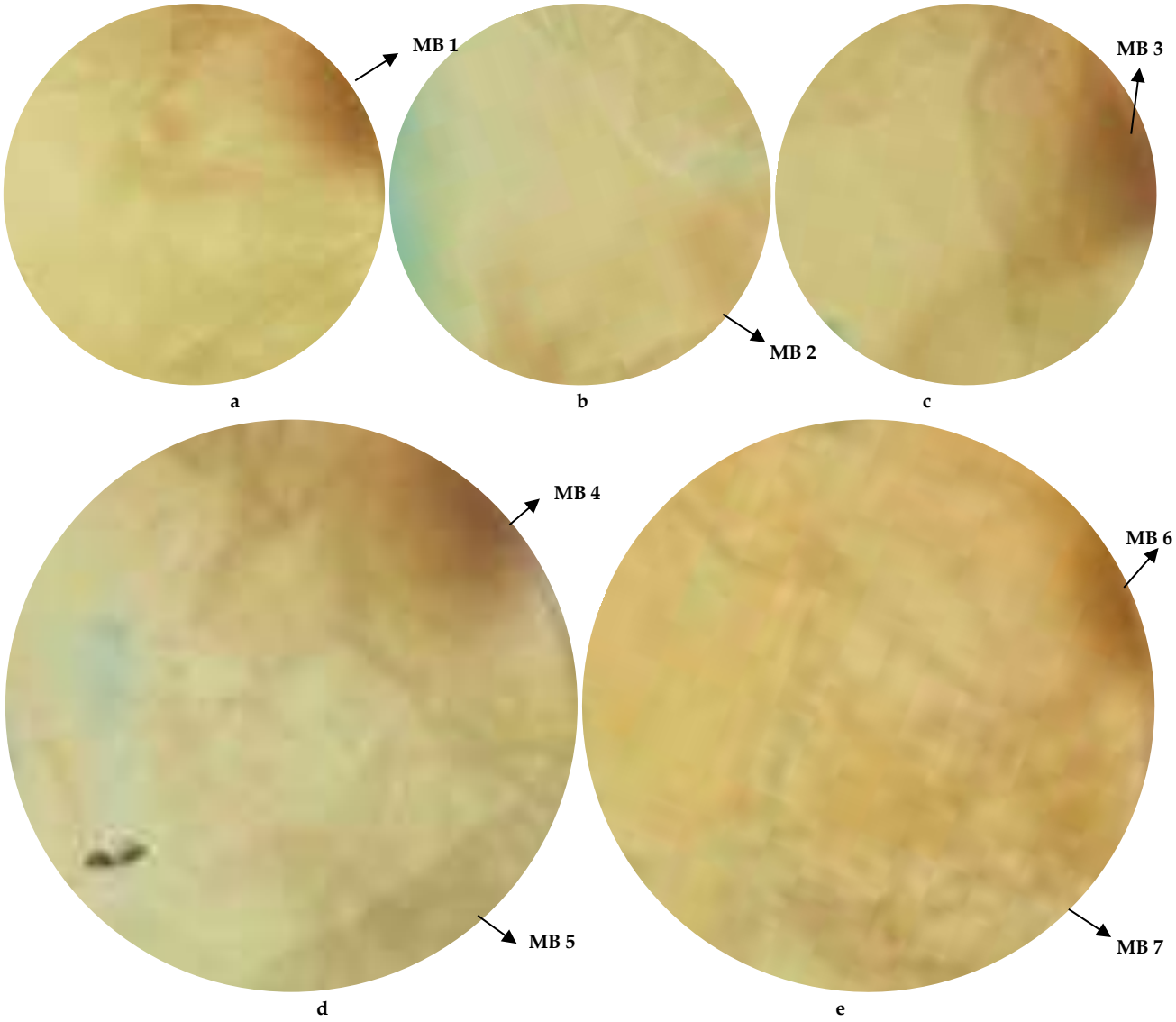
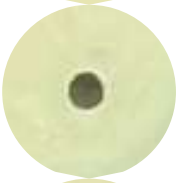
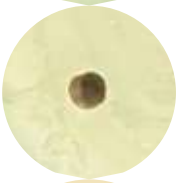
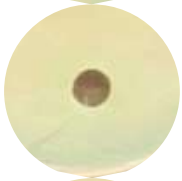
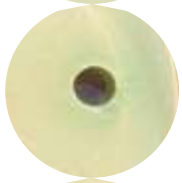
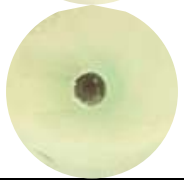


Figure 1. Marine bacterial isolation from seawater samples of the Raja Ampat Islands, West Papua. The dilution factors are (**a**) 10^{-1}, (**b**) 10^{-2}, (**c**) 10^{-3}, (**d**) 10^{-4}, and (**e**) 10^{-5}.

Table I. Characteristics of marine bacterial from seawater samples of the Raja Ampat Islands, West Papua.

Isolate	Characteristics of marine bacteria			
	Shape	Color	Elevation	Edge
MB 1	Rod	Cream	Flat	Even
MB 2	Rod	Clear White	Flat	Even
MB 3	Rod	Clear white	Flat	Even
MB 4	Rod	Cream	Flat	Even
MB 5	Rod	Cream	Flat	Even
MB 6	Rod	Clear white	Flat	Even
MB 7	Rod	Milky white	Flat	Even

Table II. Antibacterial activity screening of marine bacterial isolation from seawater samples of the Raja Ampat Islands, West Papua.

Isolates	*Salmonella typhi*		*Staphylococcus aureus*	
	Average	Figures	Average	Figures
MB 1	8.13		8.13	
MB 2	8.16		8.16	
MB 3	8.2		8.2	
MB 4	8.2		8.2	
MB 5	8.23		8.23	
MB 6	8.36		8.36	
MB 7	8.5		8.46	

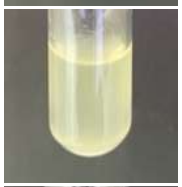
Determination of minimum inhibitory concentration

The MIC of MB 7 against *S. typhi* and *S. aureus* was determined to be 1500 µg/mL, as indicated by the absence of turbidity in the test tubes at this concentration in both cases (**Table III**). While the MIC value was the same for both bacterial species, a clearer visual observation of bacterial inhibition was noted in the *S. typhi* cultures. This observation aligns with the general principle that bacteriocins produced by Gram-positive bacteria are typically more effective against other Gram-positive bacteria, and *vice versa* for Gram-negative bacteria[18]. This phenomenon is attributed to the specific mechanisms of bacteriocin action and the differences in the cell wall structures of Gram-positive and Gram-negative bacteria. Furthermore, bacterial communication systems, such as quorum sensing, play a crucial role in regulating bacteriocin production. Quorum sensing allows bacteria to coordinate gene expression and release compounds that inhibit the growth of competing microorganisms, thereby establishing a competitive advantage within their environment[19].

Statistical analysis using the Kruskal-Wallis test revealed significant differences ($p < 0.007$) in the antibacterial activity of various concentrations of marine bacterial secondary metabolites from the Raja Ampat Islands against *S. typhi* and *S. aureus*. To further investigate these differences, post-hoc Mann-Whitney tests were conducted to compare each concentration with

the negative control. The results demonstrated that the 1500 µg/mL concentration exhibited significant antibacterial activity against both bacterial strains ($p < 0.025$), while lower concentrations (500, 700, and 1000 µg/mL) did not show significant differences compared to the control ($p > 0.05$). These findings suggest that higher concentrations of secondary metabolites extracted from marine bacteria in the Raja Ampat Islands exhibit significant antibacterial activity against *S. typhi* and *S. aureus*.

Table III. Minimum inhibitory concentration of MB 7 against *S. typhi* and *S. aureus*.

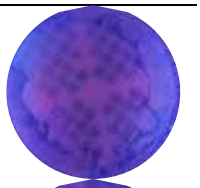
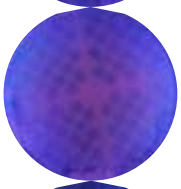
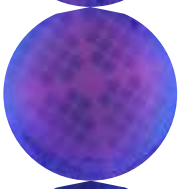
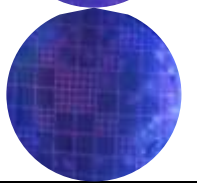
Concentration (µg/mL)	*Salmonella typhi*			*Staphylococcus aureus*		
	Turbidity	**p-value**	**Figures**	**Turbidity**	**p-value**	**Figures**
500	Cloudy	0,007[a] 1,000[b]		Cloudy	0,007[a] 1,000[b]	
700	Cloudy	0,007[a] 1,000[b]		Cloudy	0,007[a] 1,000[b]	
1000	Cloudy	0,007[a] 1,000[b]		Cloudy	0,007[a] 1,000[b]	
1500	Slightly cloudy	0,007[a] 0,025[b]		Slightly cloudy	0,007[a] 0,025[b]	
Bacterial suspension (negative control)	Cloudy	0,007[a] 1,000[b]		Cloudy	0,007[a] 1,000[b]	
Chloramphenicol (positive control)	Clear	0,007[a] 0,025[b]		Clear	0,007[a] 0,025[b]	

Notes: [a]: the significance value of the Kruskal-Wallis Test; [b] : the significance value of the Mann-Whitney Test

Determination of minimum bactericidal concentration

To further evaluate the antibacterial activity of MB 7, the MBC was determined. The MBC, which assesses the concentration of the compound required to kill the bacteria, was evaluated by subculturing samples from the MIC test onto NA plates[20]. The MBC is defined as the lowest concentration of the compound that results in no visible bacterial growth on the NA plates[21]. Our results, presented in **Table IV**, demonstrated that MB 7 from the Raja Ampat Islands of West Papua did not exhibit any bactericidal activity against either *S. typhi* or *S. aureus*. This was evidenced by the observation of bacterial growth on the NA plates at all tested concentrations of MB 7, indicating the absence of a discernible MBC value. These findings are consistent with previous studies[22,23] that also reported the lack of an MBC for certain compounds against specific bacterial strains. This suggests that while MB 7 may inhibit the growth of these bacteria at certain concentrations (as indicated by the MIC values), it may not necessarily kill them[24,25].

Table IV. Minimum bactericidal concentration of MB 7 against *S. typhi* and *S. aureus*.

Concentration (µg/mL)	*Salmonella typhi*				*Staphylococcus aureus*			
	Replication			Figures	Replication			Figures
	I	II	III		I	II	III	
1500	Grow colony	Grow colony	Grow colony		Grow colony	Grow colony	Grow colony	
Bacterial suspension (negative control)	Grow colony	Grow colony	Grow colony		Grow colony	Grow colony	Grow colony	
Chloramphenicol (positive control)	Clear	Clear	Clear		Clear	Clear	Clear	

CONCLUSION

This study successfully isolated and identified seven bacterial strains from seawater samples collected from the Raja Ampat Islands, West Papua. All isolates exhibited a rod-shaped morphology. Among these isolates, strain MB 7 demonstrated significant antibacterial activity against both *S. typhi* and *S. aureus* with moderate inhibition zones. Further analysis revealed MIC of 1500 µg/mL for both bacterial pathogens. Statistical analysis using the Kruskal-Wallis and Mann-Whitney tests confirmed the significant antibacterial activity of MB 7 secondary metabolites. However, no MBC was observed, suggesting that the observed antibacterial activity may be bacteriostatic in nature.

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AUTHORS' CONTRIBUTION

Conceptualization: Dede Mahdiyah
Data curation: Muhammad Rifqi Dharmawan
Formal analysis: Dede Mahdiyah, Muhammad Rifqi Dharmawan, Noval
Funding acquisition: -
Investigation: Muhammad Rifqi Dharmawan
Methodology: Dede Mahdiyah, Noval
Project administration: Dede Mahdiyah, Noval
Resources: Dede Mahdiyah
Software: -
Supervision: Dede Mahdiyah, Noval
Validation: Dede Mahdiyah, Noval
Visualization: Muhammad Rifqi Dharmawan
Writing - original draft: Muhammad Rifqi Dharmawan

Writing - review & editing: Dede Mahdiyah, Noval

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this study.

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Research Article

Standardized Herbal Extract for Wound Healing: A Comparative Study of *Centella asiatica*, *Curcuma domestica*, and *Heterotrigona itama* Honey Combination in Rabbits

Diniatik [1]

Paula Mariana Kustiawan [2*]

Halida Suryadini [3]

[1] Department of Pharmacy, Universitas Muhammadiyah Purwokerto, Purwokerto, Central Java, Indonesia
[2] Department of Pharmacy, Universitas Muhammadiyah Kalimantan Timur, Samarinda, East Kalimantan, Indonesia
[3] Department of Pharmacy, Universitas Muhammadiyah Palangkaraya, Palangka Raya, Central Kalimantan, Indonesia

*email: pmk195@umkt.ac.id; phone: +6281349568120

Keywords:
Centella asiatica
Curcuma domestica
Stingless bee honey
Wound Healing

Abstract

Centella asiatica and *Curcuma domestica* are recognized for their wound-healing properties. This study investigated the synergistic effects of combining these plant extracts with stingless bee (*Heterotrigona itama*) honey on wound healing in rabbits. Ethanolic extracts of *C. asiatica* and *C. domestica* were prepared in a 1 : 10 ratio. Blended formulations containing 20% plant extracts and 80% honey were created in two ratios: 1 : 1 and 2 : 1. The total flavonoid and tannin content of the plant extracts were quantified. Male rabbits were subjected to a wound-healing model, and the formulations were applied topically for seven days. Wound healing was assessed, and the results were compared to a positive control. The ethanolic extracts of *C. asiatica* and *C. domestica* were rich in flavonoids and tannins. The blended honey formulations exhibited promising wound-healing effects, with Formula 2 (2 : 1 ratio of plant extracts) demonstrating slightly superior results compared to Formula 1 and the positive control. These findings suggest the potential of the combined extracts of *C. asiatica*, *C. domestica*, and *H. itama* honey as effective wound-healing agents.

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INTRODUCTION

The skin serves as the body's primary barrier, protecting it from environmental insults and pathogens. Skin injuries, such as those caused by sharp objects, disrupt this protective barrier, leading to tissue damage and bleeding, consequently disrupting homeostasis and triggering an inflammatory response[1]. Wound healing is a complex physiological process aimed at restoring the integrity and function of damaged tissues. This multi-stage process typically involves three overlapping phases: inflammation, proliferation, and maturation[2].

The rate and quality of wound healing can vary significantly depending on factors such as the location, severity, and extent of the injury[3]. The healing process typically involves several overlapping phases, including inflammation, proliferation, and remodeling. During the remodeling phase, the initially reddish and thick scar tissue gradually transitions into a paler and thinner scar. This phase also witnesses significant wound contraction. While scar tissue plays a crucial role in wound closure, it is important to note that it rarely achieves the full tensile strength of normal, uninjured skin, typically reaching only 80% of the original strength[4].

Acute wounds, which occur in individuals with otherwise healthy tissues, usually heal predictably within a defined timeframe, restoring normal anatomical and functional integrity[5]. To facilitate the wound healing process, it is crucial to

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address the inflammatory response and mitigate the detrimental effects of reactive oxygen species (ROS). Oxidative stress plays a crucial role in various skin aging processes, including collagen degradation and wrinkle formation[6]. Natural products rich in antioxidants and immune-modulating compounds offer promising therapeutic options to support and accelerate wound healing. Antioxidants, by neutralizing free radicals, can help mitigate these effects[7]. Natural sources of antioxidants, such as plant extracts and bee products, have gained significant attention due to their potential safety and efficacy.

Centella asiatica (gotu kola), a widely used medicinal herb, is rich in bioactive compounds like saponins, flavonoids, and triterpenoids, exhibiting antioxidant, anti-inflammatory, and wound-healing properties[8]. *Curcuma domestica* (turmeric), another renowned medicinal plant, contains curcuminoids, essential oils, and other bioactive compounds with potent antioxidant and anti-inflammatory activities[9]. The presence of flavonoids and phenols in *C. asiatica* and *C. domestica* is expected to exert antioxidant effects, thereby inhibiting lipid peroxidation. This antioxidant activity may contribute to the prevention of cellular necrosis, improved vascularization, and enhanced collagen fiber viability, ultimately leading to increased strength of the dermal extracellular matrix[10]. Apart from these two natural ingredients, stingless bee honey, produced by various bee species including *Heterotrigona itama*, is also a rich source of polyphenols, flavonoids, and other bioactive compounds with antioxidant and antimicrobial properties[11].

While individual studies have investigated the antioxidant and wound-healing properties of these natural ingredients, limited research has explored their combined effects. This study aims to evaluate the potential synergistic effects of a blended extract containing *C. asiatica*, *C. domestica*, and stingless bee honey on *in vivo* model using male albino rabbits. The findings of this research will contribute to the development of novel and effective natural-based formulations for skin rejuvenation and wound healing.

MATERIALS AND METHODS

Materials

This study utilized fresh leaves of *C. asiatica* and rhizomes of *C. domestica*, both obtained from Banyumas, Central Java, Indonesia. The plant species were authenticated by the Department of Biology, Universitas Muhammadiyah Purwokerto. Stingless bee honey, specifically from the *H. itama* species, was sourced from East Kalimantan, Indonesia. Other materials used in the study included ethanol, various organic solvents, distilled water, quercetin standards, and a set of *in vivo* wound healing test kits, including male albino rabbits.

Methods

Total flavonoid and tannin contents of a blended of C. asiatica and C. domestica extracts

Total flavonoid content (TFC) was determined using the aluminum chloride colorimetric method. Briefly, 100 mg of the plant extract was subjected to acid hydrolysis. This involved refluxing the extract with 1 mL of 0.5% w/v hexamethylenetetramine, 20 mL of acetone, and 2 mL of 25% HCl in water for 30 minutes. The hydrolyzed mixture was then filtered, and the filtrate was collected in a 50 mL volumetric flask. After cooling, the volume was adjusted to 50 mL with distilled water. Subsequently, 20 mL of the hydrolyzed filtrate was extracted with ethyl acetate. The combined ethyl acetate extracts were collected in a 25 mL volumetric flask and brought to volume with ethyl acetate[12]. The TFC of the extract was determined spectrophotometrically by reacting the ethyl acetate extract with aluminum chloride. The absorbance was measured at the maximum wavelength of 430 nm, and the results were expressed as mg of quercetin equivalents per g of dry extract (mgQE/g). The TFC of each plant extract was determined and standardized according to the methods outlined in the Indonesian Herbal Pharmacopoeia[13]. In addition to TFC, total tannin content (TTC) was also measured using the UV-visible spectrophotometric method at a wavelength of 730 nm. Based on the results of TFC and TTC analysis, blended honey preparations were formulated.

Preparation of blended honey

Blended extracts were prepared by combining extracts of *C. asiatica* and *C. domestica* with stingless bee honey. The extracts were initially mixed thoroughly in a mortar and pestle to ensure homogeneity. Honey was then gradually added and mixed continuously until a smooth and uniform blend was achieved (**Figure 1**). Two different concentration ratios of the blended

extracts were utilized for further evaluation, as outlined in **Table I**. These specific ratios were selected based on the outcomes of preliminary experiments aimed at optimizing the combination of plant extracts.

Animal handling

Healthy adult male albino rabbits weighing approximately 3 kg were used in this study. The animals were housed individually in clean cages under standard laboratory conditions, including a 12-hour light/dark cycle and controlled temperature and humidity. Prior to the experiment, the rabbits were acclimatized to the laboratory environment for five days. A standard diet of kale was provided to all rabbits throughout the study. To avoid confounding effects on wound healing, carrots were excluded from the diet, as carrot tubers contain saponins, known to possess wound-healing properties[14].

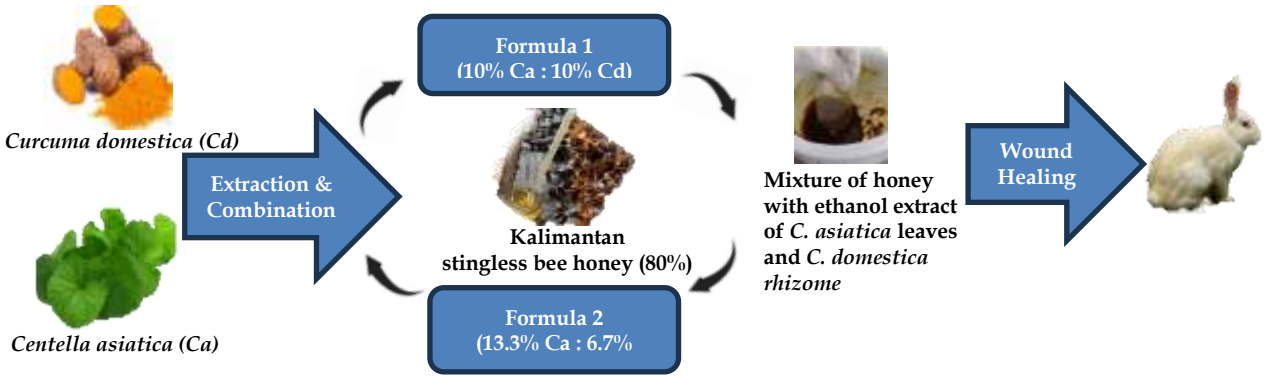

Figure 1. Scheme of mixture of stingless bee honey with ethanol extract of *C. asiatica* leaves and *C. domestica* rhizome.

Table I. Blended honey formulation.

Formula	*Centella asiatica* (%)	*Curcuma domestica* (%)	Extract ratio	Stingless bee honey (%)
F1	10	10	1:1	80
F2	13.33	6.67	2:1	80

Determination of wound healing

This *in vivo* study was conducted with ethical approval from the Health Research Ethics Committee of the Universitas Muhammadiyah Purwokerto, Indonesia (approval number KEPK/UMP/33/III/2022). The study utilized a rabbit model to evaluate wound healing efficacy. Prior to the experiment, the backs of the rabbits were shaved 24 hours beforehand. On the day of the experiment, the shaved area was cleaned with 70% alcohol. A 1.5 cm incision was then made on the subcutaneous layer of the rabbit's skin using sterile surgical instruments. This was achieved by gently lifting the skin with sterile tweezers and making a controlled incision with a sterilized scalpel[15].

Data analysis

This study employed a randomized controlled trial design. A total of three rabbits were included in the study. Each rabbit received three wounds, resulting in nine total wounds. Wound I on each rabbit served as the positive control, treated with povidone-iodine. Wound II on each rabbit was treated with varying concentrations of blended honey applied topically three times daily (every eight hours). Wound III on each rabbit served as the negative control, receiving no treatment. The progress of wound healing was monitored and the size of the wound was measured in cm using a ruler every day for eight days.

RESULTS AND DISCUSSION

Total flavonoid and tannin contents of a blended of C. asiatica and C. domestica extracts

A standard curve was generated for quercetin (**Figure 2**), yielding a linear regression equation of $y = 0.0184x + 0.5256944$ with a high coefficient of determination ($R^2 = 0.9444$, $R = 0.9716$), indicating a strong correlation between absorbance and quercetin concentration. **Table II** presents the TFC and TTC of the two ethanol extract mixtures of *C. asiatica* leaves and *C.*

domestica rhizome (F1 and F2). Based on these phytochemical analyses, the wound healing activity of both F1 and F2 was subsequently evaluated.

Determination of wound healing

The wound healing process was assessed in male albino rabbits administered with either F1 or F2, alongside negative and positive controls, as shown in **Figure 3**. The negative control group exhibited complete wound closure between days 9 and 14. In contrast, all treatment groups, including the positive control, F1, and F2, demonstrated significantly faster wound healing, with complete closure observed between days 6 and 8. Notably, rabbits treated with F2, containing a higher proportion of *C. asiatica* leaf extract, exhibited a slightly faster wound healing rate compared to those treated with F1. This observation suggests that the increased concentration of *C. asiatica* extract in F2 may contribute to enhanced wound healing activity. These findings align with previous studies that have demonstrated the potent wound healing properties of *C. asiatica*, attributed to its rich content of triterpenoids, flavonoids, and other bioactive compounds[16,17].

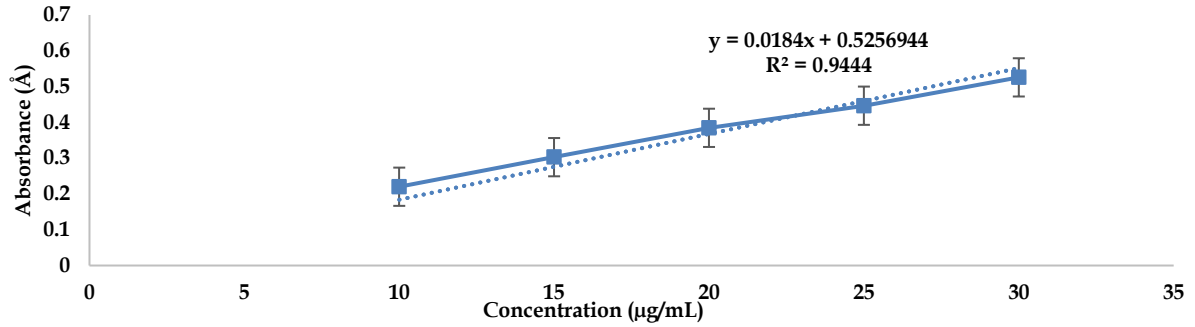

Figure 2. Quercetin standard curve.

Table II. Total flavonoid content of a blended of *C. asiatica* and *C. domestica* extracts.

Formula	TFC (mgQE/g)	TTC (mgQE/g)
F1	18.88+1.21	4.7+0.3
F2	12.57+0.81	6.48+0.12

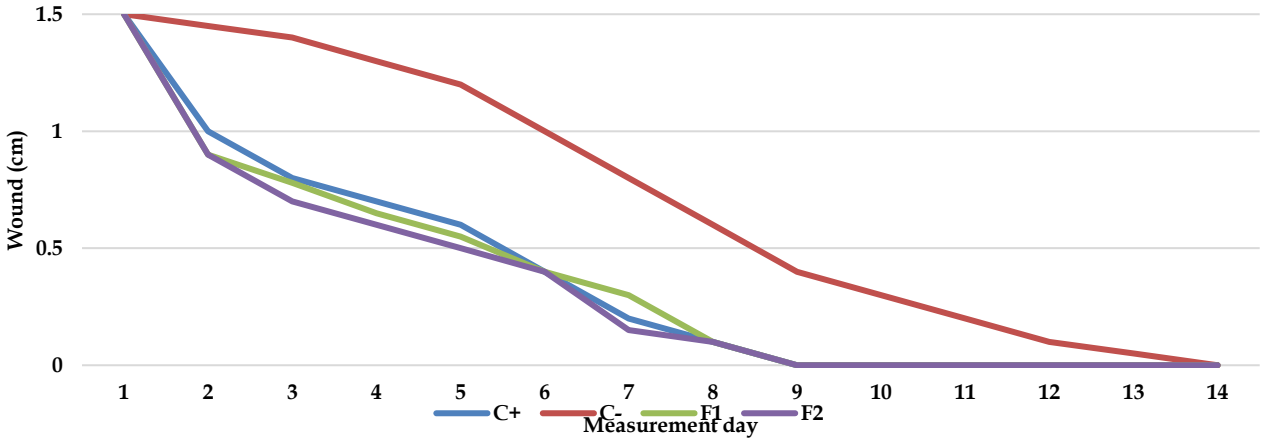

Figure 3. Wound healing activity of samples (C+: positive control; C-: negative control).

All groups exhibited an inflammatory phase on the first day, characterized by redness and swelling. The proliferative phase, characterized by granulation tissue formation and re-epithelialization, was observed from the second to the fifth day in the positive control group. Notably, the proliferative phase in the F1 and F2 groups was shorter, lasting from the second to the third day. The maturation phase, marked by collagen deposition and scar formation, commenced on the sixth day in the positive control group and on the fourth day in the F1 and F2 groups. These findings suggest that the combined preparations of *C. asiatica* leaf extract, *C. domestica* rhizome extract, and stingless bee honey demonstrate the potential to accelerate the wound healing process, particularly by shortening the proliferative phase.

The present study demonstrated promising wound healing activity from a topical formulation incorporating extracts of *C. asiatica* leaves, *C. domestica* rhizomes, and stingless bee honey. These findings align with traditional uses within the community where *C. asiatica* has been employed to treat various ailments, including infectious diseases and wounds[18]. Its therapeutic potential is attributed to a diverse array of phytochemicals, including triterpenoid saponins such as asiaticoside, madecassoside, and asiatic acid, which possess anti-inflammatory, antioxidant, and wound healing properties[19,20]. Notably, asiaticoside has been specifically recognized for its anti-leprotic activity[21]. Previous studies have confirmed the antimicrobial activity of *C. asiatica* extracts, with alcoholic extracts demonstrating greater efficacy against *Staphylococcus aureus* and *Escherichia coli* compared to aqueous infusions[22]. This finding aligns with the observed wound healing outcomes in our study. The inclusion of *C. asiatica* extract in the wound healing formulation (F2) likely contributed to its enhanced antimicrobial activity, potentially leading to a slightly faster wound healing rate compared to the formulation with a lower extract concentration (F1). This observation supports the notion that *C. asiatica* extract may play a crucial role in preventing secondary infections and promoting a more rapid healing process.

The potential for anti-inflammatory properties is also observed in *C. domestica*. A study by Fahryl and Carolia (2019) revealed that numerous bioactive compounds within *C. domestica* exhibit diverse pharmacological properties, including antioxidant, antitumor, antidiabetic, and anti-inflammatory activities[23]. These bioactive compounds encompass a range of molecules such as epigallocatechin gallate (EGCG), carnosol, hydroxitirosol, kaempferol, resveratrol, curcumin, and genistein. Notably, curcumin, a prominent phytochemical found in *C. domestica*, has demonstrated anti-inflammatory efficacy in animal models, specifically in reducing tarsal joint edema in mice. This finding further supports the potential of *C. domestica* as a source of natural compounds with anti-inflammatory properties.

Stingless bee honey, a natural product derived from plant nectar and processed by bees, exhibits a wide range of beneficial properties. It serves as a valuable source of nutrients, including vitamins (A, B_1, B_2, B_3, B_5, B_6, C, D, E, and K), flavonoids, and beta-carotene[24]. Historically, honey has been utilized in traditional medicine for treating respiratory diseases, digestive tract infections, and various other ailments. While sharing some commonalities with other honey types, stingless bee honey possesses distinct characteristics, notably its often-sour taste[25]. These unique properties, combined with its nutritional richness, make it a promising ingredient in wound healing formulations. The current study explored the efficacy of two blended formulations containing stingless bee honey, both of which demonstrated excellent wound healing activity in previous testing.

Povidone-iodine 10% served as the positive control in this study, as it is widely recognized as a gold-standard antiseptic. Povidone-iodine is a complex of polyvinylpyrrolidone and iodine, exerting its antibacterial action through the slow release of iodine upon contact with injured tissue. This released iodine disrupts bacterial metabolism by inhibiting enzymes and damaging bacterial cell structures, ultimately leading to bacterial death[26].

The combination formulations containing *C. asiatica* leaf extract, *C. domestica* rhizome extract, and stingless bee honey were hypothesized to operate through similar mechanisms. Tannins, present in the extracts, possess inherent antibacterial properties. Furthermore, curcuminoids from *C. domestica* exhibit both antibacterial and anti-inflammatory activities[27]. These combined effects within the formulations were expected to synergistically promote wound healing and accelerate the reduction of inflammation. While both formulations (F1 and F2) demonstrated significant wound healing activity, F2 exhibited a slightly faster healing rate. This minor difference might be attributed to subtle variations in the total flavonoid and tannin contents between the two formulations, although these differences were not statistically significant.

CONCLUSION

The blended F2 combination, comprising *C. asiatica* leaf extract and *C. domestica* rhizome extract with stingless bee honey, demonstrated promising wound healing activity in this study. After seven days of treatment, the wound healing effect of F2 was observed to be slightly superior to that of F1 and the positive control group. This enhanced activity may be attributed to the higher tannin content observed in F2, which is known to possess wound-healing properties. These findings suggest that the F2 combination can serve as a valuable standard blended formula for further in-depth investigations into the potential of these natural ingredients in wound healing applications.

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AUTHORS' CONTRIBUTION

Conceptualization: Diniatik
Data curation: Diniatik, Paula Mariana Kustiawan
Formal analysis: Diniatik,
Funding acquisition: Diniatik
Investigation: Diniatik, Paula Mariana Kustiawan
Methodology: Diniatik
Project administration: Diniatik
Resources: Diniatik, Paula Mariana Kustiawan
Software: Halida Suryadini
Supervision: Diniatik, Paula Mariana Kustiawan
Validation: Diniatik
Visualization: Paula Mariana Kustiawan
Writing - original draft: Diniatik, Paula Mariana Kustiawan, Halida Suryadini
Writing - review & editing: Diniatik, Paula Mariana Kustiawan

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this study.

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Research Article

Physicochemical Properties and Antioxidant Potential of Gels from Foam Mat Drying and Ethanolic Extract of Red Spinach (*Amaranthus cruentus*)

Ibrahim Arifin [1]

Khoirul Anwar [1]

Muhammad Farih Arsyada [2]

Nevy Pratiwi [2]

Ayu Shabrina [3*]

[1] Department of Chemical Pharmacy, Universitas Wahid Hasyim, Semarang, Central Java, Indonesia
[2] Undergraduate Pharmacy Program, Universitas Wahid Hasyim, Semarang, Central Java, Indonesia
[3] Department of Pharmaceutics and Pharmaceutical Technology, Universitas Wahid Hasyim, Semarang, Central Java, Indonesia

*email: shabrina@unwahas.ac.id; phone: +6287836100003

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Abstract

Red spinach (*Amaranthus cruentus*), a rich source of antioxidant anthocyanins, is susceptible to light degradation. Foam mat drying (FMD) is a promising technique to preserve these pigments. This study compared the physicochemical properties and antioxidant activity of gels incorporating FMD and ethanolic extracts of *A. cruentus* (EEAC). Both were incorporated into gels at varying concentrations (1%, 3%, and 5%). FMD gels exhibited a darker red color and significantly higher total anthocyanin content (8.33 ± 0.25/100 g) and stronger antioxidant activity (IC_{50} of 35.67 ± 1.87 ppm) compared to EEAC gels (10.45 ± 0.15/100 g and IC_{50} of 47.88 ± 2.45 ppm, respectively). Both gel types had similar pH values (5.32-5.77). Increasing the concentration of either extract affected the viscosity, spreadability, and adhesion of the gels. Importantly, FMD gels displayed significantly higher antioxidant activity (58.75 ± 2.12 to 64.72 ± 2.01%) than EEAC gels (31.75 ± 2.13 to 50.12 ± 3.01%) across all formulations. These findings suggest that FMD-based gels offer a superior delivery system for *A. cruentus* antioxidants, potentially leading to innovative food products with enhanced nutritional value and health benefits.

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INTRODUCTION

Natural plant extracts have gained significant popularity in the cosmetic industry, offering consumers a preference for products derived from natural sources. However, the incorporation of these extracts often presents challenges. One major concern is the inherent instability of many plant-based pigments, which can undergo undesirable color changes, such as darkening, during storage. This phenomenon is typically attributed to the oxidation of bioactive compounds within the extract[1]. Various approaches have been explored to enhance the stability of these natural pigments, including encapsulation, lyophilization, and oxygen exclusion[2]. While effective in some cases, these methods may not be readily applicable to all cosmetic formulations, particularly those with a gel-based consistency, and can pose challenges for large-scale industrial production.

Red spinach (*Amaranthus cruentus*) is a rich source of anthocyanins, a class of pigments with potent antioxidant properties[3,4]. Anthocyanins, naturally occurring pigments responsible for the vibrant colors in many fruits and vegetables, possess potent antioxidant properties with potential health benefits[5]. These pigments are responsible for the vibrant red color of the leaves. However, anthocyanins are susceptible to degradation due to environmental factors such as high temperature, intense light exposure, and changes in pH[6,7]. In alkaline conditions, for instance, anthocyanins undergo structural changes and form

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colorless compounds[8]. Despite these challenges, *A. cruentus* extracts have demonstrated significant antioxidant activity in various studies. Moilati *et al*.[9] reported potent antioxidant activity of *A. cruentus* ethanol extract with an IC_{50} value of 2.82 ppm. Similarly, Ni'am *et al*.[10] observed potent antioxidant activity in *A. cruentus* extract sheet masks, with an IC_{50} value of 68.55 ppm. These findings highlight the potential of *A. cruentus* extracts as a valuable source of natural antioxidants for various applications.

Extraction is a widely employed technique to isolate bioactive compounds from natural sources. However, extracted natural products often exhibit rapid color degradation, resulting in a darkening of the extract[11]. To mitigate this issue, various drying methods have been explored. Foam mat drying (FMD) has emerged as a promising technique for preserving the color and quality of natural products[12]. In FMD, maltodextrin acts as a foaming agent, enabling the formation of a foam matrix that is subsequently dried at low temperatures under vacuum conditions[13]. This gentle drying process minimizes the risk of thermal degradation and oxidation of heat-sensitive bioactive compounds[14]. Furthermore, FMD produces a dried product with desirable characteristics, such as vibrant color, appealing aroma, and pleasant taste, often closely resembling the fresh material[15]. The FMD process also offers significant advantages in terms of drying time, typically completing within 24 hours. The resulting dried product is typically a fine, crystalline powder with low moisture content and intense color[16].

Encapsulation technologies have emerged as promising approaches for improving the stability and bioavailability of these bioactive compounds. While previous research[17] has explored the microencapsulation of anthocyanins for food preservation using hydrogel matrices, their application in topical formulations has been limited. This study aims to investigate the physical characteristics and antioxidant activity of FMD containing *A. cruentus* extract encapsulated within a hydrogel matrix. By comparing the properties of these FMD with those of a *A. cruentus* extract gel, this research seeks to evaluate the potential of this encapsulation technique for developing stable and effective topical delivery systems for anthocyanin-rich extracts.

MATERIALS AND METHODS

Materials

Fresh *A. cruentus* leaves were obtained from the Bandungan area in Semarang Regency, Indonesia. The plant species was authenticated by the Herbarium of the Semarang College of Pharmaceutical Sciences (STIFAR), with a certificate number of 026/EL-AFM/II/2023. The gel base formulation consisted of carbopol 940, Tween 80, Span 80, sorbitol, liquid paraffin, triethanolamine (TEA), methylparaben, propylparaben, and distilled water, all of pharmaceutical grade and purchased from Multi Kimia Raya Chemical. Dimethyl sulfoxide (DMSO), used as a solvent, was of analytical grade and obtained from Sigma Aldrich through PT Kairos.

Methods

Extraction

Fresh *A. cruentus* leaves (1 kg) were thoroughly washed with distilled water to remove any adhering soil and debris. The washed leaves were then sorted to remove any discolored or damaged parts. Subsequently, the leaves were dried in an oven at 40°C for 8 hours until they reached a constant weight. The dried leaves were then ground into a fine powder using a blender. Maceration was employed for the extraction process. Briefly, 100 g of the powdered plant material was macerated with 500 mL of 96% ethanol for 24 hours at room temperature. This process was repeated three times (3x24 hours) with fresh ethanol each time. After each maceration step, the extract was filtered through Whatman No. 1 filter paper. The combined filtrates were then concentrated using a rotary evaporator at 50°C under reduced pressure to obtain a thick extract. The yield of the extract was calculated gravimetrically. Finally, the obtained thick ethanolic extracts of *A. cruentus* (EEAC) was incorporated into a gel base according to the formulation described by previous research[18].

Foam mat drying of A. cruentus

Fresh *A. cruentus* leaves were meticulously washed to remove any adhering dirt and potential pests. Subsequently, the leaves were air-dried under ambient conditions to eliminate excess moisture. A 1:1 (w/v) ratio of fresh leaves to distilled water was employed for the preparation of a leaf slurry. This slurry was then homogenized using a high-speed blender. To enhance foam formation, 6% (v/v) Tween 80 was incorporated into the homogenate and the mixture was vigorously mixed

for 8 minutes using a laboratory mixer. Subsequently, 15% (w/w) maltodextrin was added to the mixture and stirred for an additional 3 minutes. The resulting mixture was carefully spread onto aluminum foil trays lined with 1 mm thick aluminum foil. These trays were then placed in a tray dryer maintained at a constant temperature of 40°C for 60 minutes. The drying process was continued until a constant weight was achieved, indicating complete moisture removal. Finally, the dried *A. cruentus* leaf FMD was pulverized and sieved through a No. 60 mesh sieve to obtain a fine powder[19].

Formulation of EEAC and FMD gels

Carbopol 940 (1%) was dispersed in 10 mL of distilled water heated to 80°C in a glass beaker with constant stirring for 1 hour. The dispersion was then transferred to a mortar and neutralized with triethanolamine dropwise under continuous stirring until a transparent gel (Mixture A) was formed. Separately, hydroxypropyl methylcellulose (HPMC) (0.5%) was also dispersed in 10 mL of distilled water at 80°C for 1 hour and then transferred to a mortar. The HPMC dispersion was stirred until a transparent gel (Mixture B) was obtained. Methylparaben (0.15%) was dissolved in propylene glycol (Mixture C). Subsequently, Mixtures A and B were sequentially added to Mixture C with continuous stirring until a homogenous gel base was formed. The EEAC (1% w/w) was dissolved in 5 mL of 96% ethanol and then incorporated into the gel base with gentle stirring. For the FMD formulations, the *A. cruentus* FMD (1% w/w) was dissolved in propylene glycol and added to the gel base. Finally, distilled water was added to the mixtures to achieve the desired final volume and ensure homogeneity[2,20]. **Table I** summarizes the compositions of the EEAC and FMD gel formulations.

Table I. Formulation of EEAC and FMD gels.

Materials	Concentration (g)					
	F1	F2	F3	F4	F5	F6
Amaranthus cruentus FMD	1	3	5	-	-	-
Amaranthus cruentus EEAC	-	-	-	1	3	5
Carbopol 940	1	1	1	1	1	1
HPMC	0.5	0.5	0.5	0.5	0.5	0.5
Propylene glycol	10	10	10	10	10	10
Methylparaben	0.18	0.18	0.18	0.18	0.18	0.18
Triethanolamine	3	3	3	3	3	3
Distilled water	Up to 100					

Determination of anthocyanin levels

To assess the stability of EEAC and FMD under simulated gastric (pH 1.0) and intestinal (pH 4.5) conditions, solutions were prepared by dissolving a known amount of EEAC or FMD in respective buffer solutions. The ratio of EEAC/FMD to buffer was maintained at 1:5 (v/v). The prepared solutions were incubated at room temperature for 15 minutes. Subsequently, the absorbance of each solution was measured at the maximum wavelength of 512 nm using a spectrophotometer. To correct for any background interference, a second measurement was taken at 700 nm[21].

Physical properties of gel

Physical characterization of the gels was conducted to assess their suitability for topical application. Organoleptic properties, including color, odor, and texture, were evaluated through direct visual observation. Homogeneity was assessed by visually inspecting the gel for any visible phase separation or sedimentation after gentle shaking. pH was measured using a calibrated pH meter. Viscosity was determined using a viscometer, and spreadability as well as adhesion was evaluated by measuring the time taken (minutes) for a defined volume of gel to spread over a specified area (cm) on a glass slide[22].

Antioxidant activity test

The antioxidant activity of the EEAC, FMD, and the developed gel formulations was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. For the EEAC and FMD, a series of concentrations (50, 100, 150, 200, and 250 ppm) were prepared. One milliliter of each sample was mixed with 2 mL of 0.1 mM DPPH solution in a test tube, and the volume was adjusted to 5 mL with ethanol. The mixture was incubated for 30 minutes at room temperature in the dark. Subsequently, the absorbance of the reaction mixture was measured at 516.12 nm using a spectrophotometer. The percentage of DPPH radical scavenging activity was calculated, and the IC_{50} values, representing the concentration of the sample required to inhibit 50% of DPPH radicals, were determined[23].

For the gel samples, 1 g of each gel formulation was dissolved in 10 mL of 96% ethanol. Three milliliters of this solution was then mixed with 2 mL of 0.1 mM DPPH solution, shaken vigorously, and incubated for 30 minutes at room temperature in the dark. The absorbance of the reaction mixture was measured at 516.12 nm using a spectrophotometer. The percentage of DPPH radical scavenging activity of the gel formulations was subsequently calculated.

Data analysis

To assess the impact of the incorporated extract on the physicochemical properties of the gels, a two-way ANOVA was employed to analyze data on total anthocyanin content, pH, viscosity, stickiness, spreadability, and percentage inhibition. This statistical method allowed for the evaluation of the effects of both the gel formula (FMD vs. EEAC) on these parameters. Organoleptic properties, including appearance, odor, and texture, were evaluated and described descriptively.

RESULTS AND DISCUSSION

The extraction of *A. cruentus* yielded a 38.86% thick EEAC, characterized by a dark brown color and a distinctive *A. cruentus* aroma. In contrast, the FMD exhibited a 92.43% yield and presented as a dark red powder with a characteristic *A. cruentus* aroma. **Figure 1** visually depicts the physical appearance of both the EEAC and FMD. The significant difference in yield between the two methods can be attributed to the foaming process involved in FMD preparation, which necessitates the addition of a foaming agent to the *A. cruentus* powder. Conversely, the traditional extraction method, involving solvent evaporation, leads to a substantial loss of mass due to the removal of the solvent.

a **b**

Figure 1. Appearance of (**a**) EEAC and (**b**) FMD of *A. cruentus*.

Table II presents the total anthocyanin content of EEAC and FMD. Statistical analysis revealed a significant difference ($p < 0.05$) in anthocyanin content between the two samples, with FMD exhibiting significantly higher levels than the ethanolic extract. This discrepancy can be attributed to the inherent limitations of the conventional extraction process. Prolonged extraction times and elevated temperatures can lead to the degradation of heat-sensitive anthocyanins. Studies have shown that high temperatures accelerate the degradation of these pigments[24]. Moreover, the extraction process itself can contribute to the oxidation and degradation of flavonoid compounds, including anthocyanins[25]. Drying methods involving temperatures exceeding 60°C can further exacerbate this issue, leading to the oxidation of anthocyanins and a noticeable darkening of the sample[26].

In contrast, the FMD effectively preserves the labile anthocyanin pigments. By rapidly removing water through sublimation, FMD minimizes thermal degradation and oxidative processes, resulting in higher retention of these bioactive compounds. This finding aligns with previous research highlighting the advantages of FMD for preserving the stability and bioactivity of heat-sensitive compounds[13]. Furthermore, the FMD process offers several advantages over conventional drying methods, including lower operating costs, reduced drying time, and lower energy consumption[27]. These factors make FMD a more suitable and sustainable approach for preserving the bioactive compounds of *A. cruentus* for various applications.

Table II. Anthocyanin levels in EEAC and FMD.

Sample (n =3)	Yield (%)	Anthocyanin levels ± SD (per 100 g)	IC_{50} ± SD (ppm)
Amaranthus cruentus EEAC	38.86 ± 4.20	8.33 ± 0.25	47.88 ± 2.45
Amaranthus cruentus FMD	92.43 ± 2.21	10.45 ± 0.15	35.67 ± 1.87

The visual characteristics of the gels formulated with EEAC and FMD are depicted in **Figure 2**. The EEAC gel exhibited a dark brown color, a characteristic *A. cruentus* odor, and a sticky consistency. In contrast, the FMD gel displayed a dark red color, a distinctive *A. cruentus* scent, and a rapid film-forming property upon application to the skin.

a **b** **c** **d** **e** **f**

Figure 2. Gels made from EEAC (**a-c**) and FMD (**d-f**). (**a**) F1, (**b**) F2, (**c**) F3, (**d**) F4, (**e**) F5, and (**f**) F6.

A comprehensive overview of the physical characteristics of both gel formulations is presented in **Table III**. The pH of the formulated gels was evaluated to assess their stability and potential for topical application. No significant differences in pH were observed between the gels prepared with EEAC and those prepared with FMD. This finding suggests that the drying method did not significantly alter the acidic nature of the extract. Furthermore, an increase in the concentration of *A. cruentus* extract resulted in a decrease in the pH of the gels. This observation is consistent with the acidic nature of anthocyanins, the primary bioactive compounds in *A. cruentus*, which are known to lower the pH of solutions[16,28].

The present study investigated the influence of increasing concentrations of EEAC and FMD on the viscosity of developed hydrogels. A significant increase in viscosity was observed with increasing concentrations of both EEAC and FMD. This finding aligns with previous research, which demonstrated that an increase in the concentration of natural polymers and bioactive compounds generally leads to an increase in the viscosity of formulations[29]. Notably, within each formula (varying EEAC and FMD concentrations), a significant difference in viscosity was observed, indicating that the concentration of the active substances directly influenced this rheological property. However, statistical analysis revealed no significant difference in viscosity between gels containing the same concentration of active substances, regardless of whether the active substance was EEAC or FMD. This suggests that the viscosity of the hydrogel is primarily influenced by the overall concentration of the active ingredient, rather than the specific type of active substance.

The spreadability of the EEAC and FMD gels was evaluated at the same concentration. No significant differences in spreadability were observed between the two formulations. This finding aligns with previous research demonstrating that increasing the concentration of active substances within a gel matrix can lead to an increase in viscosity, consequently reducing its spreadability[30]. This inverse relationship between viscosity and spreadability is well-established in the literature[31].

The adhesion properties of the EEAC and FMD gels were evaluated. Notably, no significant differences in adhesion were observed between gels containing the same concentration of active ingredients, nor were any variations found between different formulations. These findings align with previous research conducted by Rahmawati *et al*.[32], which reported no discernible effect of increasing the concentration of active substances on gel adhesion. However, Puspita *et al*.[33] demonstrated that exceeding an active substance concentration of 10% can indeed influence adhesion properties. The present study suggests that within the concentration ranges investigated, the active ingredients in EEAC and FMD gels did not significantly impact their adhesive properties.

The IC_{50} values of the ethanolic extract of EEAC and FMD gels exhibited significant differences ($p < 0.05$). The FMD gel demonstrated higher IC_{50} values compared to the EEAC gel, indicating a lower antioxidant activity. These findings were consistent with the observed gel inhibition percentages. Both EEAC and FMD gels demonstrated a dose-dependent increase in antioxidant activity, aligning with previous research that higher concentrations of bioactive compounds generally correlate with increased antioxidant activity[34].

The enhanced antioxidant activity of the FMD gel can be attributed to the milder processing conditions employed during drying. The FMD process operates at lower temperatures compared to the conventional extraction method[34], which is crucial for preserving the stability of heat-sensitive compounds such as anthocyanins. Previous studies have reported that anthocyanins are susceptible to degradation at temperatures exceeding 50°C[35,36]. Furthermore, the shorter processing time of the FMD process (3 hours) compared to the 8-hour extraction process may have minimized the potential for degradation of bioactive compounds.

Table III. Physical characterization of EEAC and FMD gels.

Parameters (n = 3)	Formula					
	F1	F2	F3	F4	F5	F6
pH	5.77 ± 0.68	5.65 ± 0.72	5.45 ± 0.88	5.68 ± 0.65	5.54 ± 0.71	5.32 ± 0.84
Viscosity (cps)	98.45 ± 2.33[a]	115 ± 4.18[a]	122 ± 8.75[a]	95.21 ± 3.41[b]	120.25 ± 8.14[b]	134.15 ± 5.18[b]
Spreadability (cm)	5.63 ± 0.11[a]	4.77 ± 0.14[a]	3.81 ± 0.12[a]	5.56 ± 0.13[b]	4.68 ± 0.1[b]	3.78 ± 0.11[b]
Adhesion (minutes)	2.28 ± 0.55[a]	2.33 ± 0.61[a]	2.41± 0.54[a]	2.31 ± 0.47[b]	2.45 ± 0.63[b]	2.32± 0.48[b]
% inhibition	31.75 ± 2.13[ac]	48.66 ± 3.15[ac]	50.12 ± 3.01[ac]	58.75 ± 2.12[bc]	61.19 ± 2.35[bc]	64.72 ± 2.01[bc]

Note: [a]: significantly different between each EEAC formula; [b]: significantly different between each PMK formula; [c]: significantly different between EEAC compared to FMD at the same concentration.

Foam mat drying demonstrated significant potential in enhancing the stability of anthocyanin pigments within Amaranthus cruentus extract. The presence of maltodextrin in the foaming process likely acted as a protective agent, encapsulating the pigments and minimizing their degradation during drying[37]. This is supported by the observation that FMD-dried *A. cruentus* exhibited superior color retention compared to conventionally dried samples. Furthermore, the relatively short drying time and low-temperature processing conditions inherent to FMD contribute to its efficiency and minimize potential oxidative damage to the pigments[38]. These findings align with previous studies that have successfully employed FMD to preserve the color and bioactive compounds of various plant materials, particularly those containing sensitive phytochemicals[39,40].

The enhanced stability of anthocyanins in FMD-dried *A. cruentus* was reflected in the improved antioxidant activity observed in this study. This finding corroborates the results of Farid *et al.*[41], who reported significant antioxidant activity and flavonoid content in FMD-dried plant materials. The optimal maltodextrin concentration for maximizing anthocyanin retention in this study (10%) aligns with previous findings[42]. Moreover, the low-temperature drying conditions employed in FMD (below 50°C) are known to effectively preserve the phytochemical content of plant extracts. The observed enhancement in antioxidant activity in the *A. cruentus* gel formulated with FMD-dried extract further supports the efficacy of this drying method. The foam matrix likely provides a protective environment that minimizes the degradation of anthocyanins and other phenolic compounds during processing and storage[43].

CONCLUSION

This study demonstrated that FMD successfully preserved the bioactive properties of *A. cruentus* leaves. The FMD powder exhibited enhanced antioxidant activity compared to the conventionally dried extract, as evidenced by a lower IC_{50} value in the DPPH assay. Furthermore, the incorporation of FMD powder into gel formulations resulted in improved antioxidant activity compared to gels containing the conventionally dried extract. This finding suggests that FMD effectively preserves the bioactive compounds, particularly anthocyanins, responsible for the antioxidant activity. Importantly, the FMD process also maintained the color and overall physical characteristics of the gels, indicating its potential for producing stable and visually appealing formulations. These findings highlight the potential of FMD as a promising technique for preserving the bioactive properties and enhancing the utilization of *A. cruentus* leaves in various food and pharmaceutical applications.

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AUTHORS' CONTRIBUTION

Conceptualization: Ibrahim Arifin, Khoirul Anwar, Ayu Shabrina
Data curation: Muhammad Farih Arsyada, Nevy Pratiwi, Ayu Shabrina
Formal analysis: Ibrahim Arifin, Khoirul Anwar, Ayu Shabrina
Funding acquisition: Ibrahim Arifin, Khoirul Anwar, Ayu Shabrina
Investigation: Ibrahim Arifin, Khoirul Anwar, Ayu Shabrina
Methodology: Ibrahim Arifin, Khoirul Anwar, Ayu Shabrina
Project administration: Ibrahim Arifin, Khoirul Anwar, Ayu Shabrina
Resources: Ibrahim Arifin, Khoirul Anwar, Ayu Shabrina
Software: -
Supervision: Ibrahim Arifin, Khoirul Anwar, Ayu Shabrina
Validation: Ibrahim Arifin, Khoirul Anwar, Ayu Shabrina
Visualization: -
Writing - original draft: Muhammad Farih Arsyada, Nevy Pratiwi
Writing - review & editing: Ibrahim Arifin, Khoirul Anwar, Ayu Shabrina

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this study.

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Research Article

Rationality of Tuberculosis Treatment for Drug-Susceptible Pulmonary Tuberculosis at a Primary Care in Bandung

Alfi Nurul Islamiyah [1*]
Syarifuddin [1]
Morsalina Akhsa [1]
Iis Rukmawati [2]
Eni Margayani [1]
Linda Purnamawati Suherman [1]
Vina Septiani [1]
Robby Ramdani [1]

[1] Department of Pharmacy, Universitas Jenderal Achmad Yani, Cimahi, West Java, Indonesia
[2] Pharmacy Installation, Ibrahim Adjie Primary Care, Bandung, West Java, Indonesia

*email: alfi.nurul@lecture.unjani.ac.id; phone: +6281220542445

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Abstract

Tuberculosis (TB) remains a significant global health concern, particularly in Indonesia. Ensuring rational TB treatment is crucial for effective disease control and preventing the emergence of drug resistance. This study aimed to evaluate the rationality of TB treatment among newly diagnosed drug-susceptible pulmonary TB patients in a primary care center in Bandung. A descriptive-analytical study was conducted on 56 patients who met the inclusion and exclusion criteria. Treatment rationality was assessed based on the Indonesian Society of Respirology 2021 and the National Guidelines for Medical Services 2020. While 100% of patients received the correct medication for the right indication and dose, the duration of therapy was suboptimal for most patients (83.93%) due to drug unavailability. This resulted in a lower-than-ideal treatment regimen, potentially compromising treatment outcomes and increasing the risk of drug resistance. These findings highlight the need for improved drug supply management and adherence to treatment guidelines to optimize TB treatment outcomes and reduce the burden of TB in Indonesia.

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INTRODUCTION

Tuberculosis (TB) is an ancient and enduring global health challenge. Despite decades of treatment efforts, TB remains a significant public health concern, particularly in countries like Indonesia. With a 9.2% global burden, Indonesia ranks second globally in TB cases, following India (28%) and China (7.4%). This persistent burden underscores the urgent need for continued research and innovative approaches to combat this devastating disease[1].

The World Health Organization (WHO) Global Tuberculosis Report 2022 highlights a concerning global trend. In 2021, an estimated 10.6 million people were affected by TB, reflecting a 4.5% increase compared to 2020. This global increase was driven by factors such as the COVID-19 pandemic, which disrupted TB prevention and care services. Notably, Indonesia significantly contributed to this rise, with a 13% increase in TB incidence rates between 2020 and 2021. Geographically, the Southeast Asia region bore the heaviest burden, accounting for 45% of global TB cases in 2021. Indonesia, classified as a high TB burden country, faces significant challenges in TB control, with treatment coverage rates remaining alarmingly low, estimated to be below 50% in 2021. These alarming statistics underscore the urgent need for intensified efforts to combat TB in Indonesia and improve access to effective diagnosis and treatment[2].

Tuberculosis remains a significant public health challenge globally, including in Indonesia. According to the WHO, an estimated 969,000 TB cases were reported in Indonesia in 2021, representing an alarming 17% increase compared to the previous year. This translates to one person being infected with TB every 33 seconds[3]. In West Java province, the number of

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TB cases also showed a concerning trend, rising from 85,681 in 2021 to 106,661 in 2022[4]. Within West Java, Bogor Regency, Bandung City, and Bekasi City reported the highest burden of TB, collectively accounting for 6-14% of the total cases in the province. Specifically, Bandung City witnessed a substantial increase in TB cases, rising from 8,504 in 2021 to 14,541 in 2022[5]. These figures underscore the urgent need for effective interventions to control the ongoing TB epidemic in Indonesia, particularly in high-burden areas such as West Java.

Rational drug use is paramount for successful TB treatment, ensuring optimal therapeutic outcomes and minimizing the emergence of drug-resistant strains[6,7]. This necessitates accurate and timely diagnosis, appropriate drug selection, and adherence to prescribed regimens. A key factor influencing treatment success is the correct dosing and duration of therapy. However, studies have shown that inappropriate dosing and treatment durations are prevalent in certain settings. For instance, a study conducted at the Sikumana Primary Care clinic found that among 65 sputum smear-positive pulmonary TB patients, 8 received inappropriate doses, and 11 received treatment for an incorrect duration. Treatment outcomes in this study were varied, with 47.7% of patients achieving treatment success, 36.9% completing treatment, and a combined 13.8% experiencing treatment failure, discontinuation, or death[8]. These findings underscore the critical need for improved TB treatment practices, emphasizing the importance of adherence to standardized treatment guidelines and regular monitoring of patient outcomes. Understanding the factors that contribute to inappropriate prescribing practices is crucial for developing effective interventions to improve TB treatment outcomes and ultimately combat this global health threat[9].

This study aimed to evaluate the rationality of TB treatment regimens in patients with drug-susceptible pulmonary TB. Rational TB treatment involves administering appropriate medications at correct dosages based on well-established guidelines. This study was conducted at a Primary Care center in Bandung, Indonesia, to investigate various aspects of TB treatment utilization, including prescribing patterns, medication quality, and treatment outcomes. To assess the quality of TB treatment, this study employed an audit methodology, comparing actual treatment practices to the national TB treatment guidelines[7,10]. This approach provides valuable insights into the current state of TB treatment adherence and identifies areas for improvement in TB care delivery.

MATERIALS AND METHODS

Materials

This study utilized data from a Primary Care clinic in Bandung, Indonesia, to investigate specific aspects of TB treatment. Data were collected from patient card sheets and medical records of outpatients newly diagnosed with drug-susceptible pulmonary TB who received treatment at the clinic between January 2021 and December 2022. A non-probability purposive sampling technique was employed to select patients who met the inclusion criteria:

1. Age ≥15 years
2. Completed 6 months of TB treatment
3. Complete patient card sheets and medical records

Patients with co-morbidities and those who died, discontinued treatment, or were transferred to other facilities were excluded from the study. Data collected included patient demographics (initials, gender, age, and weight), diagnosis, laboratory examination results, and dispensing data (prescribed drug, dose, and quantity dispensed).

Methods

This retrospective cohort study aimed to describe drug use patterns, identify treatment outcomes, and assess the standard of care in drug-susceptible pulmonary TB patients. The study included 67 adult patients (≥15 years) diagnosed with pulmonary TB who received treatment at a Primary Care Center in Bandung, Indonesia, between 2021 and 2022. Eleven patients were excluded from the analysis: two due to comorbid diabetes mellitus, four due to death during treatment, two due to loss to follow-up, and three due to transfer to another facility. Thus, a total of 56 patients were research subjects, as presented in **Figure 1**. This study focused on both the processes and outcomes of drug use in patients with drug-susceptible pulmonary TB. The analysis centered on patient-level data, providing insights into drug utilization patterns, including drug selection, dosage, duration of therapy, and adherence to national guidelines. This study received ethical exemption from the Research Ethics Committee of Universitas Padjadjaran Bandung (No. 381/UN6.KEP/EC/2023).

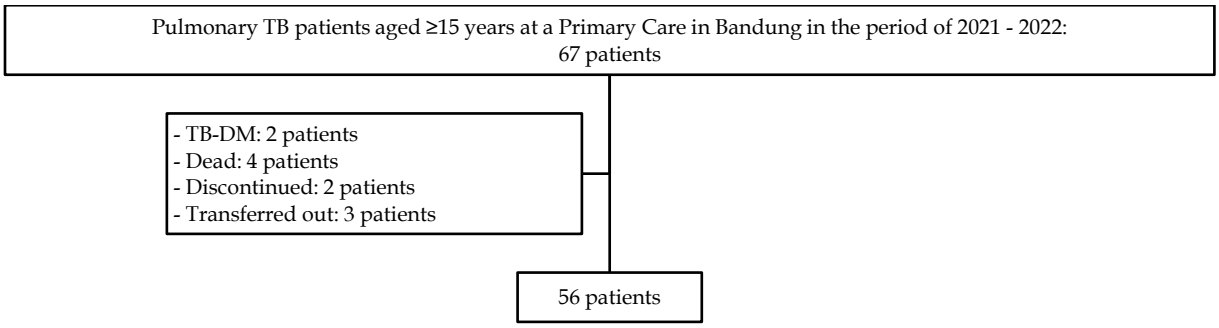

Figure 1. Research sample.

Data analysis

To assess the quality of TB treatment, drug use practices were compared against established national standards. These standards included the *Perhimpunan Dokter Paru Indonesia* (PDPI; Indonesian Society of Respirology) 2021 guidelines[6] and the *Pedoman Nasional Pelayanan Kedokteran* (PNPK; National Guidelines for Medical Services) 2020[8]. These indicators encompassed several key aspects:

1. Medication Indication: Evaluation of the appropriateness of drug prescription for the diagnosed condition.
2. Medication Selection: Assessment of adherence to recommended drug formularies and guidelines.
3. Medication Dosage: Analysis of whether prescribed dosages were appropriate for individual patients, considering factors such as age, weight, and renal/hepatic function.
4. Duration of Therapy: Evaluation of the appropriateness of the prescribed treatment duration based on clinical guidelines and patient-specific factors.
5. Treatment Outcomes: Assessment of the effectiveness of the prescribed medications in achieving desired clinical outcomes, such as symptom reduction, disease control, or cure.

RESULTS AND DISCUSSION

This study included 56 of 67 pulmonary TB patients aged ≥15 years diagnosed at a Primary Care facility in Bandung, Indonesia, between 2021 and 2022 (**Table I**). This sample size aligns with the recommended minimum of 50-75 patient records per healthcare facility for meaningful analysis of drug use and treatment evaluation[11]. The study population exhibited a female predominance, with 34 (60.71%) female patients and 22 (39.29%) male patients. This finding is partially consistent with previous research. For instance, a study in China reported a marginally higher risk of pulmonary TB in females under 40 years of age and a significantly higher risk in males over 40[12]. This disparity may be influenced by factors such as hormonal differences, occupational exposures, and social determinants of health. However, gender disparities in TB incidence are not well-documented, and further research is needed to fully understand their underlying mechanisms. A study conducted in Harare, Zimbabwe, also highlighted the nuanced role of gender in TB, demonstrating that women were more likely to develop severe forms of the disease[13]. These findings emphasize the importance of considering gender-specific factors in TB prevention and treatment strategies.

Table I. Patient demographic information.

Patient information	n (56)	%
Age (years)		
15-24 (adolescence)	19	33.93
25-34 (young adults)	10	17.86
35-44 (adults)	10	17.86
45-54 (older adults)	10	17.86
55-64 (elderly)	4	7.14
≥65 (old)	3	5.36
Gender		
Male	22	39.29
Female	34	60.71

This study found that the highest prevalence of pulmonary TB was observed in the adolescent age group (15-24 years), comprising 33.93% of the patient population. Adults aged 35-44 years and young adults aged 25-34 years followed, accounting for 17.86% and 17.86% of cases, respectively. These findings align with previous research conducted at the Oebobo Health Center in 2020, which reported a high prevalence of pulmonary TB among late adolescents (18-24 years) and young adults (25-43 years)[13]. The elevated risk of pulmonary TB across all age groups above 15 years is likely attributable to several factors, including cumulative exposure to *Mycobacterium tuberculosis*, smoking, air pollution, and other age-related conditions. Adolescents are particularly vulnerable due to factors such as increased social interactions, frequent community transmission, and potential for school-based outbreaks, all of which increase their exposure risk[15].

The cornerstone of TB management lies in the adherence to recommended treatment regimens, which are crucial for effective disease control and minimizing transmission. The WHO recommends a 6-month regimen for new pulmonary TB cases, consisting of 2 months of intensive phase treatment with rifampicin followed by 4 months of continuation phase treatment with rifampicin and isoniazid. Daily dosing is the preferred regimen for optimal treatment outcomes, as it ensures consistent drug levels and enhances treatment adherence[14]. However, this study revealed that 38 out of 53 patients (67.86%) received thrice-weekly dosing due to the unavailability of daily dose medications (**Table II**). This deviation from the recommended regimen may compromise treatment efficacy and increase the risk of treatment failure and drug resistance.

Table II. TB treatment patterns.

Regimen	n (56)	%
2HRZE/4HR*	18	33.93
2HRZE/4H3R3**	38	67.86

Note: *2HRZE/4HR regimen: isoniazid, rifampicin, pyrazinamide, and ethambutol daily for the first 2 months in the intensive phase, followed by isoniazid and rifampicin daily for the next 4 months in the continuation phase. **2HRZE/4H3R3 regimen: isoniazid, rifampicin, pyrazinamide, and ethambutol daily for the first 2 months in the intensive phase, followed by isoniazid and rifampicin three times a week for the next 4 months in the continuation phase.

The use of thrice-weekly regimens, while not the preferred option, is permissible under specific circumstances and requires close patient monitoring to ensure adherence. However, the lack of availability of daily dose medications highlights a critical gap in the current TB treatment program. The government needs to address this issue by ensuring consistent and adequate supply of all recommended medications, particularly daily dose regimens. This is crucial for optimizing treatment outcomes, preventing drug resistance, and achieving the goal of TB elimination[16].

For the treatment of drug-susceptible TB, fixed-dose combination (FDC) tablets are generally preferred over separate drug formulations. It is widely believed that FDCs enhance patient adherence by simplifying the treatment regimen, reducing pill burden, and minimizing the risk of medication omissions. This, in turn, can contribute to improved treatment outcomes and a reduced risk of drug resistance[17].

In this study, we employed two standard TB treatment regimens: 2HRZE/4HR and 2HRZE/4H3R3. The intensive phase of both regimens utilizes a daily FDC containing isoniazid 75 mg, rifampicin 150 mg, pyrazinamide 400 mg, and ethambutol 275 mg (HRZE). The continuation phase differs between the two regimens: 2HRZE/4HR utilizes a daily FDC containing isoniazid 75 mg and rifampicin 150 mg (HR), while 2HRZE/4H3R3 employs a thrice-weekly FDC containing isoniazid 150 mg and rifampicin 150 mg (H3R3). The specific dosage adjustments for each regimen, based on patient body weight, are outlined in **Table III** for 2HRZE/4HR and **Table IV** for 2HRZE/4H3R3.

Table III. 2HRZE/4HR regimen pattern.

	n (18)	%
Daily doses prescribed (tablets)		
2	11	61.11
3	3	16.67
4	4	22.22
Duration of therapy		
Intensive phase (days)		
56	16	88.89
58	1*	5.56
59	1*	5.56
Continuation phase (days)		
112	16	88.89
115	2*	11.11

Note: *inappropriate

Table IV. 2HRZE/4H3R3 regimen pattern.

	n (38)	%
Daily doses prescribed (tablets)		
2	3	7.90
3	25	65.79
4	10	26.32
Duration of therapy		
Intensive phase (days)		
56	31	81.58
57	1*	2.63
58	2*	5.26
59	2*	5.26
61	1*	2.63
66	1*	2.63
Continuation phase (weeks)		
16	38	100

Note: *inappropriate

Table V presents the results of the TB treatment use evaluation. The findings demonstrate that the indication for TB treatment and the selection of anti-TB drugs in all patients were in accordance with current treatment guidelines (100%). However, adherence to the recommended duration of therapy was observed in only 83.93% of patients. These findings are generally consistent with previous studies. For instance, a study conducted at RSUD Dr. Soedarso reported that while 100% of patients received appropriate indications and drug selections, only 85% adhered to the recommended duration of therapy[18]. Similarly, a study conducted in Oebobo Primary Care in Kupang found that while 100% of patients received the correct drug dosage, only 94.11% adhered to the recommended treatment duration[13]. These studies collectively suggest that adherence to the recommended duration of anti-TB therapy remains a significant challenge in clinical practice.

Table V. Rational use of drugs.

Criteria reviewed	n	%
Indication	56	100
Drug selection	56	100
Doses prescribed	56	100
Duration of therapy	47	83.93

The standard treatment regimen for drug-susceptible pulmonary TB typically consists of a 6-month course, divided into an intensive phase of 2 months (8 weeks or 56 days) followed by a continuation phase of 4 months (16 weeks or 112 days)[6,8]. However, our analysis revealed that 9 out of 56 patients (16.07%) received inappropriate treatment durations. Among patients receiving the 2HRZE/4HR regimen, two patients (11.11%) experienced discrepancies in both the intensive and continuation phases. Conversely, seven patients (18.42%) receiving the 2HRZE/4H3R3 regimen had inappropriate treatment durations specifically within the intensive phase. These findings highlight a significant concern regarding adherence to recommended treatment guidelines for drug-susceptible pulmonary TB within our study population.

Effective TB treatment requires a meticulously planned and consistently administered regimen, encompassing both the intensive and continuation phases. The primary objective of the intensive phase is to rapidly diminish the bacterial load within the patient's body, thereby minimizing the risk of disease transmission. This is achieved through the concurrent administration of multiple anti-TB drugs, which synergistically target and eliminate the actively multiplying bacteria[19]. Adherence to the prescribed medication regimen, including the correct dosage and frequency, is crucial. Typically, within the first two weeks of the intensive phase, the risk of transmission significantly decreases. Furthermore, this phase aims to mitigate the impact of pre-existing drug-resistant strains, ensuring that a comprehensive range of anti-TB drugs are employed from the outset. The continuation phase serves as a crucial follow-up, targeting the remaining dormant or slow-growing bacteria that may have survived the initial intensive treatment. This sequential approach is essential to prevent relapse and ensure a complete eradication of the infection[20].

Drug utilization studies play a crucial role in promoting the rational use of medications within a population. This study revealed an early signal of irrational drug use, namely inappropriate therapy duration, among TB patients. This finding underscores the critical need for pharmacist interventions to optimize TB treatment adherence and improve overall treatment outcomes. The results highlight the importance of continuing medical education programs specifically designed

for healthcare practitioners involved in TB treatment. These programs should emphasize appropriate treatment durations and address common prescribing errors. Furthermore, the implementation of regular drug utilization reviews as part of a quality improvement cycle is essential for continuous monitoring and evaluation of TB treatment practices, ultimately leading to better patient outcomes and improved public health[21].

Patient evaluation in TB treatment encompasses a multifaceted approach, including clinical assessment, bacteriologic evaluation, radiologic evaluation, monitoring of drug side effects, and assessment of treatment adherence. Among these, bacteriologic evaluation is crucial for determining the presence and clearance of *M. tuberculosis* in sputum[22]. In this study, sputum smear microscopy was performed at key intervals: at baseline (before treatment initiation), after two months of intensive phase treatment, at the end of the intensive phase if the two-month smear was positive, and at the end of the six-month treatment course (**Figure 2**). At baseline, 37 patients presented with sputum smear-positive results, while 13 patients were smear-negative. Following two months of treatment, a significant reduction in bacterial load was observed, with only two patients remaining sputum smear-positive. In the third month of treatment, these two patients achieved sputum smear negativity. By the end of the six-month treatment course, all 39 patients evaluated at this stage exhibited negative sputum smear results.

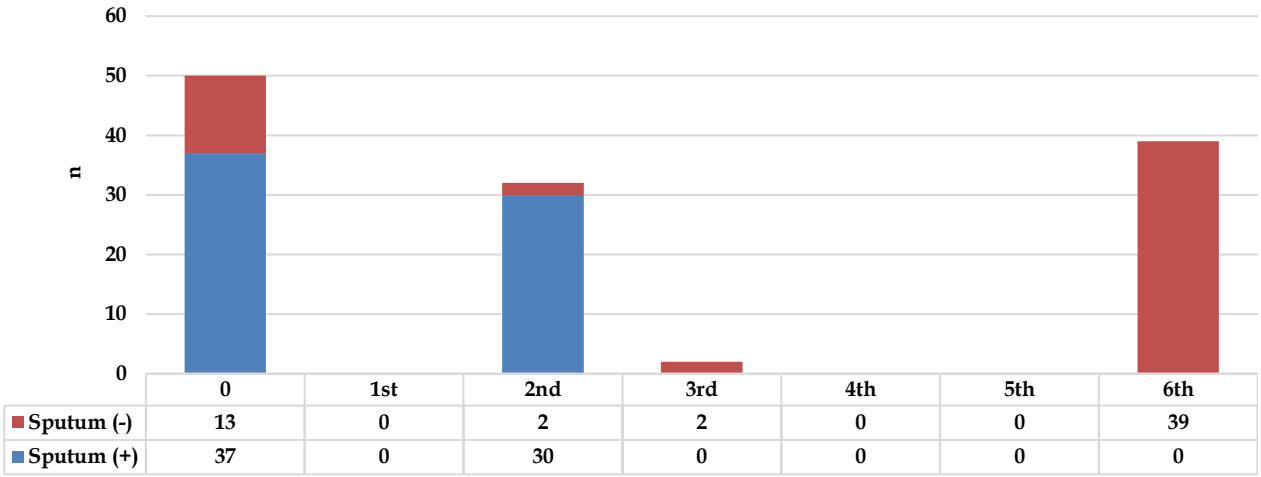

Figure 2. Bacteriological evaluation of TB treatment.

Several barriers have been identified that hinder effective TB evaluation. A study conducted in Uganda among 22 health center staff highlighted key impediments, including insufficient training for healthcare workers, heavy workloads, low staff motivation, poor coordination among healthcare services, and significant challenges faced by patients, such as time constraints, financial burdens associated with seeking and completing TB evaluations, limited health knowledge, and the stigma associated with TB[18]. Furthermore, a cohort study involving 456 participants in Ethiopia underscored the critical role of nutritional assessment, counseling, and management within TB treatment programs. This study demonstrated that incorporating these components into the treatment regimen has the potential to significantly improve treatment outcomes[23].

This study evaluated the effectiveness of anti-tuberculosis therapy by analyzing patient outcomes as presented in **Table VI**. A patient was considered "recovered" if they had a positive bacteriological test at the initiation of treatment and subsequently converted to negative at the end of the treatment course. "Complete treatment" was defined as patients who had undergone the full course of therapy with a negative bacteriological test at a previous examination but without a subsequent bacteriological evaluation at the end of treatment. Among the 56 pulmonary tuberculosis patients aged 15 years and older treated at a Primary Care Center in Bandung between 2021 and 2022, 39 patients (69.64%) were declared recovered, while 17 patients (30.36%) completed treatment. These findings are comparable to a similar study conducted at the Oebobo Primary Care Center in Kupang, which reported a treatment success rate of 57.35% and a completion rate of 36.79%[13]. However, that study also reported mortality (4.41%) and patients with no evaluation (1.47%), indicating potential gaps in patient follow-up.

Table VI. Rational use of drugs.

Outcomes of treatment	Regimen (n (%))		Total (n (%))
	2HRZE/4HR	2HRZE/4H3R3	
Recovered	11 (61.11)	28 (73.68)	39 (69.64)
Complete treatment	7 (38.89)	10 (26.32)	17 (30.36)

This study highlights a crucial observation: not all patients in the Bandung cohort underwent the recommended evaluation procedures. This underscores the critical need for enhanced education and training programs for healthcare providers to emphasize the importance of adhering to standardized treatment guidelines and conducting thorough patient evaluations for all tuberculosis cases. This will ensure accurate assessment of treatment outcomes and facilitate timely identification of patients requiring additional support or alternative treatment strategies[24,25].

CONCLUSION

This study revealed several critical shortcomings in the management of drug-susceptible pulmonary TB patients. Firstly, due to limited availability of the recommended thrice-weekly dosing regimen, a significant proportion of patients likely received suboptimal treatment. Secondly, the quality of drug use was suboptimal, characterized by irrational treatment decisions, including inappropriate durations of therapy. Finally, a concerning observation was the lack of adherence to standardized patient evaluation protocols. These findings underscore the urgent need for improvements in the management of drug-susceptible pulmonary TB, including ensuring access to recommended treatment regimens, optimizing treatment durations, and strengthening patient monitoring and evaluation systems.

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AUTHORS' CONTRIBUTION

Conceptualization: Alfi Nurul Islamiyah, Syarifuddin, Morsalina Aksha, Iis Rukmawati
Data curation: Morsalina Aksha, Iis Rukmawati
Formal analysis: Alfi Nurul Islamiyah, Syarifuddin, Morsalina Aksha, Iis Rukmawati, Eni Margayani, Linda Purnamawati Suherman, Vina Septiani, Robby Ramdani
Funding acquisition: Alfi Nurul Islamiyah, Eni Margayani
Investigation: Alfi Nurul Islamiyah, Syarifuddin, Morsalina Aksha, Iis Rukmawati
Methodology: Alfi Nurul Islamiyah, Syarifuddin, Morsalina Aksha, Iis Rukmawati
Project administration: Alfi Nurul Islamiyah, Syarifuddin, Morsalina Aksha
Resources: Alfi Nurul Islamiyah, Syarifuddin, Morsalina Aksha, Iis Rukmawati, Eni Margayani, Linda Purnamawati Suherman, Vina Septiani, Robby Ramdani
Software: -
Supervision: Alfi Nurul Islamiyah, Syarifuddin, Iis Rukmawati
Validation: Eni Margayani, Linda Purnamawati Suherman, Vina Septiani, Robby Ramdani
Visualization: -
Writing - original draft: Alfi Nurul Islamiyah, Morsalina Aksha, Iis Rukmawati
Writing - review & editing: Eni Margayani, Linda Purnamawati Suherman, Vina Septiani, Robby Ramdani

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this study.

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