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Adverse Drug Reactions to Antituberculosis Drugs in Tuberculosis Patients Using the Naranjo Algorithm at a Regional Hospital in Mataram

Reaksi Obat yang Merugikan terhadap Obat Antituberkulosis pada Pasien Tuberkulosis Menggunakan Algoritma Naranjo di Rumah Sakit Regional di Mataram

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Abstract

Tuberculosis (TB) remains one of the major health problems in Indonesia. As of October 2023, 67,770 TB cases were reported in West Nusa Tenggara Province (NTB). TB treatment requires long-term therapy using combinations of antituberculosis drugs (ATBD), which have the potential to cause Adverse Drug Reactions (ADRs). ADRs that are not properly identified and managed can increase morbidity and mortality in TB patients and negatively affect treatment outcomes. This study aimed to determine the incidence of ADRs related to the use of ATBD in TB patients at Mataram City Regional General Hospital, NTB, from February to June 2023. This observational study employed a cross-sectional design. Data were collected through structured interviews, and the Naranjo Algorithm was used to assess the causality between reported symptoms and drug use. A total of 66 patients were included in the study. The most common definite ADRs was associated with rifampicin, characterized by reddish discoloration of urine (45.70%), followed by isoniazid-induced fatigue (34%). Probable ADRs were observed with streptomycin, isoniazid, rifampicin, and pyrazinamide, mainly presenting as itching or skin rash (11.10%). Possible ADRs included headache (4.90%) and pain (4.30%), predominantly related to rifampicin use. In conclusion, ADRs among TB patients receiving ATBD at Mataram City Regional General Hospital were diverse, with rifampicin-related urine discoloration being the most prevalent.

Abstrak

Tuberkulosis (TB) tetap menjadi salah satu masalah kesehatan utama di Indonesia. Hingga Oktober 2023, dilaporkan 67.770 kasus TB di Provinsi Nusa Tenggara Barat (NTB). Pengobatan TB membutuhkan terapi jangka panjang menggunakan kombinasi obat antituberkulosis (ATBD), yang berpotensi menyebabkan Reaksi Obat yang Merugikan (Adverse Drug Reactions/ADR). ADR yang tidak diidentifikasi dan dikelola dengan baik dapat meningkatkan morbiditas dan mortalitas pada pasien TB dan berdampak negatif pada hasil pengobatan. Penelitian ini bertujuan untuk mengetahui insiden ADR yang berkaitan dengan penggunaan ATBD pada pasien TB di Rumah Sakit Umum Daerah Kota Mataram, NTB, dari Februari hingga Juni 2023. Studi observasional ini menggunakan desain cross-sectional. Data dikumpulkan melalui wawancara terstruktur, dan Algoritma Naranjo digunakan untuk menilai kausalitas antara gejala yang dilaporkan dan penggunaan obat. Sebanyak 66 pasien dimasukkan dalam penelitian ini. Efek samping obat (ADR) yang paling umum dan pasti dikaitkan dengan rifampisin, ditandai dengan perubahan warna urin menjadi kemerahan (45,70%), diikuti oleh kelelahan akibat isoniazid (34%). ADR yang mungkin terjadi diamati dengan streptomisin, isoniazid, rifampisin, dan pirazinamid, terutama berupa gatal atau ruam kulit (11,10%). ADR yang mungkin terjadi meliputi sakit kepala (4,90%) dan nyeri (4,30%), terutama terkait dengan penggunaan rifampisin. Kesimpulannya, ADR di antara pasien TB yang menerima ATBD di Rumah Sakit Umum Daerah Kota Mataram beragam, dengan perubahan warna urin terkait rifampisin sebagai yang paling umum.

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INTRODUCTION

Tuberculosis (TB) is a group of chronic infectious diseases caused by the bacterium *Mycobacterium tuberculosis*, which causes infection in the lungs and leads to pulmonary TB. In addition, *Mycobacterium tuberculosis* bacteria can also infect other parts of the body, such as the pleura, lymph nodes, bones, and other extrapulmonary organs, which is often referred to as extrapulmonary TB¹. According to 2021 data from the World Health Organization (WHO), TB causes two million deaths each year, and it is estimated that around one billion people are infected with pulmonary tuberculosis². Highest incidence of tuberculosis occurs in Southeast Asia (11%), Africa (47%), the Western Pacific (34%) and Europe (40%)³. Based on data from the Indonesian Ministry of Health in 2022, the estimated incidence of TB in Indonesia is 969.000 or 354 per 100.000 population, with estimated 144.000 deaths from TB or 52 per 100.000 population⁴. According to the West Nusa Tenggara Health Office, the number of TB cases in West Nusa Tenggara as of October 2023 was 67.770 cases (67.86%) According to the NTB Health Office, the number of TB cases in West Nusa Tenggara (NTB) as of October 2023 was 67,770 cases (67.86%)⁵.

The TB treatment procedure consists of two stages: an intensive stages phase lasting two months and a continuation phase lasting four to seven months⁶. The TB treatment therapy used is combination therapy with various types of drugs in sufficient quantities and appropriate doses according to each treatment group, such as the first line drug group, which includes Isoniazide (H), Ethambutol (E), Pyrazinamide (Z), Rifampicin (R), and Streptomycin (S). While for TB-Drug Resistant (DR) treatment, it consists of Kanamycin (Km), Ethionamide (Eto) or Prothionamide (Pto), High-dose Isoniazide (HD-H), Moxifloxacin (Mfx), Clofazimine (Cfz), Ethambutol (E), Pyrazinamide (Z)⁷. The administration of this combination therapy must be considered carefully, taking into account the organ function of TB patients, as it will affect the pharmacodynamic and pharmacokinetic effects of the drugs, especially in geriatric TB patients or TB patients with comorbidities such as diabetes or HIV. Failure in TB treatment can be caused by Adverse Drug Reactions (ADRs) or pharmacological side effects of drugs, leading to treatment non compliance by patient⁸. Other studies also explain that some TB patients experience mild to severe ADRs or side effects during TB treatment⁹. Side effects include anorexia, nausea, vomiting, abdominal pain, vertigo, cephalalgia, pruritus, visual disturbances, ototoxicity, hematuria, etc¹⁰. Therefore, the occurrence of ADRs from the use of TB drugs will increase morbidity and mortality⁹. It is very important to accurately identify ADRs from antituberculosis drugs so that appropriate treatment can be given and patient compliance can be improved during the treatment period. Therefore, it is necessary to adequately identify and address potential ADRs^{11 12}.

The Naranjo Algorithm is used to support the monitoring of potential ADRs. This method is supported by the Ministry of Health to identify drug side effects. The Naranjo questionnaire is intended to determine whether the observed side effects are caused by antituberculosis drugs or whether are other variables that contribute to the side effects¹³. Indonesia has committed to achieving the goal of eliminating tuberculosis by 2030 with a target incidence of 65 per 100.000 population and a mortality rate 6 per 100.000 population⁴. To support government's program, it is very important to conduct pharmacovigilance studies, especially on adverse drug reactions. The consequences of adverse drug reactions include treatment failure, the development of *Mycobacterium tuberculosis* resistance, and reduced treatment effectiveness. Other studies also explain that ADRs incidents cause a 3%-12% increase the number of patients and a 5%-10% increase in treatment costs in Western countries¹⁴. The main reason TB patients discontinue their treatment is the of ADRs. 38% of patients discontinue treatment due to unwanted side effects¹⁵. Therefore the purpose of this study is to determine the incidence of antituberculosis Adverse Drug Reactions in TB patients.

METHODS

Research Design

This study is an observational study with across-sectional design, conducted from February to June 2023 at the outpatient facility of the Mataram City Regional General Hospital. This study has obtained research permission from Mataram City Regional General Hospital (NO: 445/0463.2/RSUD/II/2023).

Data Collection

Respondents for this study were sampled using purposive sampling based on the inclusion and exclusion criteria. Inclusion criteria for this study were patients, both male and female, diagnosed with tuberculosis and receiving antituberculosis therapy, patients with complete medical records, willing to participate in the study, and able to communicate effectively. Exclusion criteria for this study were pregnant or breastfeeding women. Data on antituberculosis therapy was obtained from secondary data, namely patient medical records, while primary data was obtained from Naranjo Algorithm questionnaire, which was administered concurrently through interviews with patients during routine check-up which was administered concurrently through interviews with patients during routine check-ups or while waiting to collect their medication. TB patients who were willing to be respondents filled out the Naranjo Algorithm questionnaire provided. The data collection period was five months.

Research Instrument

The instrument used in this study was the Naranjo Algorithm, a validated causality assessment tool widely used to evaluate the likelihood of adverse drug reactions (ADRs). The algorithm consists of a structured questionnaire with a specific scoring system that categorizes ADRs probability into definite, probable, possible, or doubtful. This instrument was selected because it enables qualitative and semi quantitative assessment of ADRs occurrences.

Research Sample

The sample size in this study was calculated based on the Slovin formula, namely:

$$n = \frac{N}{1 + N(d)^2} \dots\dots\dots (1)$$

N= required sample size; N = total population size ; d = precision level of 10% (0.1)

Substituting the values:

$$n = \frac{188}{1 + 188(0,1)^2}$$

$$n = 65,28 \sim 66$$

Therefore, the minimum required sample size was 66 patients

Data Analysis

The analysis was conducted to determine the demographic and clinical characteristics of the patients. Furthermore, data were collected based on patients complaints. The collected data were assessed for causality using the Naranjo Algorithm, and the ADRs probability scores were calculated. The scoring categories were as follows: 1-3 (possible ADRs); 4-8 (probable ADRs); ≥ 9 (definite ADRs).

RESULT AND DISCUSSION

Based on the data presented in Table 1, the majority of patients in this study were aged <60 years (77%), indicating that TB cases were predominately found in the productive group. This finding is consistent with previous study by Putri (20220 and Oktaviani (2022), which reported that TB is more commonly observed among adolescents and young adults. Individuals in this group may be more vulnerable to immunological and hormonal changes occurring during adolescence and early adulthood. In addition, several known risk factors may contribute to TB development, including malnutrition, immunosuppression, diabetes mellitus, obesity, smoking, and alcohol consumption. Behavioral and environmental factors may also play an important role. Adolescents and young adults tend to spend considerable time in school or workplace settings, were prolonged close contact in enclosed spaces with inadequate ventilation can facilitate TB transmission. Moreover, pulmonary TB patients in this age group often have relatively high transmission potential^{16 17}.

Several studies have reported that nutritional status is significantly associated with the incidence of pulmonary TB. Individuals with poor nutritional status are at a higher risk of developing TB infection. Nutritional status plays a crucial role in maintaining immune function, as adequate nutrient intake is essential for optimal immune response. An imbalance between nutrient intake and the body's requirements may impair immune competence, particularly in the formation and function of antibodies and lymphocytes. Proteins and carbohydrates are essential for the development of these immune components, which serve as key defense mechanism against infectious agents, including *Mycobacterium tuberculosis*, the causative agent of pulmonary TB¹⁸.

Table 1. Demographic and Clinical Characteristics of Tuberculosis (TB) Patients (n= 75)

No.	Variable	Category	n (%)
1	Age	< 60 years	58 (77%)
		≥ 60 years	17 (23%)
2.	Gender	Male	37 (49%)
		Female	38 (51%)
3.	Treatment Phase	Intensive Stage	63 (84%)
		Follow-up Phase	12 (16%)
4	Comorbidity Status	With comorbidities	46 (61%)
		Without Comorbidities	29 (39%)

In this study, the proportion of TB cases among females (51%) and males (49%) was nearly equal, indicating no substantial sex disparity in the study population. This finding noteworthy, as TB is often reported to be more prevalent among males in many epidemiological studies. However, Osman in 2022 reported that the risk of TB among females may peak during mid-adolescence compared to males. This pattern has been associated with higher TB-HIV co-infection rates and increased TB related mortality among adolescent girls. In addition, several biological and physiological factors may contribute to increased susceptibility among females including hormonal changes during menarche, pregnancy-related immunological alterations, and the interaction between TB and HIV infection¹⁹.

Additionally, behavioral factors such as smoking and alcohol consumption, which are more prevalent among men may contribute to increased susceptibility to pulmonary TB²⁰. Both behaviors are known to impair immune function and compromise respiratory defense mechanisms. Cigarette smoke contains harmful substances that can damage the structure and function of the respiratory epithelium, including impairment of ciliary activity in the airway. This disruption reduces the effectiveness of mucociliary clearance, thereby facilitating the entry and persistence of *Mycobacterium tuberculosis* in the respiratory tract. Furthermore, smoking has been reported to alter both innate and adaptive immune responses, particularly affecting macrophage and leukocyte function, which play essential role in host defense againsts TB infection¹⁸.

In a study conducted by Kumarjit Sinha involving 102 patients, the majority of ADRs cases were observed in the 30-40 year age group (27.45%). However, the highest proportion of ADRs was reported in patients aged <20 years, while the lowest incidence was found in the 51-60 year age group. The differences in ADRs occurrence across age groups were statistically significant ($p < 0.001$). Furthermore, female TB patients experienced ADRs more frequently than males (91.67% vs. 62.82%), and this difference was also statically significant ($p < 0.001$)²¹. In the present study, most TB patients (84%) were undergoing treatment in the intensive phase. This finding is consistent with previous research reporting that greater proportion of TB patients were in intensive treatment phase (57.70%) compared to the continuation phase (42.30%)²². The intensive phase of TB treatment is administered for the first two months with daily dosing and requires close monitoring to ensure treatment adherence and prevent drug resistance. This is followed by the continuation phase, which is typically administered for four months to achieve sustained bacterial eradication and prevent relapse⁷.

Table 1 shows that the majority of patients in this study had comorbidities (61%). The most common comorbidity was diabetes mellitus (34 cases), followed by diabetes with pneumonia (4 cases), HIV with diabetes (3 cases), Chronic Obstructive Pulmonary Disease (COPD) (3 cases), and diabetes with COPD (2 cases) (Figure 1). These findings are consistent with the study by Wang (2024), which reported that 849 (19.91%) individuals in the case group and 1.141 (6.69%) in the control group experienced multimorbidity. Patients with one, two, or ≥ 3 comorbidities had an increased risk of developing pulmonary TB, with adjusted Odds Ratio (aOR) ranging from 2.85 to 5.16. This suggests that the presence and number of comorbid conditions may significantly increase susceptibility to pulmonary TB. Comorbidities frequently associated with increased TB risk include diabetes, chronic lower respiratory tract diseases (such as COPD, emphysema, and bronchitis) hypertension and cerebrovascular disease²³.

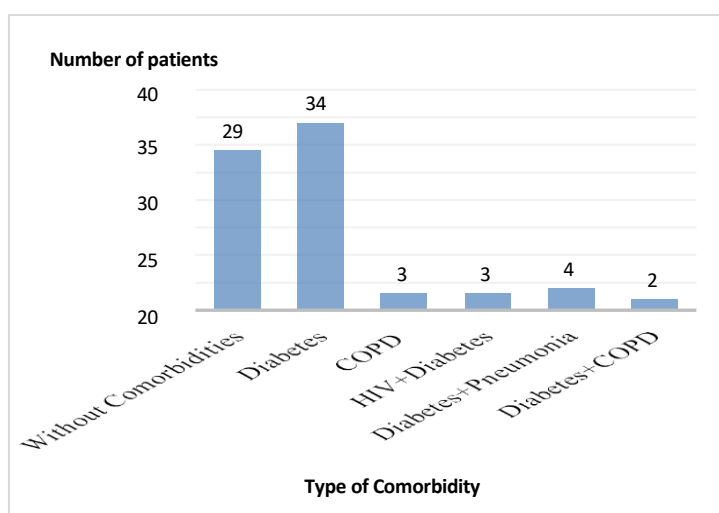


Figure 1. Distribution of Comorbidities Among Tuberculosis (TB) Patients

Diabetes mellitus is defined as a condition characterized by elevated blood glucose level (≥ 126 mg/dL). Although the exact impact of diabetes on TB severity remains under investigation, diabetes has been shown to negatively affect antituberculosis treatment outcomes. One proposed mechanism involves altered pharmacokinetics of anti-TB drugs. Studies have reported difference in plasma rifampicin concentrations between TB patients with and without diabetes. Rifampicin is potent inducer of hepatic enzymes, particularly cytochrome P450 (CYP) 3A4 and CYP2C, leading to reduce plasma concentrations of several co-administered drugs. This includes (S)-warfarin and first and second generation sulfonylurea antidiabetic agents such as tolbutamide, glibenclamide (glyburide), glimepiride and glipizide^{24 25}.

Pulmonary TB itself may also lead to structural lung damage, affecting the pulmonary vasculature. Damage to the lung parenchyma can result in vasculitis and endarteritis, potentially reducing the cross-sectional area of pulmonary blood vessels and contributing to pulmonary hypertension²⁶. Furthermore, previous studies have demonstrated that patients with diabetes, HIV infection, alcoholism, substance abuse, immunosuppressive therapy, or malignancy tend to have lower treatment success rates and higher mortality compared to TB patients without these conditions ($p < 0.001$)²⁷. Globally, the prevalence of ADRs among TB patients ranges from 8% to 85%. The occurrence of ADRs may be influenced by several factors, including diabetes, HIV co-infection, variations in anti-TB dosing, and pharmacological interactions with antiretroviral and oral antidiabetic medications²⁸.

The Naranjo Algorithm is used to evaluate the likelihood of ADRs associated with medication use. This method employs a structured questionnaire to systematically assess the probability of drug-related adverse events. In this study, causality assessment using the Naranjo Algorithm was performed for each patient to determine the likelihood that the reported adverse events were related to antituberculosis therapy¹⁰. Causality assessment is essential in clinical practice and pharmacovigilance, as it supports appropriate therapeutic decision making and drug safety monitoring. The results of ADRs analysis among TB patients, as presented in Table 2 and Figure 2, indicated that red colored urine (45.70%), headache (4.90%), and pain (4.30%) were most frequently associated with rifampicin use. Fatigue or drowsiness (34%) was predominantly observed in patients receiving isoniazid. Meanwhile, itching or skin rash (11.10%) was reported in patients receiving combination anti-TB therapy, including streptomycin, isoniazid, rifampicin and pyrazinamide.

Tabel 2. Naranjo-Based Causality Assessment of ADRs

Drug Name	Number of ESO Events (n=162)	Manifestation	Naranjo Scale	(%)
Rifampicin	74	Red- colored urine	definite	45.7
	8	Headache	possible	4.9
	7	Pain	possible	4.3
Isoniazid	55	Fatigue /drowsiness	definite	34
Streptomycin,isoniazid, Rifampicin, pyrazinamide	18	Itching/ skin rash	probable	11.10

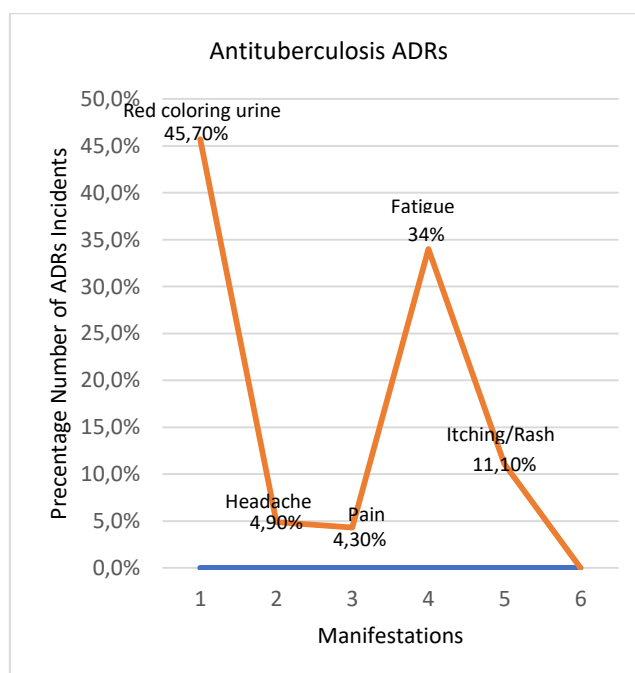


Figure 2. Frequency Distribution of ADRs Among Tuberculosis (TB) Patients

In line with the findings of this study, the Ministry of Health Guidelines for Tuberculosis Mangement (2020) acknowledge that anti-TB drugs are associated with various adverse effects. Skin rash has been reported with the use of streptomycin, isoniazid, rifampicin and pyrazinamide. Therefore, clinicians should carefully monitor patients who develop severe adverse reactions. Rifampicin, in particular is known to cause red-colored urine due to its pharmacological properties. Patients should be informed of this effect prior to treatment initiation, as it is considered a harmless and unexpected reaction ⁷. Previous studies have also reported that adverse effects frequently occur during TB treatment. During the first month of therapy, headache was among the most commonly reported symptoms (54,5%). In the second month, itching (72.7%), headache (72.7%), and nausea (72.7%) were frequently observed ¹⁰. Drug induced hepatotoxicity is recognized as a serious adverse effect of isoniazid. According to Kim (2021), the incidence of isoniazid related hepatotoxicity in combination anti-TB regimens ranges from 1.6% to 2.7%. Rifampicin and pyrazinamide have also been implicated in hepatotoxic reactions. In addition, pyrazinamide and ethambutol have been reported to cause hyperuricemia in patients without prior gout, which may result in arthralgia ²⁹. Furthermore, another study involving 511 TB patients reported that 93 patients (18.20%) experienced adverse drug reactions during anti-TB therapy, most commonly gastrointestinal disturbances, skin hypersensitivity reactions, and hepatotoxicity ²¹.

Based on data from Korea Adverse Event Reporting System (KIDS-KAERS) between 2009 and 2018, a total of 17.843 Adverse Drug Reaction (ADR) cases were reported for first line anti-TB drugs (1.14%). The highest proportion of ADRs was associated with rifampicin (28%), followed by isoniazid (24.0%), pyrazinamide (23.9%) and ethambutol (23.4%). The most commonly affected organ system were gastrointestinal tract (32%), skin and appendages (25.9%), and the liver and biliary system (14.2%). Nausea was the most frequently reported ADRs (14.6%), followed by elevated liver enzymes (14.2%), rash (11.7%), pruritus (9.1%), vomiting (8.9%), and urticaria (4.2%). Most ADRs occurred within the first month of treatment initiation. However, certain reactions such as neuropathy, paresthesia, hematological abnormalities, renal function impairment, and liver enzyme abnormalities were also frequently reported after two months of therapy ³⁰.

Limitation

This study has several limitations that should be considered when interpreting the findings. First, the cross sectional design limits the ability to establish temporal relationship and causal inferences between anti-TB drugs and ADRs. Second, the study was conducted in a single hospital with relatively small sample sized (n=66), which may limit the generalizability of the results to broader TB populations in other region. Third the data were collected through patient interviews and medical records, which may introduce recall bias and information bias. Additionally, laboratory confirmation for certain ADRs, such as hepatotoxicity or renal impairment, was not comprehensively evaluated, and causality assessment relied solely on the Naranjo Algorithm without comparison to other validated causality tools. Future studies with larger multicenter cohort, prospective design and comprehensive clinical and laboratory monitoring are recommended tp strengthen the evidance regarding ADRs patterns in TB patients.

CONCLUSION

Adverse Drug Reactions (ADRs) were commonly identified among tuberculosis patients receiving first line anti-TB therapy, with rifampicin associated red colored urine being the most frequent reaction (45,70%), followed by headache (4.90%) and pain (4.30%). Isoniazid was primarily associated with fatigue or drowsiness (34%), while itching or skin rash (11.10%) was observed in patients receiving combination therapy with streptomycin, isoniazid, rifampicin, and pyrazinamide. These findings emphasize the importance of systematic ADRs monitoring snd early detection using structured causality assessment tools to support patient safety, maintain treatment adherence, and optimize therapeutic outcomes in tuberculosis management.

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AUTHOR CONTRIBUTION STATEMENT

FA contributed to conceptualization, data collection, data analysis, and drafting of the manuscript. **ROU** contributed to study design, supervision, data interpretation, critical revision of the manuscript, and served as the corresponding author. **PMDR** contributed to methodology development and data validation. **AM** contributed to data analysis and manuscript review.

CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest

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Phytochemical Screening and Total Flavonoid Content of *Annona squamosa* and *Centella asiatica* Ethanolic Extracts for Diabetic Wound Patch

Pemeriksaan Fitokimia dan Kandungan Flavonoid Total Ekstrak Etanol *Annona squamosa* dan *Centella asiatica* untuk Plester Luka Diabetes

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Abstract

Diabetic wounds are one of the chronic complications of diabetes mellitus, characterized by delayed healing and a high risk of bacterial infection. The development of natural product-based pharmaceutical preparations is considered a promising alternative approach due to their relative safety and wide availability. Soursop leaves (*Annona squamosa* L.) and gotu kola leaves (*Centella asiatica* L.) are known to contain various secondary metabolites that contribute to antibacterial activity and wound healing. This study aimed to perform phytochemical screening and determine the total flavonoid content of ethanolic extracts of soursop leaves and gotu kola leaves as a scientific basis for the development of an antibacterial patch for diabetic wound management. Extraction was conducted using maceration with 96% ethanol. Qualitative phytochemical screening was performed to identify flavonoids, phenolics, tannins, saponins, steroids, and alkaloids. Total flavonoid content was determined using UV-Vis spectrophotometry with quercetin as the reference standard at a maximum wavelength of 427 nm. The results showed that the extraction yields of soursop leaf and gotu kola leaf ethanolic extracts were 14.64% and 14.72%, respectively. Both extracts contained flavonoids, phenolics, tannins, steroids, and alkaloids, while saponins were detected only in the gotu kola leaf extract. The total flavonoid contents of soursop leaf and gotu kola leaf extracts were 1.5% and 1.12%, respectively. In conclusion, ethanolic extracts of soursop leaves and gotu kola leaves possess phytochemical profiles that support antibacterial and wound-healing activities, indicating their potential as active ingredients in antibacterial patch formulations for diabetic wounds.

Abstrak

Luka diabetes merupakan salah satu komplikasi kronis diabetes melitus, yang ditandai dengan penyembuhan yang lambat dan risiko infeksi bakteri yang tinggi. Pengembangan sediaan farmasi berbasis produk alami dianggap sebagai pendekatan alternatif yang menjanjikan karena relatif aman dan tersedia secara luas. Daun sirsak (*Annona squamosa* L.) dan daun gotu kola (*Centella asiatica* L.) diketahui mengandung berbagai metabolit sekunder yang berkontribusi pada aktivitas antibakteri dan penyembuhan luka. Studi ini bertujuan untuk melakukan skrining fitokimia dan menentukan kandungan flavonoid total dari ekstrak etanol daun sirsak dan daun gotu kola sebagai dasar ilmiah untuk pengembangan plester antibakteri untuk manajemen luka diabetes. Ekstraksi dilakukan menggunakan maserasi dengan etanol 96%. Skrining fitokimia kualitatif dilakukan untuk mengidentifikasi flavonoid, fenolik, tanin, saponin, steroid, dan alkaloid. Kandungan flavonoid total ditentukan menggunakan spektrofotometri UV-Vis dengan kuersetin sebagai standar referensi pada panjang gelombang maksimum 427 nm. Hasil penelitian menunjukkan bahwa rendemen ekstraksi etanol daun sirsak dan daun gotu kola masing-masing sebesar 14,64% dan 14,72%. Kedua ekstrak tersebut mengandung flavonoid, fenolik, tanin, steroid, dan alkaloid, sedangkan saponin hanya terdeteksi pada ekstrak daun gotu kola. Kandungan flavonoid total ekstrak daun sirsak dan daun gotu kola masing-masing sebesar 1,5% dan 1,12%. Kesimpulannya, ekstrak etanol daun sirsak dan daun gotu kola memiliki profil fitokimia yang mendukung aktivitas antibakteri dan penyembuhan luka, menunjukkan potensinya sebagai bahan aktif dalam formulasi plester antibakteri untuk luka diabetes.

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INTRODUCTION

Diabetes mellitus is a chronic metabolic disease with increasing global prevalence and remains a major public health problem ¹. One of its most serious complications is diabetic wound or diabetic foot ulcer (DFU), which is characterized by delayed healing, prolonged inflammation, and high risk of infection. Diabetic wounds are closely associated with impaired tissue perfusion, peripheral neuropathy, and microbial infection ¹. These factors contribute to a prolonged wound-healing process and significantly increase the risk of lower-extremity amputation, thereby reducing the patient's quality of life and increasing healthcare costs ².

In recent years, the development of natural product-based pharmaceutical preparations has become a promising approach due to their broad pharmacological activities and relatively minimal side effects compared to conventional synthetic agents ³. Traditional medicinal plants have been utilized for generations in wound care, and many have been scientifically investigated for their healing potential through in vitro, in vivo, and clinical studies ⁴. Several medicinal plants have been reported to show beneficial activity in the management of gangrenous wounds, including *Annona reticulata*, *Carica papaya*, *Aloe vera*, *Centella asiatica*, *Punica granatum*, and *Anredera cordifolia* ⁴. These findings support the idea that medicinal plants may serve as important sources of bioactive compounds for the development of modern wound care products, particularly for chronic and difficult-to-heal wounds such as diabetic ulcers ^{4,3}.

Among various medicinal plants, soursop (*Annona squamosa* L.) and gotu kola (*Centella asiatica* L.) are considered potential candidates due to their reported pharmacological properties related to wound healing ^{3,5,6}. According to previous studies, gotu kola has been reported to be effective for grade IIa wounds, while soursop was effective for grade IIb wounds, with a reported healing percentage of 100% ³. These results suggest that both plants may be explored as alternative therapeutic materials for wound healing applications ³.

Soursop leaves (*Annona squamosa* L.) have been reported to possess antibacterial and wound-healing activity, which may be associated with their phytochemical constituents, including flavonoids and phenolic compounds ^{5,7}. The presence of antibacterial activity is particularly important for diabetic wounds because infection is one of the most common causes of delayed healing and wound deterioration ^{1,4}. In addition, antioxidant activity contributes to wound healing by reducing oxidative stress that may damage cells and prolong inflammation, thereby interfering with tissue regeneration and wound closure ^{1,7}. A previous study also confirmed that *A. squamosa* leaves exhibited antibacterial and wound-healing activities, supporting their potential development in topical wound therapy ⁵.

Gotu kola (*Centella asiatica* L.) is a traditional medicinal plant that promotes wound healing by stimulating fibroblast proliferation and collagen synthesis, which are crucial for extracellular matrix formation and tissue repair during the proliferation phase. Experimental excision wound models have also confirmed the wound-healing potential of *C. asiatica* extract, supporting its development as a topical active ingredient, particularly for chronic wounds with impaired tissue repair ⁶.

Flavonoids are major phenolic compounds that support wound healing through antioxidant, anti-inflammatory, and antibacterial activities ⁷. These effects help reduce oxidative damage, regulate inflammation in chronic wounds, and control infection, which is important in diabetic ulcer management ^{5,7}. Therefore, phytochemical screening and total flavonoid content determination are essential for the preliminary evaluation of wound-healing plant extracts ⁷.

Based on the above considerations, this study focuses on phytochemical screening and the determination of total flavonoid content of ethanolic extracts of soursop leaves (*Annona squamosa* L.) and gotu kola leaves (*Centella asiatica* L.) as candidate active ingredients for the development of an antibacterial patch for diabetic wound treatment ⁷.

METHODS

Materials

The materials used in this study were dried soursop leaves (*Annona squamosa* L.) and dried gotu kola leaves (*Centella asiatica* L.), 96% ethanol, methanol, concentrated hydrochloric acid (HCl), magnesium powder, 10% aluminum chloride (AlCl₃) solution, and quercetin as a reference standard.

Research Procedure

Extraction Procedure: Extraction was carried out using the maceration method with 96% ethanol for 72 h under periodic stirring. The filtrate was then concentrated using a rotary evaporator to obtain a viscous crude extract ⁸.

Phytochemical Screening: Qualitative phytochemical screening was performed using standard color reaction tests to identify the presence of flavonoids, phenolics, tannins, saponins, steroids, and alkaloids ⁹.

Determination of Total Flavonoid Content (TFC)

Total flavonoid content was determined using UV–Vis spectrophotometry with quercetin as the reference standard at a maximum wavelength range of 400–450 nm. The results were expressed as percentage of quercetin equivalent (% w/w) ¹⁰.

a. Preparation of Quercetin Standard Solutions

Quercetin powder (50 mg) was dissolved in 96% ethanol and diluted to 50 mL to obtain a 1000 ppm stock solution (Stock 1). Stock 2 (100 ppm) was prepared by transferring 5 mL of Stock 1 and diluting with 96% ethanol to 50 mL. Standard series solutions were prepared by pipetting 5, 6, 7, 8, and 9 mL of Stock 2 into separate 50 mL volumetric flasks and diluting with 96% ethanol to obtain concentrations of 10, 12, 14, 16, and 18 ppm, respectively.

b. Determination of Maximum Wavelength

The UV–Vis spectrophotometer was set to scan in the range of 400–450 nm using 96% ethanol as blank. A 10 ppm quercetin solution was then measured to determine the maximum wavelength.

c. Construction of the Quercetin Calibration Curve

Each standard solution (10–18 ppm) was pipetted (1 mL) and mixed with 1 mL of 2% AlCl₃ and 1 mL of 120 mM potassium acetate, followed by incubation for 30–60 min. The absorbance was then measured using UV–Vis spectrophotometry to obtain the calibration curve.

d. Preparation of Sample Solutions

Each extract sample (75 mg) was dissolved in ethanol and diluted to 50 mL to obtain a sample concentration of 1500 ppm.

e. Measurement of Total Flavonoid Content

One milliliter of the sample solution was mixed with 1 mL of 2% AlCl_3 and 1 mL of 120 mM potassium acetate, followed by incubation for 30–60 min. The absorbance was measured using UV–Vis spectrophotometry, and total flavonoid content was calculated using the linear regression equation obtained from the quercetin calibration curve

RESULT AND DISCUSSION

Soursop leaves (*Annona squamosa* L.) and gotu kola leaves (*Centella asiatica* L.) are known to contain various phytochemical constituents with potential antibacterial, antioxidant, and wound-healing activities ⁶. The maceration method using ethanol was selected due to its ability to extract a wide range of phytochemical compounds efficiently while maintaining the structural integrity of bioactive molecules. Flavonoids are phenolic compounds that are soluble in 70% ethanol; therefore, the maceration approach applied in this study was considered appropriate for extracting flavonoid-rich fractions ¹¹.

A. Extraction Yield

Table 1. Summarizes the extraction yield

Material	Soursop leaf extract (<i>A. squamosa</i> L.)	Gotu kola leaf extract (<i>C. asiatica</i> L.)
Sample weight	1 kg	0.5 kg
Extract weight	146.41 g	73.6 g
Yield (%)	14.64%	14.72%

The extraction yields of ethanolic extracts of *Annona squamosa* and *Centella asiatica* were 14.64% and 14.72%, respectively. These findings indicate that 96% ethanol effectively extracted polar and semi-polar phytochemical constituents.

B. Phytochemical Screening

Tabel 2. Qualitative phytochemical screening results

Material	Soursop leaf extract (<i>A. squamosa</i> L.)	Gotu kola leaf extract (<i>C. asiatica</i> L.)
Saponins	Not detected (–)	Detected (+)
Phenolics	Detected (+)	Detected (+)
Tannins	Detected (+)	Detected (+)
Flavonoids	Detected (+)	Detected (+)
Steroids	Detected (+)	Detected (+)
Alkaloids	Moderate (+2)	Weak (+1)

Notes: (–) not detected; (+) detected. Alkaloid intensity: (+1) weak reaction, (+2) moderate reaction, (+3) strong reaction.

Phytochemical screening demonstrated that both extracts contained flavonoids, phenolics, tannins, steroids, and alkaloids. Saponins were detected only in *Centella asiatica* extract.

C. Total Flavonoid Content

Table 3. Total flavonoid content of the ethanolic extracts of sour sop leaves and gotu kola leaves

Parameter	Soursop leaf extract (<i>A. squamosa</i> L.)	Gotu kola leaf extract (<i>C. asiatica</i> L.)
Sample weight (mg)	75	75
Absorbance	0.2788	0.2095
Concentration (mg/L)	229.852	169.741
Total flavonoid content (%)	1.50	1.12

The total flavonoid contents of *Annona squamosa* and *Centella asiatica* extracts were 1.50% and 1.12%, respectively. The higher flavonoid level observed in *Annona squamosa* may contribute to its stronger antioxidant and antibacterial activities.

DISCUSSION

A. Extraction Yield

The extraction yield is closely related to the amount of bioactive compounds obtained from the raw material. A higher yield indicates a greater amount of extracted constituents, suggesting better extraction efficiency. In this study, the extraction yields were considered satisfactory because both extracts showed yields above 10%¹². The extraction yields of sour sop leaf extract and gotu kola leaf extract were 14.64% and 14.72%, respectively, indicating that 96% ethanol was effective in extracting polar and semi-polar secondary metabolites¹³.

B. Phytochemical Screening

Qualitative phytochemical screening demonstrated that both extracts contained flavonoids, phenolics, tannins, steroids, and alkaloids, while saponins were detected only in the gotu kola leaf extract. These secondary metabolites play important roles in antibacterial activity and wound healing processes¹⁴. *Annona squamosa* leaves have been reported to contain various phytochemicals, including glycosides, phenolics, flavonoids, alkaloids, and phytosterols, and acetogenins such as squamocin and annonacin are known to exhibit strong antibacterial and antioxidant activities¹³. Meanwhile, *Centella asiatica* is rich in triterpenoids (asiaticoside, madecassoside, and asiatic acid) as well as flavonoids, which may synergistically contribute to skin regeneration and wound healing¹.

C. Total Flavonoid Content

Since the sour sop leaf extract was confirmed to contain flavonoids, total flavonoid content (TFC) was determined based on the formation of a complex compound with $AlCl_3$. This reaction occurs due to the interaction between the C-4 keto group and the adjacent hydroxyl group at C-3 or C-5, resulting in a bathochromic shift toward the visible region, which produces a yellow color in the solution. Quercetin was used as the standard reference for TFC determination because it represents a common flavonoid compound⁷. The results showed that the total flavonoid content of sour sop leaf extract (1.50%) was higher than that of gotu kola leaf extract (1.12%). Several related studies

reported similar findings, supporting the results obtained in this research. For comparison, a mulberry leaf extract patch formulated using a PVP–EC polymer matrix showed a yield of 12% and a flavonoid content of 1.8% ¹⁵.

Flavonoids act as strong antioxidants by scavenging free radicals, inhibiting lipid peroxidation, and supporting tissue regeneration. The higher flavonoid content in soursop extract compared to gotu kola may explain its stronger antibacterial potential ¹⁶. However, gotu kola contains higher levels of triterpenoids and therefore may provide superior effects in skin regeneration.

A combination of both extracts is expected to provide a synergistic effect between the antibacterial activity of soursop and the tissue-healing activity of gotu kola. This combined extract profile supports synergistic mechanisms, where soursop phenolics and flavonoids may inhibit wound-infecting microbes such as *Staphylococcus aureus* and *Escherichia coli* in an animal wound model ⁵, while gotu kola saponins may stimulate fibroblast proliferation and enhance collagen synthesis, thereby accelerating epithelialization ^{6,16}.

CONCLUSION

The ethanolic extracts of soursop leaves and gotu kola leaves contained secondary metabolites that support antibacterial activity and wound healing. The total flavonoid contents of 1.50% (soursop) and 1.12% (gotu kola) indicate promising pharmacological potential. Therefore, both extracts have potential to be developed as active ingredients in antibacterial patch formulations for diabetic wound treatment.

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AUTHOR CONTRIBUTION STATEMENT

J contributed to conceptualization, data collection, data analysis, and drafting of the manuscript. **YE** contributed to study design, supervision, data interpretation, critical revision of the manuscript, and served as the corresponding author. **US** contributed to methodology development and data validation and contributed to data analysis and manuscript review.

CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest

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The Effect Of Drying Method On The Yield Of Kecombrang Leaves (*Etilngera Elatoir*) Using The Maceration Method With 70% Ethanol Solvent

Pengaruh Metode Pengeringan Terhadap Hasil Rendemen Daun Kecombrang (*Etilngera Elatoir*) Menggunakan Metode Maserasi Dengan Pelarut Etanol 70%

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Abstract

Kecombrang leaves (*Etlingera elatior*) have potential as traditional medicinal materials due to their secondary metabolite content, which exhibits antioxidant and antibacterial activities. Drying is an important step in the processing of plant simplicia. This study aimed to determine the effect of different drying methods (sun drying, drying cabinet, and shade drying/room temperature) on moisture loss and extract yield of kecombrang leaves using the maceration method with 70% ethanol as the solvent. This study employed a completely randomized design with 18 samples. The simplicia were dried according to each method, macerated for 3 days, filtered, and concentrated using a water bath. Data were analyzed using normality and homogeneity tests, followed by One-Way ANOVA and Post Hoc Tukey HSD. The results showed that all methods met the acceptable moisture loss requirement (<10%), with the lowest values obtained from the drying cabinet method (6.53–8.02%) and the highest from shade drying (9.67–9.99%). The highest extract yield was obtained from the drying cabinet method (12–18%), followed by sun drying (8–16%), while shade drying produced the lowest yield (4–12%). One-Way ANOVA indicated a significant difference among the drying methods ($p = 0.001$). Post Hoc Tukey HSD showed that the drying cabinet method was significantly different from sun drying ($p = 0.031$) and shade drying ($p = 0.002$), while no significant difference was found between sun drying and shade drying ($p = 0.411$). In conclusion, the drying cabinet method is the most effective method for producing higher extract yield of kecombrang leaves.

Abstrak

Daun kecombrang (*Etlingera elatior*) memiliki potensi sebagai bahan obat tradisional karena kandungan metabolit sekundernya yang menunjukkan aktivitas antioksidan dan antibakteri. Pengeringan merupakan langkah penting dalam pengolahan simplisia tanaman. Penelitian ini bertujuan untuk menentukan pengaruh berbagai metode pengeringan (pengeringan matahari, pengeringan kabinet, dan pengeringan di tempat teduh/suhu ruangan) terhadap kehilangan air dan rendemen ekstrak daun kecombrang menggunakan metode maserasi dengan etanol 70% sebagai pelarut. Penelitian ini menggunakan rancangan acak lengkap dengan 18 sampel. Simplisia dikeringkan sesuai dengan masing-masing metode, dimaserasi selama 3 hari, disaring, dan dipekatkan menggunakan penangas air. Data dianalisis menggunakan uji normalitas dan homogenitas, diikuti dengan ANOVA Satu Arah dan Post Hoc Tukey HSD. Hasil menunjukkan bahwa semua metode memenuhi persyaratan kehilangan air yang dapat diterima (<10%), dengan nilai terendah diperoleh dari metode pengeringan kabinet (6,53–8,02%) dan nilai tertinggi dari pengeringan di tempat teduh (9,67–9,99%). Hasil ekstrak tertinggi diperoleh dari metode lemari pengering (12–18%), diikuti oleh pengeringan matahari (8–16%), sedangkan pengeringan di tempat teduh menghasilkan hasil terendah (4–12%). ANOVA Satu Arah menunjukkan perbedaan signifikan di antara metode pengeringan ($p = 0,001$). Post Hoc Tukey HSD menunjukkan bahwa metode lemari pengering berbeda secara signifikan dari pengeringan matahari ($p = 0,031$) dan pengeringan di tempat teduh ($p = 0,002$), sedangkan tidak ditemukan

perbedaan signifikan antara pengeringan matahari dan pengeringan di tempat teduh ($p = 0,411$). Kesimpulannya, metode lemari pengering adalah metode yang paling efektif untuk menghasilkan hasil ekstrak daun kecombrang yang lebih tinggi.

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INTRODUCTION

Indonesia is a country with high biodiversity, one of which is the kecombrang plant (*Etlingera elatior*), which is widely utilized by the community as a food ingredient and traditional medicine¹. Kecombrang leaves are known to contain various secondary metabolite compounds such as flavonoids, phenolics, and tannins that have the potential to be antioxidants and antibacterials². This potential makes kecombrang leaves attractive to be developed as raw materials for pharmaceutical preparations based on natural ingredients. The processing of natural materials into *simplicia*, the drying process is an important stage that aims to reduce moisture content, prevent the growth of microorganisms, and maintain the stability of active compounds³. Different drying methods, such as sun drying, drying in the shade, and drying using a dryer, can have different influences on the characteristics of *simplicia*⁴. Differences in temperature and drying time have the potential to lead to degradation of the active compound or changes in the physical properties of the material, which can further affect the extraction results⁵. The drying process of *simplicia* plays a very crucial role in the herbal medicine production chain because it functions to stop enzymatic activity that can degrade active compounds, while lowering the moisture content to a minimum limit to prevent the growth of contaminant microorganisms during the storage period⁶. In addition to ensuring the chemical stability and microbiological safety of raw materials, optimal drying also changes the physical characteristics of the plant to be more fragile, thus simplifying the pollination process and maximizing the efficiency of the filtration of active substances during extraction⁷.

Extraction is the process of separating active compounds from natural materials using certain solvents. The maceration method with 70% ethanol solvent is widely used because it is relatively safe, easy to apply, and able to extract polar and semipolar compounds optimally⁸. One of the important parameters in the extraction process is yield, which describes the efficiency of the extraction process and is influenced by the quality of the *simplicia*, the drying method, as well as the extraction conditions. Some previous studies have reported that drying methods can affect the active compound content and yield of medicinal plant extracts⁹.

Research on post-harvest optimization of *Etlingera elatior* has been conducted by Firdhauzi *et al.* (2024)¹⁰ and Alwi *et al.* (2025)¹¹, but the focus of the evaluation of the drying method is still limited to flower organs. On the other hand, Sartono *et al.* (2025)¹² have extracted the leaf part using 70% ethanol and obtained a fairly high yield (15.94%), but the *simplicia* used have not been evaluated based on the variety of drying methods. Although some previous studies have reported on the pharmacological activity of the kecombrang plant (*Etlingera elatior*), most of these studies are still limited to the use of flower and rhizome parts. Research on post-harvest optimization of leaves, especially related to the effect of drying methods on extraction efficiency, is still very rare. Based on this description, this study was conducted to examine the effect of the drying method on the yield of kecombrang leaf extract (*Etlingera elatior*) using the maceration method with 70% ethanol solvent, so that it is expected to provide scientific

information as a basis for selecting the right simplicia processing method in the development of natural medicinal materials.

METHODS

Tools and Materials

This study uses the following laboratory equipment, namely, YATT® brand analytical digital scales with an accuracy of 0.01g/ 0.001 oz, Pyrex® brand beaker glass (50 mL, 100 mL, and 250 mL), maceration containers made of glass covered with Iwaki® brand filters and Whatman® No.1 filter paper, heating devices in the form of manual water baths (no brand) with a temperature range of 30–100°C; Samsung® Galaxy A-series digital camera for documentation, AND® MX-50 brand moisture analyzer with a moisture content measurement range of 0–100%, Pyrex® brand measuring cups (100 mL and 500 mL); National omega® brand blender with 220W power, GEA® brand chemical thermometer with temperature range of 10^{0C} up to 110°C; as well as porcelain cups (no brand) 50ml, 60ml 75ml and 100ml¹³.

The materials used in this study include fresh kecombrang leaves (*Etlingera elatior*), 70% technical ethanol brand JK CARE®, Whatman® No.1 filter paper, and aluminum foil.

Classification

The following is a complete scientific classification of kecombrang plants :

Kingdom	: <i>Plantae</i>
Divisi	: <i>Magnoliophyta</i>
Class	: <i>Liliopsida</i>
Ordo	: <i>Zingiberales</i>
Family	: <i>Zingiberaceae</i>
Genus	: <i>Etlingera</i>
Species	: <i>Etlingera elatior</i> ¹⁴

Research Procedure

Preparation of Kecombrang Leaf Sempel (*Etlingera elatior*)

The research began with the preparation of samples in the form of fresh kecombrang leaves (*Etlingera elatior*) selected based on bright green and undamaged criteria. The sample was then wet sorted to remove impurities, washed using running water, drained, and cut to a uniform size to speed up the drying process¹⁵.

Simplicia Drying Process

The drying process of simplicia is carried out using three different methods, namely, Drying sunlight is carried out by drying kecombrang leaves in direct sunlight at 11.00–15.00 WIB with an average temperature of ±37°C until the leaves are dry. Wind drying is carried out at a room temperature of around ±27°C without exposure to direct sunlight for 3-10 days. Meanwhile, drying using a dryer is carried out at a temperature of ±60°C for 1–2 days until a constant weight is obtained. Each drying method was carried out six times¹⁶.

Determination of Simplicia Moisture Content

The resulting dry simplicia was then analyzed for moisture content using a moisture analyzer. Prepare the moisture analyzer tool and click the tare button on the tool. Then the simplicia sample is weighed as much as 1 gram, placed evenly on the weighing container, and then set the heating temperature (105) by the automatic stop method.

It is then analyzed until the moisture content is automatically displayed by the appliance as the drying shrinkage value¹⁷.

Simplisia Powder Manufacturing

The dried simplisia is then mashed using a blender until a homogeneous powder is obtained. Simplisia powder was weighed as much as 5 grams and extracted using the maceration method with 70% ethanol solvent at a material-to-solvent ratio of 1:20 (b/v). The maceration process is carried out for 3×24 hours in a closed container and protected from light, with stirring every 24 hours to improve contact between the sample and the solvent¹⁸.

Filtration and Thickening of Extracts

After the maceration process is complete, the solution is filtered using filter paper to obtain the extracted filtrate. The filtrate is then evaporated using a water bath until a thick extract is obtained.

Extract Yield Calculation

Extract yield is calculated based on the ratio of the weight of the thick extract to the weight of the initial simplisia powder and is expressed in percent (%), according to the method of determining the yield of natural material extract¹⁹.

Data Analysis

The data obtained from this study were in the form of drying shrinkage value, simplisia moisture content, and yield of kecombrang leaf extract after drying treatment with different methods. All data were analyzed using the IBM SPSS Statistics statistical application version 26 (IBM Corp., Armonk, NY, USA) which is commonly used in quantitative research to test differences between treatments.

Data analysis begins with testing the normality and homogeneity of the data to ensure the suitability of basic statistical assumptions. Once met, the statistical test used is *Analysis of Variance* (ANOVA) one-way to determine whether there was a significant difference between the three drying methods to the response variables (drying shrinkage, moisture content, and extract yield). In ANOVA, the significance level is $\alpha = 0.05$. The P-value < 0.05 indicates a significant difference between the treatments, while the P-value > 0.05 indicates no significant difference between the drying methods²⁰.

RESULT AND DISCUSSION

This study aims to determine the effect of different drying methods on the yield of kecombrang leaf extract (*Etlingera elatior*) extracted using the maceration method with 70% ethanol solvent. The drying methods used include sun drying, shade drying, and dryer cabinets. The results of the study show that the whole drying method meets the moisture content requirements, a good requirement for drying shrinkage is less than 10%⁹, with the lowest moisture content obtained in the dryer method (6.53–8.02%) and the highest in the shade drying method (9.67–9.99%). The yield of kecombrang leaf extract from each drying method can be seen in figure 1.

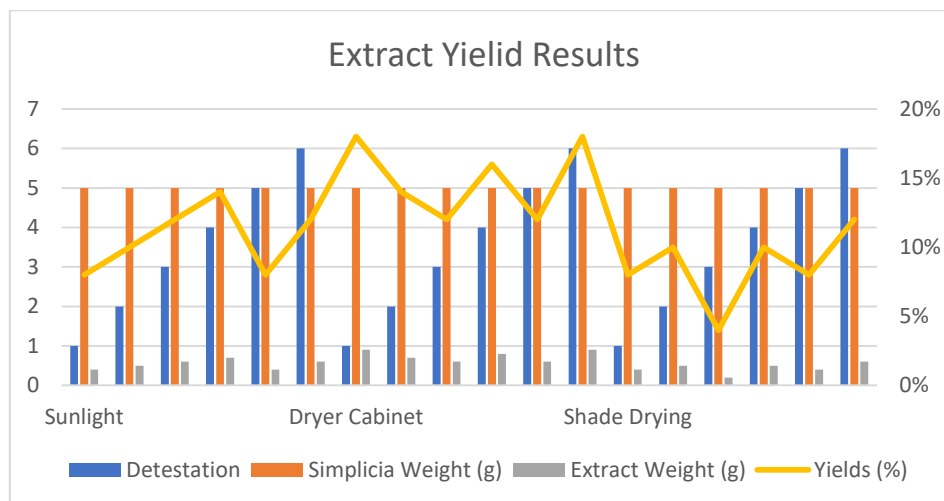


Figure 1. Yield of Kecombrang Leaf Extract Based on Drying Method

Based on the data presented in Figure 1, the average yield of kecombrang leaf extract is different in each drying method. The dryer method produced the highest average yield (15%), followed by sun drying (10.67%), and shade drying (8.67%). The difference in average values is quite clear showing that drying conditions affect the amount of extracts obtained. Drying using a dryer is carried out for 2 days at a temperature of 60°C and produces the highest yield. Stable temperatures and shorter drying times allow for more optimal moisture content reduction, thereby increasing the efficiency of the extraction process using 70% ethanol⁹.

Sun-drying is carried out for 3 days at temperatures ranging from 27–31°C and results in lower efficiency than a dryer. This is likely due to uncontrolled environmental conditions as well as temperature fluctuations. Meanwhile, drying the shade is carried out for 10 days at a temperature of 22–27°C and produces the lowest yield. Longer drying times at lower temperatures are thought to lead to less effective dehydration reductions, thus impacting extraction efficiency. Therefore, the difference in temperature and length of drying time contributed to the variation in the average yield of the extract, with the dryer method showing the most optimal results in this study²¹.

Table 1. Statistical test results

Perlakuan	Normalitas(p-value)	Homogenitas(p-value)	Anova(p-value)
SL	.415	.871	.003
DC	.191		
SD	.554		

Description :

SL : Sunlight

LP : Dryer cabinet

SD : Shade Drying

Normality test: p-value exceeding 0.05 indicates a normal distribution

Homogeneity test: p value exceeding 0.05 indicates homogeneous data

ANOVA test: p-value less than 0.05 indicates significant differences between data

Based on the results of the data analysis that has been carried out, the first stage is a normality test to find out whether the research data is normally distributed as one of the conditions for using parametric tests. The normality test was carried out using the Shapiro-Wilk test because the number of samples in each group was less than 50. The test results showed that the significance value (*Sig.*) was greater than 0.05, so it can be concluded that the data on each treatment were normally distributed²². The variance homogeneity test was carried out using the Levene test to find out whether the variance between treatment groups had similarities (homogeneous). Based on the results of the Levene test, a significance value of 0.871 was obtained. Since the significance value is greater than 0.05 ($0.871 > 0.05$), it can be concluded that the variance between treatment groups is homogeneous²³. The results of the analysis showed an F value of 17.432 with a significance value of 0.000. Since the significance value is less than 0.05 ($0.000 < 0.05$), it can be concluded that there is a significant difference in mean between the treatments given. This shows that different treatments have different effects on the value produced²⁴.

Multiple Comparisons

Dependent Variable: Turkey HSD Value

Table 2. Results of post hoc analysis

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Sunlight	Dryer Cabinet	-4.33333*	1.52510	.031	-8.2947	-.3719
	Shade Drying	2.00000	1.52510	.411	-1.9614	5.9614
Dryer Cabinet	Sunlight	4.33333*	1.52510	.031	.3719	8.2947
	Shade Drying	6.33333*	1.52510	.002	2.3719	10.2947
Shade Drying	Sunlight	-2.00000	1.52510	.411	-5.9614	1.9614
	Dryer Cabinet	-6.33333*	1.52510	.002	-10.2947	-2.3719

Sunlight Drying Method vs Dryer Cabinet Drying Method, the average sunlight method value is lower by -4.33333 than the dryer method. This difference is statistically significant with a p value = 0.031 (< 0.05). The 95% confidence interval is in the range of -8.2947 to -0.3719, which does not go past zero, thus reinforcing the existence of a real difference²⁵. Sunlight Drying Method vs Shade Drying Method, the average value of the sunlight method is higher by 2.00000 than the Shade Drying method. However this difference was not significant ($p = 0.411$), with a 95% confidence interval between -1.9614 to 5.9614, which passed zero. Dryer Cabinet Drying Method vs Sunlight Drying Method, the average value of the dryer method is higher at 4.33333 than the sunlight method. This difference was significant ($p = 0.031$) with a 95% confidence interval between 0.3719 to 8.2947, which did not go past zero²⁶. Dryer Cabinet Drying Method vs Shade Drying Method, the average value of the dryer method is higher by 6.33333 than the Shade Drying method. This difference was statistically significant with a value of $p = 0.002$ (< 0.05). The 95% confidence interval is in the range of 2.3719 to 10.2947, which does not cross the zero mark²⁷. Shade Drying Method

vs Sunlight Drying Method, the average value of the room temperature method is lower by -2.00000 than the sunlight method. However this difference was not significant ($p = 0.411$), with a 95% confidence interval between -5.9614 to 1.9614, which passed zero.

Shade Drying Method vs Drying Cabinet Drying Method, the average value of the Shade Drying method is lower by -6.33333 compared to the dryer method. This difference was significant ($p = 0.002$) with a 95% confidence interval between -10.2947 to -2.3719, all of which were below zero. Based on the results of the Post Hoc Tukey HSD test, significant differences were found in:

Sunlight vs Dryer Cabinets → Sunlight is lower

Dryer Cabinet vs Sunlight → Dryer Cabinet is higher

Dryer Cabinet vs Shade Drying → Dryer Cabinets are higher

Shade Drying vs Dryer Cabinet → Shade Drying is lower

From these results, it can be concluded that the drying method of drying the dryer produces the highest value and is significantly different from the sunlight and room temperature methods. Meanwhile, the sunlight and room temperature methods did not differ significantly from each other, so they had relatively equal but lower results than the dryer method.

Limitation

This study has several limitations that need to be considered in interpreting the results of the study. The parameters analyzed were only limited to the moisture content and yield of the extract, so they did not fully represent the overall quality and content of specific bioactive compounds of kecombrang leaf extract²⁸. In addition, stability testing of active compounds during the drying process was also not carried out. In addition, the environmental conditions in the sunlight drying and shade drying methods, such as temperature, humidity, and light intensity, are not strictly controlled, which can affect the consistency of the research results²⁹. This study used only one extraction method (maceration) and one type of solvent (70% ethanol), thus limiting the generalization of results to extraction methods or other types of solvents. Further research is recommended to include phytochemical analysis, stability tests, and comparisons with various extraction methods to obtain more comprehensive and representative results³⁰.

CONCLUSION

Based on the results of the study, the drying method affects the moisture content and yield of kecombrang leaf extract (*Etlingera elatior*). All drying methods meet acceptable moisture content requirements (<10%); However, the dryer method produced the lowest moisture content and highest extract yield (15%), compared to sun drying (10.67%) and shade drying (8.67%). The difference in temperature and length of drying time affects the efficiency of the extraction process. Drying using a dryer for 2 days at 60°C gives the most optimal results compared to drying in the sun for 3 days (27–31°C) and drying in the shade for 10 days (22–27°C). Therefore, the dryer method is considered the most effective approach to increase the yield of kecombrang leaf extract by using the maceration method and 70% ethanol solvent.

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AUTHOR CONTRIBUTION STATEMENT

TUTI ALAWIYAH: Conceptualization, Methodology, Investigation, Data Curation, Formal Analysis, Writing—Original Drafts, Writing—Review and Editing, Visualization, Supervision, Validation.

CONFLICT OF INTEREST DECLARATION

The author states that there is no conflict of interest.

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Analysis of Antioxidant Activity and Total Flavonoid Content of *Averrhoa bilimbi* L. Leaf Ethanol Extract Using the DPPH Method

Analisis Aktivitas Antioksidan dan Kandungan Flavonoid Total Ekstrak Etanol Daun *Averrhoa bilimbi* L. Menggunakan Metode DPPH

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Abstract

Starfruit (*Averrhoa bilimbi* L.) is not only a commonly consumed as a fruit but is also widely used in traditional medicine. The plant has long been utilized by the communities to help treat various ailments, including coughs and rectal inflammation. This study aimed to determine the total flavonoid content and antioxidant activity of the ethanol extracts of *Averrhoa bilimbi* leaves fractionated using n-hexane. The extraction process was carried out using the maceration method with 70% ethanol, followed by fractionation with 95% n-hexane to separate active compounds from the crude extract. The total flavonoid content in the n-hexane fraction of the ethanol extract was measured using a UV-Vis spectrophotometer at a wavelength of 229-230 nm, while antioxidant activity was evaluated using the DPPH method at 515 nm. The results showed that the total flavonoid content of the n-hexane fraction of the ethanol extract was 37.3 mgQE/g extract. The antioxidant activity test produced an IC₅₀ value of 6.58 µg/mL, compared to a standard IC₅₀ value of 2.45 µg/mL for Ascorbat acid. These findings indicate that the n-hexane fraction of *Averrhoa bilimbi* leaves ethanol extract possesses very strong antioxidant properties.

Abstrak

Belimbing (*Averrhoa bilimbi* L.) tidak hanya umum dikonsumsi sebagai buah tetapi juga banyak digunakan dalam pengobatan tradisional. Tanaman ini telah lama dimanfaatkan oleh masyarakat untuk membantu mengobati berbagai penyakit, termasuk batuk dan radang dubur. Penelitian ini bertujuan untuk menentukan kandungan flavonoid total dan aktivitas antioksidan dari ekstrak etanol daun *Averrhoa bilimbi* yang difraksinasi menggunakan n-heksana. Proses ekstraksi dilakukan menggunakan metode maserasi dengan etanol 70%, diikuti dengan fraksinasi dengan n-heksana 95% untuk memisahkan senyawa aktif dari ekstrak kasar. Kandungan flavonoid total dalam fraksi n-heksana dari ekstrak etanol diukur menggunakan spektrofotometer UV-Vis pada panjang gelombang 229-230 nm, sedangkan aktivitas antioksidan dievaluasi menggunakan metode DPPH pada 515 nm. Hasil menunjukkan bahwa kandungan flavonoid total fraksi n-heksana dari ekstrak etanol adalah 37,3 mgQE/g ekstrak. Uji aktivitas antioksidan menghasilkan nilai IC₅₀ sebesar 6,58 µg/mL, dibandingkan dengan nilai IC₅₀ standar sebesar 2,45 µg/mL untuk asam Askorbat. Temuan ini menunjukkan bahwa fraksi n-heksana dari ekstrak etanol daun *Averrhoa bilimbi* memiliki sifat antioksidan yang sangat kuat.

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INTRODUCTION

One of the major causes of non-communicable diseases is oxidative stress, a condition characterized by an imbalance between free radicals and antioxidants in the body. Free radicals are naturally produced during metabolic processes. However, excessive or uncontrolled free radical formation may cause oxidative damage to cells and increase the risk of degenerative diseases such as atherosclerosis, heart failure, cancer, and Alzheimer's disease.

Various health problems, long predating formal healthcare and modern medicine, have been identified. Many people are increasingly using plants as traditional medicines, citing their reduced side effects compared to synthetic drugs and their ability to maintain the immune system. Free radicals in the human body originate from endogenous and exogenous sources. Endogenous sources include autoxidation, enzymatic oxidation, and respiratory burst processes, while exogenous sources include medications, radiation, cigarette smoke, and air pollutants.^{14 15} Antioxidants play an important role in neutralizing free radicals and interrupting oxidation chain reactions, thereby protecting the body from oxidative damage.

Medicinal plants have long been used as traditional remedies because they are considered to have fewer side effects than synthetic drugs. One plant reported to possess antioxidant potential is *Averrhoa bilimbi* L. Previous studies demonstrated that ethanol extracts and fractions of *Averrhoa bilimbi* leaves exhibit hypoglycemic and hypolipidemic activities in diabetic rat models.⁴ In addition, purified ethanol extracts have shown potential antihypertensive activity.¹⁷

Antioxidant activity can be evaluated using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method, which measures the ability of compounds to scavenge stable free radicals.¹⁸ Reported that ethanol extract of *Averrhoa bilimbi* leaves exhibited very strong antioxidant activity with an IC_{50} value of $16.99 \pm 0.12 \mu\text{g/mL}$. Another study reported that 70% ethanol extract of *Averrhoa bilimbi* fruit showed strong antioxidant activity with an IC_{50} value of 74.625 ppm.³

Although several studies have reported the antioxidant activity of *Averrhoa bilimbi* leaves, most investigations have focused on crude ethanol extracts without further fractionation. Differences in extraction and fractionation methods may influence the composition of secondary metabolites and the biological activity of the resulting extracts. Limited information is available regarding the antioxidant activity and total flavonoid content of the n-hexane fraction of *Averrhoa bilimbi* leaves ethanol extract. Fractionation using n-hexane is important for separating compounds based on polarity and identifying fractions with potential bioactive properties. Therefore, this study aimed to determine the antioxidant activity based on IC_{50} values and the total flavonoid content of the n-hexane fraction of *Averrhoa bilimbi* leaves ethanol extract using the DPPH method.

METHODS

The type of research used is experimental research. To determine the total flavonoid levels and antioxidant activity of *Averrhoa bilimbi* leaves extract. This research was conducted at the Health Chemistry Laboratory, Faculty of Health, Nahdlatul Ulama University Surabaya. The research population was *Averrhoa bilimbi* plants obtained in Sekaran District, Lamongan, and the samples used were *Averrhoa bilimbi* leaves that were neither too old nor too young; there were no yellow spots, indicating that the leaves were not old. The plant samples have been identified/determined based on their scientific names at BRIN Cibinong Bogor, *Averrhoa bilimbi* leaves (*Averrhoa bilimbi* L), family *Oxalidacea*.

Tools used: in this research were a beaker glass, measuring cup, test tube, petri dish, volume pipette, dropper pipette, analytical balance, spatula, stirring rod, test tube and rack, vortex, bottle, set of extraction tools, and UV-Vis Spectrophotometer. **Materials:** used were *Averrhoa bilimbi* leaves, acetic acid, chloroform, ammonia, FeCl_3 , PbAcetate 5%, H_2SO_4 2 M, methanol, ethanol, n-hexane, gallic acid, sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl or DPPH, Tris-HCl buffer 1 M (pH 7.4), distilled water, quercetin standard, AlCl_3 10%, and ascorbic acid.

Research Procedure

Sample Preparation

One kg of washed *Averrhoa bilimbi* leaves was dried and blended. The leaves were extracted by maceration with 70% ethanol in a 1:10 ratio. The sample was isolated for 3 days at room temperature, away from sunlight, and stirred every 24 hours for 5 minutes. Filtering was performed with filter paper and a vacuum to separate the pulp and filtrate. Concentration was carried out using a rotary evaporator until the solvent separated. A thick ethanol extract of *Averrhoa bilimbi* leaves was obtained. Fractionation was performed using 95% n-hexane. The thick ethanol extract of *Averrhoa bilimbi* leaves was partitioned using a separating funnel using hexane. The fraction was evaporated using a rotary evaporator to obtain the hexane extract.

Determination of Total Flavonoids

- Qualitative Flavonoid Test: *Averrhoa bilimbi* leaves extract was dissolved with a suitable solvent (Aquadest) in two test tubes. 3-4 drops of FeCl_3 , and 5% Pb Acetate were added.¹⁹
- Quantitative Flavonoid Test:
 - A. Preparation of a standard quercetin solution: Weigh 25 mg of the standard quercetin solution. Dissolve it in 25 ml of 96% ethanol to obtain 1000 ppm. Take 1 ml using a pipette to obtain 1000 ppm, and add 10 ml of 96% ethanol to obtain 100 ppm.
 - B. Determination of Operating Time and Maximum Wavelength: Take 1 ml of the 100 ppm quercetin solution. Add 1 ml of 10% AlCl_3 and 8 ml of 5% acetic acid. The absorbance is measured at a wavelength of 200-600 nm.
 - C. Preparation of a quercetin standard curve: A series of concentration solutions was prepared using quercetin as the standard. These concentrations were 40, 60, 80, 100, and 120 ppm. A 1 mL solution of each concentration was reacted with 0.2 mL of 10% AlCl_3 , 0.2 mL of 10% acetic acid, 3 mL of 96% ethanol, and 5.6 mL of distilled water. The absorbance of the series was read using UV-Vis spectrophotometry at the maximum wavelength.
 - D. Determination of flavonoid content: *Averrhoa bilimbi* leaf extract was prepared at a concentration of 1000 ppm. A 1 mL sample of each 1000 ppm extract solution was taken. Variations in concentrations were made at 40 ppm, 60 ppm, 80 ppm, 100 ppm, and 120 ppm. 0.2 mL AlCl_3 , 0.2 mL 10% acetic acid, 3 mL 96% Ethanol, and 5.6 ml Aquades were added. The absorbance reading was carried out at the maximum wavelength.¹⁹ The absorbance results obtained were then calculated using Microsoft Excel.

Antioxidant Activity Test using DPPH method

- Preparation of *Averrhoa bilimbi* leaves extract: Prepare 25 mg of *Averrhoa bilimbi* leaves extract. Then dissolve it using 50 ml of methanol p.a. in a beaker.
- Preparation of DPPH solution: Weigh 10 mg of DPPH. Dissolve it in 34 ml of methanol p.a. to the mark using a 100 ml volumetric flask (100 ppm) as a stock solution. Then, place it in a dark glass bottle.
- Preparation of standard ascorbic acid solution as a control: Weigh 10 mg of ascorbic acid. Dissolve it in 100 ml of methanol p.a. using a 100 ml volumetric flask to obtain a stock solution with a concentration of 1000 ppm. The test is performed by making six serial dilutions of the ascorbic acid reference solution: 2 ppm, 3 ppm, 4 ppm, 5 ppm, and 6 ppm.
- Determining the maximum DPPH wavelength: 1 ml of DPPH solution was placed in a test tube. Add methanol to a total volume of 5 ml, then homogenize and incubate at 37°C for 30 minutes. Measure the solution using a UV-Vis spectrophotometer at a wavelength of 515-518 nm and determine the maximum wavelength.
- Preparation of Blank: A blank 1 ml of DPPH solution was placed in a test tube, methanol was added to a total volume of 5 ml, and then covered with aluminum foil. The mixture was homogenized and incubated in the dark at 37°C for 30 minutes. Measure the blank solution using a UV-Vis spectrophotometer at a wavelength of 517 nm.
- Control measurements: 1 ml of each sample concentration was placed in a test tube. Add 1 ml of DPPH solution and 3 ml of p.a. methanol. Homogenize the solution. Incubate in a dark room at 37°C for 30 minutes. The absorbance of each sample was measured using a UV-Vis spectrophotometer at a wavelength of 517 nm.
- Antioxidant Activity Measurement: From each stock solution, 1 ml was pipetted to obtain concentrations of 25 ppm, 50 ppm, 75 ppm, 100 ppm, 125 ppm, and 150 ppm. Each concentration was made up to volume with methanol p.a. in a 5 ml volumetric flask. Then, 1 ml of each concentration was taken, and 2 ml of DPPH solution was added. The mixture was vortexed and incubated for 30 minutes at room temperature. The absorbance of each solution was measured at a wavelength of 515 nm, with four replicates. The absorbance results were then calculated using Microsoft Excel for IC₅₀.¹⁰

Data Analysis

Data analysis in the DPPH method antioxidant activity test was carried out by calculating the percentage of DPPH free radical inhibition, which was calculated using the formula:

$$\% \text{ Inhibisi} = \frac{\text{Abs Blank} - \text{Abs sample}}{\text{Abs Blank}} \times 100$$

RESULT AND DISCUSSION

The extraction results from 1000 ml of *Averrhoa bilimbi* leaves extract, using a 70% ethanol solvent concentrated with a rotary evaporator, were in the form of a 200 ml thick extract. Then, it was fractionated using 96% n-hexane and reconstituted with a rotary evaporator to obtain a 20 ml thick extract.

Total Flavonoid Content Results

The qualitative flavonoid test aims to determine the presence or absence of flavonoids in *Averrhoa bilimbi* leaves extract.

Table 1. Qualitative Test of Flavonoids

Test	Reagent	Observation	Result
Flavonoid	FeCl ₃	Violet	+

The test showed a positive result for flavonoids. A positive result with FeCl₃ indicated a purple color change, formed due to a complex reaction between the Fe metal from FeCl₃ and the flavonoid hydroxyl group. The operating time was determined to determine the length of time required for the solution to reach constant absorbance. This was determined by measuring the absorbance of the quercetin standard solution at its maximum wavelength using a UV-Vis spectrophotometer. This was done by taking 1 mL of the quercetin solution and adding 1 mL of 10% AlCl₃ and 8 mL of 5% acetic acid. The study obtained a constant absorbance operating time. Analysis of the wavelength determination using a concentration of 100 ppm yielded a maximum wavelength of 229 nm. This maximum wavelength must be determined for use in sample content determination because it is at this wavelength that the maximum absorbance value is obtained, where the absorption change due to concentration is also maximum.¹¹

Table 2. Results of determining operating time

Minutes to	Absorbance (229 λ)
0	0.229
1	0.228
2	0.227
3	0.229
4	0.229

Table 3. Absorbance value of quercetin standard solution

No.	Concentration (ppm)	Absorbance Average
1	40	0.237
2	60	0.238
3	80	0.511
4	100	0.546
5	120	0.774

The standard curve is intended to calculate the concentration of *Averrhoa bilimbi* leaves. The determination of the standard curve is obtained by reading the absorbance value of the quercetin standard solution with concentrations of 40 ppm, 60 ppm, 80 ppm, 100 ppm, and 120 ppm. The absorbance value of the quercetin standard solution can be seen in Table 3. Based on Table 3, the linear regression equation is obtained, namely $Y = 0.0069x - 0.0916$, and the correlation coefficient (r) is 0.92.

Determination of Flavonoid Content in *Averrhoa bilimbi* leaves

Flavonoids in *Averrhoa bilimbi* leaves extract were determined by entering absorbance values into a quercetin standard curve, resulting in an average total flavonoid content of 37.3 mgQE/g extract. The higher the flavonoid content, the greater the flavonoid's antioxidant benefits.¹²

Results of Antioxidant Activity Testing with a UV-Vis Spectrophotometer

Measurement of antioxidant activity using the DPPH method utilizes spectrophotometric principles. A compound with antioxidant activity will donate its hydrogen atom to bind with DPPH, forming reduced DPPH, indicated by a loss of purple color to pale yellow, accompanied by a decrease in absorbance. Absorbance

measurements in this study were performed at a maximum wavelength (λ_{max}) of 515 nm for the DPPH solution, with absorbance being the maximum wavelength that can be used for measurements using the DPPH method as stated by.⁵ The measurement data in Table 4 indicates DPPH radical scavenging activity, as evidenced by a decrease in DPPH radical absorbance values caused by the test samples at various concentrations and an increase in the percentage (%) of antioxidant activity. This is also visible with the naked eye, as the purple color fades and becomes slightly yellowish after a 30-minute incubation period.⁷

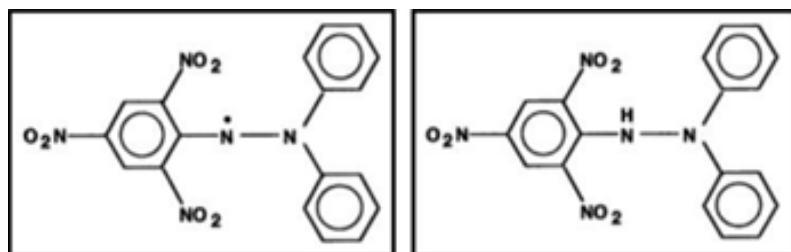


Figure 1. DPPH radical inhibition reaction (Molyneux, 2004)

This color change occurs due to the presence of compounds in the sample that donate hydrogen atoms to the DPPH radical so that it is reduced to a more stable form, namely DPPH-H (1,1-diphenyl-2-picrylhydrazine) (Figure 1) from the results of measuring the n-hexane fraction of *Averrhoa bilimbi* leaves extract shows that the higher the sample concentration, the lower the absorbance value of the DPPH solution in methanol.¹

Table 4. Results of Antioxidant Activity Measurement of *Averrhoa bilimbi* leaves Extract Samples in UV-Vis Spectrophotometry

Concentration (ppm)	Abs Average (4 Replicate)	% Inhibition	Regression Equation	IC ₅₀ (µg/mL)
Blanko	0.012	0	Y= 0.3862x + 7.5293	6.58
25	0.223	20.63%	R= 0.956	
50	0.215	22.94%		
75	0.215	25.26%		
100	0.141	49.73%		
125	0.138	50.04%		
150	0.088	68.58%		

The regression equation between the concentration of *Averrhoa bilimbi* leaves extract sample (x-axis) and the percentage (%) of antioxidant activity (y-axis) is $y = 0.3862x + 7.5293$ with a correlation coefficient value (R^2) = 0.956. The positive correlation coefficient value illustrates that with increasing concentration of n-hexane fraction, the antioxidant activity is also greater. This relationship can be seen in the picture.⁸

The parameter used in testing antioxidant activity by capturing DPPH radicals is the Inhibitory Concentration (IC₅₀) value. The smaller the IC₅₀ value, the more active the sample is as an antioxidant. Based on the linear regression equation obtained, the IC₅₀ value or the concentration required to capture 50% of the DPPH radicals from the n-hexane fraction is 6.58 µg/mL. This value indicates that the n-hexane fraction of *Averrhoa bilimbi* leaves extract has very strong antioxidant activity, because it has an IC₅₀ value ranging from 0-50 µg/mL. The positive control used in this study was Ascorbic acid. The use of a positive control was intended to compare how strong the antioxidant potential

contained in the n-hexane fraction of *Averrhoa bilimbi* leaves extract is with the antioxidants contained in the n-hexane fraction of *Averrhoa bilimbi* leaves extract.

Table 5. Results of Measurement of Antioxidant Activity of Ascorbic acid in UV-Vis Spectrophotometry

Concentration (ppm)	Abs Average (4 Replicate)	% Inhibition	Regression Equation	IC ₅₀ (µg/mL)
Blanko	0.012	0	Y= 0.0552x + 20.309	2.45
25	0.221	21.88%	R= 0.9883	
50	0.234	22.86%		
75	0.211	24.90%		
100	0.208	25.97%		
125	0.208	26.57%		
150	0.201	28.28%		

The linear regression equation obtained from the results of ascorbic acid antioxidant activity measurements in table 5 is $y = 0.0552x + 20.309$ with a correlation coefficient value of 0.9883. The IC₅₀ value obtained from the results of ascorbate acid measurements is 2.45 µg/mL. This value indicates that ascorbic acid has very strong antioxidant activity, because it has an IC₅₀ value of less than 50 µg/mL. The relationship between concentration and percentage inhibition of ascorbate acid can be seen in table 5.

The antioxidant activity test showed that the n-hexane fraction of *Averrhoa bilimbi* leaf ethanol extract exhibited very strong antioxidant activity, with an IC₅₀ value of 6.58 µg/mL. In comparison, ascorbic acid used as the positive control showed an IC₅₀ value of 2.45 µg/mL, indicating stronger antioxidant activity than the sample fraction. According to antioxidant activity classification, compounds with IC₅₀ values below 50 µg/mL are categorized as very strong antioxidants. Therefore, both the n-hexane fraction and ascorbic acid were classified as very strong antioxidants, although ascorbic acid exhibited higher antioxidant potency due to its lower IC₅₀ value.

The antioxidant activity observed in this study may be associated with the presence of flavonoid compounds and other phytochemical constituents in the extract. Flavonoids are known to act as free radical scavengers by donating hydrogen atoms or electrons to stabilize free radicals and inhibit oxidative chain reactions. The total flavonoid content obtained in this study was 37.3 mg QE/g extract, suggesting that flavonoid compounds may contribute to the antioxidant activity of the n-hexane fraction.

The antioxidant activity obtained in the present study was stronger than that reported by ¹⁸, who found that ethanol extract of *Averrhoa bilimbi* leaves had an IC₅₀ value of 16.99 µg/mL. Similarly, ³ reported that 70% ethanol extract of *Averrhoa bilimbi* fruit exhibited an IC₅₀ value of 74.63 ppm, which was categorized as a strong antioxidant. The lower IC₅₀ value obtained in the present study indicates higher antioxidant potency compared to those previous studies. ⁹

Differences in antioxidant activity among studies may be influenced by several factors, including plant part used, extraction solvent, fractionation process, phytochemical composition, environmental growing conditions, and extraction methods. In this study, fractionation using n-hexane may have concentrated certain bioactive compounds

responsible for antioxidant activity, resulting in stronger free radical scavenging activity. Fractionation separates compounds based on polarity, allowing specific metabolites to become enriched in particular fractions.²

Several phytochemical compounds reported in *Averrhoa bilimbi* leaves, including flavonoids, phenolics, tannins, and terpenoids, are known to possess antioxidant properties. These compounds can donate electrons to neutralize DPPH free radicals, thereby reducing oxidative stress. The strong antioxidant activity demonstrated by the n-hexane fraction suggests that semi-nonpolar compounds contained in the fraction may contribute significantly to its antioxidant potential.

The strong antioxidant activity observed in the n-hexane fraction may also be associated with the presence of less polar flavonoid compounds, such as flavonoid aglycones. Compared with glycosylated flavonoids, flavonoid aglycones generally possess higher lipophilicity, allowing better interaction with free radicals in lipid environments and improved membrane permeability. In addition, hydroxyl groups present in flavonoid structures play an important role in hydrogen donation and radical stabilization mechanisms. Therefore, the flavonoid content and other semi-nonpolar phytochemicals present in the n-hexane fraction may contribute synergistically to the antioxidant activity observed in the DPPH assay.

Overall, the findings of this study indicate that the n-hexane fraction of *Averrhoa bilimbi* leaf ethanol extract has promising potential as a natural antioxidant source and may be further developed for pharmaceutical, nutraceutical, or functional food applications. However, further studies are needed to identify specific active compounds responsible for the antioxidant activity and to evaluate their biological effects through in vivo studies.

LIMITATION

This study has several limitations. First, the antioxidant activity evaluation was conducted only using the DPPH method, which measures free radical scavenging activity in vitro and may not fully represent antioxidant activity under biological conditions. Second, the study focused only on the n-hexane fraction of *Averrhoa bilimbi* leaf ethanol extract without identification or characterization of specific active compounds responsible for the observed antioxidant activity. Third, the total flavonoid content was measured quantitatively, but individual flavonoid compounds were not analyzed using advanced analytical techniques such as HPLC or LC-MS. In addition, this study did not evaluate other phytochemical constituents that may also contribute to antioxidant activity. Finally, the biological activity of the extract was not assessed through in vivo studies; therefore, further research is needed to confirm its pharmacological potential, safety, and mechanism of action in biological systems.

CONCLUSION

Based on the research that has been done, it can be concluded that the total flavonoid content of the n-hexane extract of *Averrhoa bilimbi* leaves (*Averrhoa bilimbi* L.) quantitatively ranges from 37.5 mgQE/g extract or 3.75%. The n-hexane fraction of *Averrhoa bilimbi* leaves extract has antioxidant activity, which is indicated by an IC₅₀ value of 6.58 µg/mL, while ascorbic acid, as a positive control, has an IC₅₀ value of 2.45 µg/mL.

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AUTHOR CONTRIBUTION STATEMENT

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Overview Of Toddlers Diarrhea Self-Medication At Pharmacy "X", Sidoarjo, January-March 2025

Tinjauan Umum Pengobatan Mandiri Diare Pada Balita Di Apotek "X", Sidoarjo, Januari-Maret 2025

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Abstract

Diarrhea is one of the common health problems in toddlers and is often treated through self-medication by the community. Choosing the right medicine is very important to avoid complications, considering the low immune system of toddlers and the potential risks of using drugs without a doctor's prescription. This study used a qualitative descriptive design, the study was conducted at the "X" Pharmacy, Sidoarjo District, Sidoarjo Regency from January to March 2025. The sample consisted of 90 parents/caregivers of toddlers who bought diarrhea medicine for their children and met the inclusion criteria. The results showed that the types of drugs most often chosen for self-medication for diarrhea in toddlers were probiotics (46%), zinc supplements (41%), Oral Rehydration Solution (ORS) (11%), and a combination of kaolin-pectin (2%). The characteristics of the respondents were mostly aged 20-25 years and had a high school or diploma education level. The choice of diarrhea medicine in self-medication in toddlers was dominated using probiotics and zinc, in accordance with the recommendations of the Indonesian Ministry of Health in handling diarrhea. However, there is still a need to improve education to the public regarding the rational use of drugs and clinical indications for giving antibiotics. An active role is needed for health workers, especially pharmacists, in providing accurate information to the public to prevent the negative impacts of inappropriate self-medication.

Abstrak

Diare merupakan salah satu masalah kesehatan umum pada balita dan seringkali diobati secara mandiri oleh masyarakat. Memilih obat yang tepat sangat penting untuk menghindari komplikasi, mengingat sistem imun balita yang masih lemah dan potensi risiko penggunaan obat tanpa resep dokter. Penelitian ini menggunakan desain deskriptif kualitatif, penelitian dilakukan di Apotek "X", Kecamatan Sidoarjo, Kabupaten Sidoarjo dari Januari hingga Maret 2025. Sampel terdiri dari 90 orang tua/pengasuh balita yang membeli obat diare untuk anak mereka dan memenuhi kriteria inklusi. Hasil penelitian menunjukkan bahwa jenis obat yang paling sering dipilih untuk pengobatan mandiri diare pada balita adalah probiotik (46%), suplemen seng (41%), Oral Rehydration Solution (ORS) (11%), dan kombinasi kaolin-pektin (2%). Karakteristik responden sebagian besar berusia 20-25 tahun dan memiliki tingkat pendidikan SMA atau diploma. Pilihan obat diare dalam pengobatan mandiri pada balita didominasi oleh penggunaan probiotik dan seng, sesuai dengan rekomendasi Kementerian Kesehatan Indonesia dalam penanganan diare. Namun, masih perlu peningkatan edukasi kepada masyarakat mengenai penggunaan obat yang rasional dan indikasi klinis pemberian antibiotik. Peran aktif diperlukan dari petugas kesehatan, khususnya apoteker, dalam memberikan informasi yang akurat kepada masyarakat untuk mencegah dampak negatif dari pengobatan mandiri yang tidak tepat.

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INTRODUCTION

Indonesians frequently resort to self-medication as a preliminary measure in addressing health concerns when symptoms manifest. Self-medication is defined as the practice of treating oneself by selecting and using medications available without a physician's prescription, provided that the medication is deemed safe and appropriate for the symptoms being experienced^{1,2,3}. Proper self-medication can provide several benefits, including reducing healthcare costs, shortening waiting times for treatment, and decreasing the workload of healthcare professionals, especially for minor illnesses^{2,4}.

However, inappropriate self-medication practices due to limited knowledge regarding dosage, indications, and drug selection may lead to adverse effects, inappropriate therapy, and delayed diagnosis^{1,4,5}. Therefore, adequate public education regarding rational drug use is essential, particularly for diseases that are commonly treated without medical supervision^{6,7}. One of the diseases frequently treated through self-medication is diarrhea. Diarrhea is considered a common illness that can often be managed independently if there are no severe complications^{6,8}.

Diarrhea is characterized by an increase in the frequency of bowel movements with a soft to liquid consistency. This ailment is among those with a high incidence rate, particularly among young children. A multitude of reports indicate that diarrhea persists as a primary cause of mortality among children, predominantly attributable to the rapid onset of dehydration^{9,10}. Clinically, diarrhea is characterized by an increased frequency of bowel movements with soft or liquid stool consistency. Despite being common, diarrhea remains a significant public health problem, especially among infants and toddlers, due to the high risk of dehydration and nutritional disturbances^{9,10}. Several studies have also reported that recurrent diarrhea during early childhood is associated with impaired growth and an increased risk of stunting^{11,12}.

The causes of diarrhea are multifactorial and include infections caused by bacteria, viruses, and parasites, as well as non-infectious causes such as food poisoning and adverse effects of certain medications. The spread of diarrheal diseases is closely related to environmental sanitation, access to clean water, personal hygiene, and food safety practices^{13,14}. Poor sanitation conditions and unhealthy living environments contribute significantly to the transmission of diarrhea, particularly in densely populated communities. Therefore, diarrhea management should not only focus on pharmacological therapy but also involve preventive efforts through the implementation of clean and healthy living behaviors^{4,15-17}.

Management of diarrhea in children generally includes fluid and electrolyte replacement through oral rehydration solution, zinc supplementation, adequate nutritional intake, and the selective use of antibiotics when clinically indicated. Early and proper management is important because toddlers are highly vulnerable to dehydration due to their immature immune systems^{18,19}. In addition, children with poor nutritional status may experience worsening health conditions if diarrhea is not treated appropriately and promptly²⁰⁻²². For this reason, the role of parents or caregivers in selecting appropriate medication during self-medication practices becomes very important.

This study was conducted in an area characterized by high population density and environmental conditions that may increase the risk of diarrhea transmission, such as rice fields, swamps, and drainage areas^{17,23}. Pharmacy "X" in Sidoarjo District is frequently visited by families with toddlers, making self-medication practices for childhood diarrhea relatively common in this setting^{16,17,24}. Research regarding the pattern of self-medication for diarrhea in toddlers is important to provide information about the types of medications commonly used by the community and to evaluate whether these practices are in line with current recommendations²³.

Therefore, this study aimed to describe the pattern of self-medication for diarrhea in toddlers at Pharmacy "X" in Sidoarjo District during January–March 2025. The findings of this study are expected to provide additional information regarding the use of diarrhea medication in toddlers, serve as reference material for educational institutions, and improve public understanding of the rational and safe use of medicines for childhood diarrhea²⁵. Furthermore, this study is expected to provide practical information for families in managing childhood diarrhea safely and in accordance with recommended treatment guidelines.

METHODS

The present study employed a descriptive qualitative design, incorporating a structured interview approach, to obtain an overview of self-medication practices for treating diarrhea in toddlers at Pharmacy "X" in Sidoarjo District. The data collection process was executed concurrently, thereby ensuring the collection of results that reflected the prevailing circumstances in the field. The data collection procedure commenced with the procurement of research authorization, subsequently followed by the selection of respondents based on predetermined inclusion criteria. Respondents who met the established criteria were administered a questionnaire. Subsequent to the collection of data, the researcher examined the data, coded it, transferred it to a master table, and cleaned it to ensure the validity and consistency of the information. The research was conducted at Pharmacy "X" in Sidoarjo District, Sidoarjo Regency. The data collection phase was conducted from January to March of 2025.

Research Variables

The present study incorporated two primary variables: independent and dependent variables. The independent variable in this study was the selection of diarrhea medication for toddlers by parents or caregivers. The dependent variable in this study was a description of self-medication practices for diarrhea in toddlers at Pharmacy "X." Each variable is described based on characteristics that can be measured through a questionnaire. The age of the infant's mother is measured using an interval scale, the age of the infant using a nominal scale, and the level of education using an ordinal scale. The therapy given uses a nominal scale with categories of drug choices such as kaolin-pectin, probiotics, zinc supplementation, and oral rehydration solution.

Population and Sample

Inclusion criteria

inclusion criteria, namely parents who purchased medication for toddlers aged 1–5 years with diarrhea and were willing to be respondents.

Exclusion criteria

Exclusion criteria included parents who could not read and write, spoke languages other than Indonesian, or parents of toddlers who did not have diarrhea.

Sampling Techniques

The sampling technique employed nonprobability sampling in conjunction with the purposive sampling method, a technique that determines the sample based on specific criteria deemed pertinent to the research objectives. The sample size was determined using the Slovin formula with a 10% margin of error and a 90% confidence level, as follows:

$$n = \frac{N}{1 + Ne^2}$$

Information:

n = sample size

N = population size

e = margin of error (10% or 0.1)

Upon entering a population value of 42, the following results were obtained:

$$n = \frac{42}{1 + 42(0,1)^2}$$
$$n = \frac{42}{1 + 0,42}$$
$$n = \frac{42}{1,42} = 29,57 \approx 30$$

This number was then multiplied by three according to the research requirements, resulting in a total sample of 90 respondents.

Instruments and Data Collection

The research instrument employed a structured questionnaire administered through face-to-face interviews with respondents. The questionnaire was designed to identify the types of drugs chosen and the factors influencing self-medication decisions. Primary data were obtained from interviews and questionnaires, while secondary data were obtained from records of the number of toddlers suffering from diarrhea at Pharmacy "X".

Data Analysis

The data obtained from the questionnaire were analyzed using descriptive univariate analysis. The analysis was conducted to describe respondents' characteristics, including parents' age, toddlers' age, education level, and the types of drugs used for self-medication of diarrhea in toddlers. The data were presented in the form of frequency distribution tables and percentages. Percentage calculations were performed using the following formula:

$$P = \frac{f}{n} \times 100\%$$

Where:

- P = Percentage
- f = Frequency
- n = Total number of respondents

The results were interpreted descriptively according to the objectives of the study.

RESULT AND DISCUSSION

The present study was conducted over a period of three months at Pharmacy "X" in Sidoarjo District, Sidoarjo Regency, from January to March 2025. The data presented herein was obtained through the administration of questionnaires to parents or caregivers of toddlers with diarrhea.

Respondent Characteristics

The respondents' characteristics in this study include their age, the age of the toddlers they care for, and their level of education. A comprehensive demographic overview of the respondents is presented in Table 2. The total number of respondents in this study was 90 people.

Table 1. Respondent Characteristics

Characteristics	Categories	Frequency (n)	Percentage (%)
Respondent Age	20-25 Years Old	50	55%
	26-30 Years Old	25	27%
	31-35 Years Old	15	17%
	36-40 Years Old	0	1%
Toddler Age	1 Years Old	27	32%
	2 Years Old	27	32%
	3 Years Old	29	35%
	4 Years Old	7	1%
Respondent Education	Junior High School	7	8%
	Senior High School	48	53%
	Diploma / College	35	39%
Amount		90	100%

As indicated by the data presented in Table 1, the majority of respondents fell within the 20–25 age range (55%), with a significant proportion comprising those between the ages of 26 and 30 (27%), followed by those between 31 and 35 (17%), and finally those between 36 and 40 (1%). The age of respondents constitutes a salient factor that can influence their capacity to comprehend health information, including in determining medication choices for self-medication. In the context of toddler age characteristics, the 3-year-old age group exhibits the highest prevalence (35%), followed by 1 and 2 years old (32% each) and 4 years old (1%). Regarding educational attainment, the data indicated that more than half of the respondents had completed high school, accounting for 53% of the sample. This was followed by individuals who had obtained a diploma or college degree, constituting 39% of the respondents. Notably, only 8% of the respondents had graduated from junior high school, and no respondents reported not having completed high school. The respondents' level of education has been demonstrated to influence their capacity to receive and process information related to the management of diarrhea.

Type of Medication Administration

The therapeutic approach to diarrhea encompasses non-pharmacological interventions, such as fluid and electrolyte replacement, dietary modifications, and pharmacological prevention and therapy. This latter category includes the use of antidiarrheal medications and antibiotics. The administration of antibiotics is reserved for specific conditions, including bloody diarrhea, diarrhea caused by cholera, and diarrhea accompanied by comorbidities²⁸ The present study documented the various categories of pharmaceuticals and nutritional supplements procured by respondents during the designated study period.

Table 2. Types of Medication Selection

Type of Medicine	Number of Patients	Percentage (%)
Probiotics	30	46%
Zinc	27	41%
Oral Rehydration Solution	26	11%
Kaolin–Pectin	7	2%

Amount	90	100
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The most prevalent type of medication administered was probiotics, given to 30 infants (46%). Probiotics, such as *Lactobacillus casei* (L-Bio) and a combination of *Lactobacillus acidophilus*–*Bifidobacterium longum*–*Streptococcus thermophilus* (Lacto-B), have been shown to aid in the absorption of nutrients, regulate water absorption, and enhance stool consistency. Probiotics have been demonstrated to reduce intestinal pH through the production of lactic acid, thereby impeding the proliferation of pathogenic bacteria. Probiotics have been demonstrated to be efficacious in reducing the frequency and duration of acute diarrhea in children. Zinc supplements were utilized by 27 infants (41%) This finding is consistent with Nasif ⁷, who reported that probiotics were among the most commonly selected self-medication choices for diarrhea in community pharmacies in Padang, Indonesia. Similarly, Nuzhat et al emphasized the growing community preference for probiotic-based interventions in managing childhood diarrhea in Southeast Asia. Zinc has been demonstrated to play a role in DNA formation, boosts the immune system, and supports protein synthesis. As posited by Wulandari ²³, the regular administration of zinc supplements for a period of ten days has been demonstrated to reduce the severity of diarrhea, enhance immune system function, and prevent recurrent bouts of diarrhea. It has also been reported that zinc effectively reduces the frequency of defecation and the duration of acute diarrhea in toddlers. In line with the current study, Legesse et al¹⁹ found that zinc supplementation was widely used in combination with ORS in treating diarrhea among under-five children in East Africa, highlighting its universal role in diarrhea management across low-middle income countries.

Furthermore, oral rehydration solution was utilized by 26 toddlers (11%). An oral rehydration solution is a medical intervention employed to restore fluid and electrolyte levels in patients suffering from diarrhea. The administration of oral rehydration solution (ORS) can reduce stool volume by 25%, reduce nausea and vomiting by 30%, and reduce the need for intravenous fluids by up to 33%²⁹. The efficacy of the WHO-recommended ORS in accelerating the recovery of diarrhea has been demonstrated by numerous studies, which have also noted the potential for imbalances in sodium and potassium levels following rehydration. The relatively low use of ORS (11%) in this study contrasts with WHO recommendations and findings by Legesse ¹⁹, who reported higher ORS co-utilization rates in structured health settings. This gap suggests a need for community-level health promotion to increase ORS awareness among caregivers.

In Another medication employed in this study is a combination of kaolin-pectin, administered to 7 toddlers (2%). According to Walker, kaolin-pectin functions by adsorbing bacterial toxins and binding water, thereby reducing intestinal peristalsis and improving stool consistency²⁵. The minimal use of kaolin-pectin (2%) in this study aligns with the declining recommendation of this drug class in current clinical guidelines, as it is no longer a first-line treatment for childhood diarrhea²⁷.

CONCLUSION

A study of the description of self-medication in the management of diarrhea in toddlers at Pharmacy "X" in Sidoarjo District from January to March 2025 was conducted. The results of the study indicated that the most commonly used drug group by respondents was probiotics (46%), followed by zinc supplements (41%), oral rehydration solution (11%), and kaolin-pectin combination (2%). These findings suggest that the medication selected by the community does not yet fully align with current WHO recommendations²⁷. The aforementioned recommendations underscore the significance of zinc and oral rehydration solution as the primary therapeutic

modalities for treating diarrhea in children. Zinc has been demonstrated to play a role in reducing the frequency of bowel movements, improving stool consistency, and accelerating the healing process. In addition, oral rehydration solutions serve to replace fluid and electrolyte loss, thereby preventing dehydration..

In light of these findings, it is recommended that Pharmacy "X" in Sidoarjo District increase its educational and outreach activities to the community regarding the proper use of medications for self-medication of diarrhea in toddlers, in accordance with the recommendations of international health organizations. Furthermore, concerted promotional efforts are required to prevent diarrhea through various measures, including the promotion of clean and healthy living behaviors, maintenance of environmental hygiene, hand-washing before eating, exclusive breastfeeding, provision of complementary foods at 6-9 months of age, utilization of proper sanitation facilities, and provision of immunizations, including measles immunization. It is anticipated that these initiatives will enhance public awareness and contribute to a reduction in the prevalence of diarrhea among toddlers.

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AUTHOR CONTRIBUTION STATEMENT

APH: Supervision, Guidance, Conceptualization; **LS:** Data Collection, Pharmacy Permission Processing, Investigation; **AZF:** Data Curation, Analysis, Writing Original Draft Preparation

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Activity Test Of Papaya Fruit Extract And *Curcuma Mangga* As An Appetite Enhancer With Anorexia Test Model On Marshmallow Preparation Innovation

Uji Aktivitas Ekstrak Buah Pepaya Dan *Curcuma Mangga* Sebagai Peningkat Nafsu Makan Dengan Model Uji Anoreksia Pada Inovasi Produk Marshmallow

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Abstract

Feeding difficulties in children are becoming an increasing global problem. Natural alternatives such as *Carica papaya* and *Curcuma mangga* are believed to increase appetite. This study tested the combination of the two as an anorexia agent in the form of marshmallows, as an attractive, nutritious, and attractive delivery innovation for active substances that children like. The study used a combination of *Carica papaya* and *Curcuma mangga* extracts in marshmallow form to treat anorexia. The process includes extraction, phytochemical testing, rat testing, marshmallow formulation, and organoleptic evaluation, weight uniformity, and hedonic test for children and adults to assess its effectiveness and acceptability. Phytochemical screening showed *Carica papaya* and *Curcuma mangga* extracts contained alkaloids, steroids, polyphenols, and vitamin C. The anorexia test had a p-value of 0.05. The anorexia test has a p value (>0.05) so it has an insignificant value, but the extract still increases appetite. The formulated marshmallow has a chewy texture, a favorable aroma, but the taste and texture are less attractive to panelists. *Carica papaya* and *Curcuma mangga* extracts contain active compounds and showed appetite- enhancing effects although not statistically significant. Marshmallow has a vanilla aroma that is preferred, but the taste and shape are less than optimal. The study is limited in number of samples, further quantitative analysis and formulation evaluation are recommended.

Abstrak

Kesulitan makan pada anak-anak menjadi masalah global yang semakin meningkat. Alternatif alami seperti *Carica papaya* dan *Curcuma mangga* dipercaya dapat meningkatkan nafsu makan. Studi ini menguji kombinasi keduanya sebagai agen anoreksia dalam bentuk marshmallow, sebagai inovasi penyampaian zat aktif yang menarik, bergizi, dan disukai anak-anak. Studi ini menggunakan kombinasi ekstrak *Carica papaya* dan *Curcuma mangga* dalam bentuk marshmallow untuk mengobati anoreksia. Prosesnya meliputi ekstraksi, pengujian fitokimia, pengujian pada tikus, formulasi *marshmallow*, dan evaluasi organoleptik, keseragaman berat, dan uji hedonik untuk anak-anak dan orang dewasa untuk menilai efektivitas dan penerimaannya. Skrining fitokimia menunjukkan ekstrak Carica papaya dan Curcuma mangga mengandung alkaloid, steroid, polifenol, dan vitamin C. Uji anoreksia memiliki nilai p 0,05. Uji anoreksia memiliki nilai p (>0,05) sehingga nilainya tidak signifikan, tetapi ekstrak tersebut tetap meningkatkan nafsu makan. Marshmallow yang diformulasikan memiliki tekstur kenyal, aroma yang menyenangkan, tetapi rasa dan teksturnya kurang menarik bagi panelis. Ekstrak *Carica papaya* dan *Curcuma mangga* mengandung senyawa aktif dan menunjukkan efek meningkatkan nafsu makan meskipun tidak signifikan secara statistik. *Marshmallow* memiliki aroma vanila yang disukai, tetapi rasa dan bentuknya kurang optimal. Studi ini terbatas pada jumlah sampel, analisis kuantitatif lebih lanjut dan evaluasi formulasi

direkomendasikan.

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INTRODUCTION

Difficulty eating or decreased appetite is a common problem in children that has an impact on nutritional fulfillment, and is a global health issue¹¹. Children who experience eating disorders usually show behaviors such as refusing to eat, choosing certain foods, eating too long, or not finishing their portion of food¹⁵. This problem can be experienced by anyone regardless of age, gender, or social background³⁰. In the past two decades, the prevalence of eating disorders has steadily increased globally, from 3.4% in 2000 to 7.8% in 2018. A systematic review noted that between 1999-2022, approximately 22.36% of children and adolescents exhibited disordered eating behaviors²⁰. In Southeast Asia, the prevalence of eating disorders reached 11.5%, and in Indonesia it was recorded at 7.4%²¹.

Various efforts that have been made by every parent are making food variations, both in terms of shape and menu, to make it look more attractive; giving appetite-enhancing drugs or vitamins purchased at the pharmacy; even giving herbal medicine as a traditional medicine that is considered natural²³. Another alternative that can be considered is taking nonpharmacological multivitamins. One source of natural multivitamins is fruits that have high nutritional value, sweet taste, affordable price, and easy to obtain, such as papaya (*Carica papaya*)²⁶.

Papaya (*Carica papaya*) is a tropical fruit that is widely found in Indonesia, with a sweet and refreshing taste due to its high water content⁶. This fruit is believed to help increase children's appetite and accelerate the absorption of nutrients due to its vitamin content which is important for metabolism and body growth⁵. Papaya fruit contains the enzyme papain, which has the ability to accelerate the digestion of proteins. Papain can help achieve better food digestion, thereby enhancing the immune system. stimulating the hypothalamus to increase appetite³³. Meanwhile, *Curcuma mangga* is an annual shrub plant with bright yellow rhizomes and a distinctive aroma like mango, which is known to have benefits for increasing appetite and functions as an antipyretic, antitoxin, laxative, anticancer, and antioxidant¹⁰. The rhizome of mango ginger belongs to the Zingiberaceae family and contains secondary metabolites such as flavonoids and curcumin, which are polyphenolic compounds that can increase appetite³⁴.

Marshmallow candy is a snack that is quite popular in Indonesia and is favored by various groups, especially children⁹. The manufacturing process does not require high temperatures, making it suitable for mixing vitamin-containing ingredients without causing nutritional damage¹⁸. According literature³ showed that marshmallows can be added with functional ingredients to increase nutritional value without reducing texture and taste, making it an effective food for the delivery of active substances in a form preferred by consumers or children so that marshmallows are suitable to be added with papaya juice and *Curcuma mangga*.

Based on this background, this study aims to test the activity of a combination of papaya juice extract and *Curcuma mangga* as an anorexia agent using the anorexia test model, and develop an innovative preparation based on marshmallows as a form of active ingredient delivery. The novelty of this research lies in the combination of natural ingredients that have not been widely studied from the use of marshmallows as a natural ingredient-based orexigenic delivery medium.

METHODS

Materials

The materials used are papaya juice (Sweet Mulan), *Curcuma mangga* extract (Balitro), avicel (Brataco Chemical), distilled water (Brataco Chemical), gelatin (Brataco Chemical), dextrose (Brataco Chemical), na-cmc (Brataco Chemical), corn starch (Brataco Chemical), sucrose (Brataco Chemical), vanilli (koepoe-koepoe).

Research Procedure

Step I: Extraction

Making papaya juice extract begins with taking 6 kg of fruit, then washing the fruit thoroughly. Next, the papaya fruit is blended and separated with a filter. After that, freeze drying is carried out to get the extract²⁷. *Curcuma mangga* extraction was carried out with water solvent and stirred at 50 rpm at room temperature. Then filtering is done with a filter cloth²⁸.

Step II: Phytochemical Screening

Phytochemical Screening of Papaya Extract

Alkaloids

1 gram of extract was added to enough chloroform, then added 10 mL of ammonia and 10 mL of chloroform. Then filtered into a test tube and filtrate added 10 drops of H₂SO₄. Then divided into two different test tubes, in test tube I plus Dragendorff reagent. Contains alkaloids if an orange precipitate is formed. After that test tube II plus Mayer reagent the formation of a white precipitate indicates the presence of alkaloids¹⁴.

Flavonoids

1 mL extract plus a few drops of concentrated HCl. Then 0.2 g of Mg powder was added. Positive results are indicated by the onset of a deep red color for 3 minutes¹⁴.

Polyphenols

1 mL extract was added with a few drops of FeCl₃ 10%. Positive results form a blue to purple color²⁹.

Vitamin C

Extract as much as 1 mL added 5 ml of distilled water shaken and then added KMnO₄ 1% 10 ml. If a brown color appears, it indicates the presence of vitamin C compounds³¹.

Steroids

A total of 2 mL of extract was put into a test tube. Then added with 0.5 mL chloroform and 0.5 mL Liebermann Burchard reagent. If there is a brownish red or violet color change the sample contains terpenoids, while if it shows a greenish blue color change the sample contains steroids²⁹.

Phytochemical Screening of *Curcuma mangga* Extract

Alkaloids

A total of 2 ml of extract was added with 2 ml of HCl and 4 ml of methanol, then heated for 5 minutes, after which it was cooled and filtered. Filtrate is used for testing. Test I: 1 mL of filtrate plus 2 drops of Mayer reagent, if a white precipitate is formed, then the sample contains alkaloids. Test II: 1 mL, filtrate plus 2 drops of Dragendorff reagent, if an orange-brown precipitate forms, then the sample contains alkaloids¹⁶.

Flavonoids

1 mL extract plus 1 mL methanol, then filter. then the filtrate is added a few drops of concentrated HCl, then add Mg metal powder and amyl alcohol. Positive results are indicated by the formation of a red/orange precipitate¹⁶.

Polyphenols

1 mL extract is added with a few drops of FeCl_3 10%. Positive results form green, blue or purple color ¹⁶.

Vitamin C

Extract as much as 1 mL added 5 ml of distilled water shaken and then added KMnO_4 1% 10 ml. If a brown color appears, it indicates the presence of vitamin C compounds ³¹.

Steroids

A total of 1 mL of extract plus 2 drops of acetic acid anhydride and 2 drops of concentrated sulfuric acid. If a blue or green color forms, the sample contains steroids ¹⁶.

Anorexia Testing (*Rattus Norvegicus*)

Preparation

The study was conducted at the Pharmacy Laboratory of Muhammadiyah University of Bandung for 3 months with permission from the ethics committee of Ahmad Dahlan University with SK number 022406072. Test animals used male white rats (*Rattus norvegicus*) with 2-3 months of age and weights of 75-100 grams. Test animals were acclimatized for 1 week so that they could adjust to the surrounding environment ¹.

Classification

The test animals were divided into three groups, each consisting of five rats. The negative control group was administered Na-CMC, while the positive control group received a suspension of cyproheptadine. The test group was treated with a suspension containing papaya fruit extract and *Curcuma mangga*.

Anorexia testing

The test animals were divided into 3 groups with each group consisting of 5 rats. Then, anorexia treatment was made by giving unlimited access to the test animals using a walking wheel and a treatment was made by limiting the rats' feeding hours for 7 days. Then, cyproheptadine suspension solution was given to the positive group, papaya and *Curcuma mangga* juice solution was given to the test group, and the negative group was only given na-cmc suspension. After that, anorexia activity was observed in the test animals for 7 days (Foldi, 2024). Data from the anorexia test were analyzed using the One Way ANOVA method with a significance value of 0.05 ²².

Formulation of Marshmallow

Table 1. Marshmallow preparation formula

No.	Formula	Concentration (%)	Usability
1.	<i>Carica papaya</i> fruit extract	33.7	Active substance
2.	<i>Curcuma mangga</i> extract	6.3	Active substance
3.	Dextrose	38.1	Filler
4.	Sucrose	4.6	Sweetener
5.	Gelatin	5	Fastener
6.	Corn starch	9.3	Lubricant
7.	Avicel	2	Fastener
8.	Vanillin	1	Flavouring

First the two extracts are heated, then add the gelatin and stir until the gelatin is soft. Add dextrose syrup 114.3 g, sucrose 13.8 g, then stir. After that the dough is poured into a mixer, beaten until fluffy. Combine starch, avicel and vanilla in the mixer container and pour the batter into the pan. Let stand at room temperature for 16 hours.

After 16 hours of standing, the marshmallows are removed from the mold. Before cutting, the marshmallows were smeared with dextrose and corn starch to prevent sticking. The marshmallow preparation is ready for evaluation test².

Evaluation test of Marshmallow

Organoleptic

Organoleptic tests were conducted by analyzing the shape, smell, and taste of the marshmallows.

Uniformity of Weight

10 marshmallow preparations that have been cut, weighed and calculated the average weight of each preparation, The results of weighing should not be more than two preparations with weights deviating from the average weight of column A, then there is no one preparation with weights deviating from the average weight of column B (MOH RI, 2020).

Hedonic

The study was conducted at the Pharmacy Laboratory of Universitas Muhammadiyah Bandung with permission from the Ahmad Dahlan University ethics committee with SK number 022407076. The favorability test was conducted on 10 children and 10 adult respondents with parameters including taste, aroma, and shape & texture¹⁸.

Data Analyze

The data analysis in this study was conducted using descriptive analysis methods. For the anorexia test, the data were analyzed using the One-Way ANOVA method. Prior to performing the One-Way ANOVA, a normality test was conducted using the Shapiro–Wilk test, and a homogeneity test was carried out using Levene's test. After these assumptions were met, the One-Way ANOVA was performed using SPSS software version 22.

RESULT AND DISCUSSION

Table 2: Phytochemical Screening Results of papaya and *Curcuma mangga* fruit extracts.

No.	Phytochemical test	Papaya	<i>Curcuma mangga</i>
1.	Alkaloid	+	+
2.	Flavonoid	-	+
3.	Vitamin C	+	+
4.	Steroid	+	+
5.	Polyphenol	+	+

Phytochemical screening is the initial stage in phytochemical research which intends to provide an overview of plant compound groups⁸. This method is carried out qualitatively and quantitatively. Qualitative phytochemical screening is carried out showing color testing reactions with a color reagent, and using thin layer chromatography. Meanwhile, quantitative phytochemical screening is carried out using UV-Visible Spectrophotometry instruments¹².

The results of phytochemical screening on *Curcuma mangga* based on table 1. showed positive results in the alkaloid test with a red color change, then in the steroid test obtained the results of the formation of an orange ring, and obtained negative results in the flavonoid test with a yellow color, where the flavonoid test results will produce a positive reaction in red. This is because magnesium powder does not provide a reduction reaction of flavonoid compounds so that the test solution does not produce the desired color change²⁴. Then the results of the phytochemical screening of papaya fruit show in table 1. with the alkaloid test producing a positive reaction with a red

color change, the flavonoid test produces a negative, vitamin C, steroids, and polyphenols produce a positive reaction³¹.

Table 3. Anorexia Test in White Rats (*Rattus norvegicus*)

Group Animal Test	Mean $\pm$ SD	p-Value
Negative group	91 $\pm$ 13.46	0,960
Positive group	93 $\pm$ 12.52	
Testing group	92 $\pm$ 9.45	

The results of the anorexia test with the One Way ANOVA method where the results showed a $p > 0.05$ value, so there were no significant differences with each group. This is because the three test materials used can increase appetite in test animals. The negative group given Na- CMC gave the effect of weight gain in the test animals. Na-CMC is not the main factor as an appetite enhancer, but the hungry phase due to activity-based anorexia stress as the main factor in increasing the appetite of test animals. Then in the positive group given ciproheptadine has the side effect of increasing appetite in test rats. The test group showed that *Curcuma mangga* can increase appetite due to the content of curcumin compounds in the rhizome, which Curcumin is one of the active compounds of the phenolic group with yellow pigments which are found in the rhizomes of curcuma from the Zingiberaceae family. Curcumin also has many potentials such as appetite enhancers, antioxidants, antitumors and for other body health. while papaya fruit can increase appetite due to the high content of nutrients and vitamins such as Vitamin C, Vitamin A, and Vitamin E which are very good at increasing appetite³². The results of the anorexia test showed no statistically significant difference between the treatment groups ($p > 0.05$). However, descriptively, the test group given the combination of papaya and ginger extracts showed average values close to those of the positive control group. This insignificant result may be influenced by the limited number of test animals, resulting in low statistical power and greater variation in biological responses between animals. Furthermore, the activity-based anorexia model used can trigger a physiological adaptation mechanism in the form of a compensatory feeding response after the stressful phase of food restriction, resulting in all groups, including the negative control, still showing increased appetite³⁵.

The positive control group given cyproheptadine showed the highest increase in appetite compared to the other groups. This is consistent with cyproheptadine's mechanism of action as a serotonin antagonist that can increase eating behavior. Meanwhile, the test group given the combination of papaya and ginger extracts showed results close to those of the positive control, indicating the potential orexigenic activity of these two natural ingredients. Mango ginger is known to contain curcumin and phenolic compounds that play a role in improving digestive function, increasing bile secretion, and supporting gastrointestinal health, thus helping to increase appetite³⁷. Papaya also contains vitamin C, vitamin A, antioxidants, and the enzyme papain, which aids digestion and nutrient absorption. These compounds are thought to contribute to increased metabolism and improved appetite in test animals³⁸. Although statistical results did not show a significant difference, the average values of the test groups, which were close to those of the positive control, indicate that the combination of papaya and mango ginger extracts still has biological potential as an appetite enhancer. Therefore, further research is needed with a larger sample size, longer treatment duration, and measurement of additional parameters such as daily feed intake and body weight changes to strengthen these results³⁷.

In the Marshmallow formulation, the results of the preparation were obtained with a chewy texture, sweet taste, distinctive vanilla odor and homogeneous beige color. Gelatin has an influence on marshmallows as a gelling agent, so that the higher the concentration of gelatin, the more gel will be formed so that the texture is more chewy and elastic².

In the Marshmallow formulation, the results are obtained with a sweet taste, the sweet taste in marshmallows is due to the dominant sugar content in the preparation. Has a distinctive vanilla aroma, marshmallow preparations

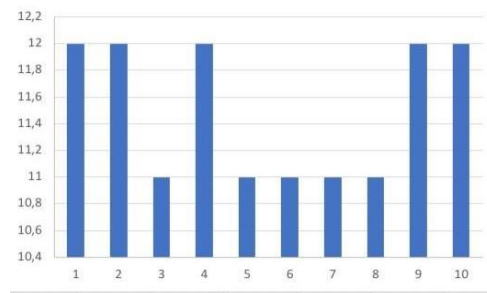


Figure 1. Weight uniformity test of marshmallow preparation

have a fragrant aroma due to the heating of sugar which is the basic ingredient of the preparation⁹. Has a beige color, due to the amount of papaya fruit extract used, the more extract, the decrease in the brightness of the preparation color¹³. This is triggered by papaya fruit which produces a yellow color⁶. And has a chewy texture, gelatin has an important role because gelatin has characteristics that depend on the concentration of gelatin used, the higher the concentration of gelatin used, the harder it is, and vice versa¹³.

The weight uniformity test on marshmallow preparations aims as the main parameter to determine the uniformity of the active substance content in this context with regard to the correct and even distribution of each marshmallow¹⁸. Based on Figure 1. Shows the results of the evaluation of the weight uniformity of papaya extract marshmallows and *Curcuma mangga* shows that each marshmallow has a weight that meets the requirements, obtained an average weight of 11.5 gr, with a good range value in accordance with the requirements of namely, 10.64-12.36 gr⁴.

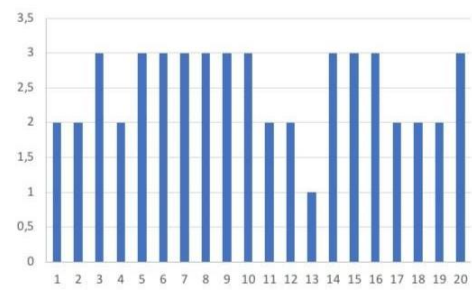


Figure 2. Hedonic test of aroma parameters of marshmallow preparations

Furthermore, the hedonic test was conducted using aroma parameters, where the aroma hedonic test is an organoleptic assessment method used to determine the level of preference or acceptance of the panelists towards the aroma of a product. This test uses a rating scale with a value of 1-3. Based on Figure 2, the results of the hedonic test conducted on 20 panelists, the average numerical value for aroma was obtained at 2.5 which can be concluded

that the panelists liked the aroma of marshmallows, this is because the marshmallow preparation made has a distinctive vanilla aroma so that the aroma of *Curcuma mangga* which is not preferred is covered by the vanilla aroma. Then the aroma that the panelists liked is thought to come from the addition of vanillin which provides a sweet and distinctive aroma so that it is able to cover the natural aroma of *Curcuma mangga* which tends to be sharp and not preferred. The use of vanilla flavor is known to increase the sensory acceptance of the product because it gives the impression of a softer, sweeter, and familiar aroma to consumers, especially children. In addition, aroma is one of the important parameters in determining the level of acceptance of a food product through the hedonic test. In addition, the heating process of sucrose and dextrose during marshmallow formulation also produces a distinctive sweet aroma that supports the increase in panelists acceptance of the product³⁵. The combination of vanilla aroma and sugar components creates a more appealing aroma characteristic, thus increasing the acceptability of marshmallow preparations as a delivery medium for active herbal ingredients. These results indicate that the aroma formulation in marshmallows is quite successful in reducing the herbal aroma characteristics of papaya and mango ginger extracts without losing the product's identity. Other studies have also shown that aroma attributes have a significant influence on panelists' preference for marshmallow products and other functional foods.

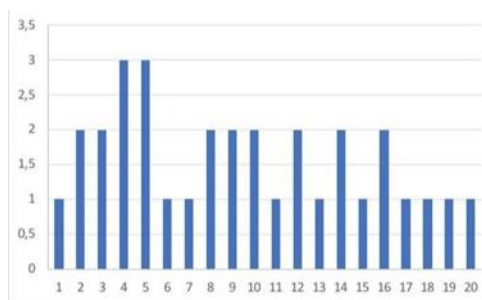


Figure 3. Hedonic Test of Flavor Parameters of Marshmallow preparations.

A hedonic test was then conducted using taste parameters. The hedonic taste test is a method for measuring the level of preference or acceptance of a product's taste by panelists or consumers. This test uses a rating scale of 1-3. Based on Figure 3, the taste parameter had an average score of 1.6, indicating that the panelists disliked the taste. This is because the gelatin taste was unpleasant and too sweet, leading respondents to dislike the product. This low preference rating is thought to be due to the overly sweet taste resulting from the use of relatively high amounts of sucrose and dextrose in the formulation. Furthermore, the distinctive flavors of the turmeric extract and gelatin may also have influenced panelists acceptance of the product. Herbal extracts generally have a distinctive aftertaste, which can reduce sensory acceptance if the flavor formulation is not properly optimized³⁶.

Taste parameters are a key factor in determining consumer acceptance of functional food products. Panelists tend to prefer products with a balanced taste, not too sweet, and without an aftertaste. In this marshmallow formulation, the combination of the sweetness of sugar and the herbal flavor of ginger may produce a taste sensation unfamiliar to panelists, especially children. Furthermore, the use of gelatin in certain concentrations can also produce a distinctive taste that affects consumption. Therefore, further formulation optimization is needed, such as reducing the sugar content, adding natural flavors, or using masking agents to improve the acceptability of the marshmallow flavor.

These results align with several previous studies that suggest that the addition of herbal ingredients or natural active ingredients to food products often affects sensory characteristics, particularly taste parameters. The

stronger the flavor of the active ingredient used, the lower the panelists preference level can be if not balanced with the addition of appropriate additives⁷.

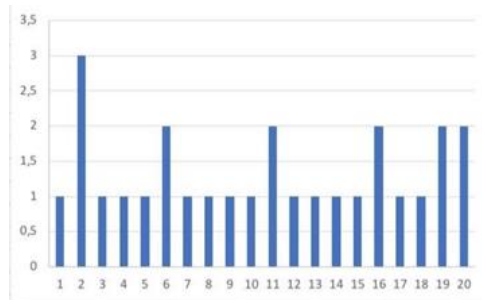


Figure 4. Hedonic Test of Shape & Texture Parameters of Marshmallow preparation.

A hedonic test was then conducted using shape and texture parameters. The hedonic test for shape and texture is a sensory evaluation used to measure consumer preference for a product's shape and texture. This test uses a rating scale of 1-3. Based on Figure 4, for the taste parameter, the average shape and texture score was 1.35, indicating that the shape and texture were less appealing to panelists. This is because panelists disliked the appearance of marshmallows, which had a less appealing color, making them less appealing to them. The low panel preference rating is thought to be influenced by the marshmallow's less appealing beige-brown color, resulting from the addition of a significant amount of papaya and mango extract. The color of a food product is one of the initial factors that attracts consumer interest before trying the product, especially marshmallows, which are generally associated with bright colors and are attractive to children.

In addition to color, the texture of the marshmallow also influenced panelists acceptance. The resulting marshmallow had a chewy texture due to the use of gelatin as a gelling agent. Gelatin has the ability to form an elastic gel and maintain the marshmallow's structure. Therefore, the higher the gelatin concentration used, the denser and chewier the product texture will be. However, a texture that is too dense or elastic can cause discomfort when chewing, thus decreasing panelists' preference. Furthermore, the use of Avicel and corn starch in the formulation can also affect the texture characteristics, making it denser and less soft.

A less uniform marshmallow shape may also influence panelists' assessment of the product. An unattractive physical appearance can reduce visual appeal and affect consumers' perceptions of the product's overall quality. Therefore, optimization of the formulation and molding process is necessary to produce a more attractive shape, brighter color, and a softer and more elastic texture to increase panelists' acceptance of this herbal extract-based marshmallow.

The results of this study align with several previous studies that have shown that visual and textural attributes are important factors in hedonic testing of food products. Products with textures that are too hard, too chewy, or less attractive in color tend to receive lower preference ratings than products with better sensory characteristics¹⁷.

LIMITATION

The researcher acknowledges that there are still many limitations in this study, including the anorexia test results, which showed no significant differences, and the flavonoid test results on papaya fruit, which yielded a

negative reaction. The author hopes that future research will produce better results than those obtained in the present study.

CONCLUSION

Based on the results obtained, it shows that both extracts contain active compounds such as alkaloids, vitamin C, steroids, and polyphenols. The anorexia test did not show significant differences between groups ($p > 0.05$), but the combination of extracts still showed a positive effect comparable to the positive control. The marshmallow preparation has a chewy texture and a flavourable vanilla aroma, but is still lacking in terms of taste and shape. Limitations of this study include the limited number of test animals and panelists and no quantitative analysis of active substances. Suggestions for further research can be done by observing specific eating behavior, daily food intake, as well as optimizing formulations and further effectiveness tests, including quantitative analysis of active substance content.

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AUTHOR CONTRIBUTION STATEMENT

RM: wrote the results and discussion sections, **RRHS:** wrote the methods section and references, **RIH:** wrote the introduction and conclusion sections, **RB:** wrote the results and discussion sections, **Z:** supervised the research and the writing of the scientific article, **FNS:** supervised the research and the writing of the scientific article.

CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest.

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Development Of Papaya Leaf Extract/Pva-Based Nanofibers Using Electrospinning Method For Medical Appliation As A Wound Dressing

Pengembangan Nanofiber Berbasis Ekstrak Daun Pepaya/PVA Menggunakan Metode Elektrosinning untuk Aplikasi Medis sebagai Pembalut Luka

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Abstract

The nanofiber manufacturing process is carried out using the electrospinning method, which produces nanometer-sized fibers. This study used a mice test animal model, where wounds in mice were treated with papaya leaf extract/PVA nanofibers, then compared with a control group using commercial wound dressings. Evaluation was carried out based on wound healing parameters, such as the speed of wound closure and the ability to form an inhibition zone on *E. Coli* bacteria. Based on the results of SEM testing, it was seen that the morphology of papaya leaf extract/PVA nanofibers had been successfully synthesized with the formation of fibers. The results of observations for the inhibition of *E. Coli* bacteria for extract concentrations of 5% and 10% for 7 and 24 hours were 13.33 and 12.43 mm. The application of 10% papaya leaf extract/PVA nanofibers was then tested as a wound dressing on test mice. The results showed that after 9 days, 10% papaya leaf extract/PVA was able to accelerate wound healing in mice. Overall, this research can contribute to the development of nanotechnology-based wound dressing materials that utilize natural resources for medical applications.

Abstrak

Proses pembuatan nanofiber dilakukan menggunakan metode elektrosinning, yang menghasilkan serat berukuran nanometer. Studi ini menggunakan model hewan uji tikus, di mana luka pada tikus diobati dengan nanofiber ekstrak daun pepaya/PVA, kemudian dibandingkan dengan kelompok kontrol yang menggunakan perban luka komersial. Evaluasi dilakukan berdasarkan parameter penyembuhan luka, seperti kecepatan penutupan luka dan kemampuan membentuk zona inhibisi pada bakteri *E. Coli*. Berdasarkan hasil pengujian SEM, terlihat bahwa morfologi nanofiber ekstrak daun pepaya/PVA telah berhasil disintesis dengan pembentukan serat. Hasil pengamatan untuk inhibisi bakteri *E. Coli* untuk konsentrasi ekstrak 5% dan 10% selama 7 dan 24 jam adalah 13,33 dan 12,43 mm. Aplikasi nanofiber ekstrak daun pepaya/PVA 10% kemudian diuji sebagai perban luka pada tikus uji. Hasil penelitian menunjukkan bahwa setelah 9 hari, ekstrak daun pepaya 10%/PVA mampu mempercepat penyembuhan luka pada tikus. Secara keseluruhan, penelitian ini dapat berkontribusi pada pengembangan bahan penutup luka berbasis nanoteknologi yang memanfaatkan sumber daya alam untuk aplikasi medis.

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INTRODUCTION

Papaya leaves (*Carica papaya*) are a traditional herbal plant that has long been used to aid wound healing. Papaya leaves are typically crushed and applied directly to the injured skin. Scientifically, papaya leaves have been shown to have anti-inflammatory effects. Their flavonoid, alkaloid, tannin, and saponin content produces

antimicrobial and antioxidant properties, accelerating wound epithelial formation by stimulating collagen production¹. Traditional methods such as applying papaya leaves directly to wounds have limitations in terms of hygiene and clinical effectiveness. Conventional bandages only serve to cover wounds without providing antibacterial effects or accelerating tissue regeneration. Therefore, the development of more modern, effective, and safe wound dressing materials is needed to support the wound healing process.

One technology currently being widely developed in the field of biomaterials is electrospinning-based nanofibers. Nanofibers are fascinating materials produced from various polymers and have demonstrated remarkable properties in a variety of biomedical and engineering applications. The high porosity and surface area offered by nanofibers' structural arrangement make them widely used in the medical field². Nanofibers are known to promote tissue formation, enhance blood vessel regeneration, and accelerate wound healing. Compared to conventional wound dressing methods, nanofibers provide better protection against bacterial infection and maintain wound moisture, thus optimizing tissue regeneration.

Nanofiber manufacturing methods have been developed using various techniques, such as electrospinning, template synthesis, centrifugal spinning, blow spinning, self-assembly, and microfluidic spinning. However, compared to other methods, electrospinning is considered the simplest, lowest-cost, and most practical technique for producing micro- to nano-sized fibers³⁻⁵. In the electrospinning process, a polymer solution is subjected to a high electrical voltage to form nanofibers that are collected in a collector. Some synthetic polymers commonly used in nanofiber production include polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), gelatin, and chitosan. Among these polymers, PVA is widely used because it is biocompatible, non-toxic, easily soluble in water, and able to form nanofibers with good stability for medical applications⁶.

Previous research has shown that the combination of natural extracts with synthetic polymers can increase the effectiveness of nanofiber-based wound dressings. Ahlawat, et al⁷ used papaya leaves that had previously been treated by drying them into powder, then added with PVA/gelatin to make nanofibers through the electrospinning method to be applied as wound dressings. The results showed that the formed nanofibers had antibacterial properties and for testing this nanofiber wound dressing also showed good potential for use. In addition Huang Z, et al⁸, it was reported that the combination of PVA polymer with sodium alginate produced a wound dressing with good antibacterial properties.

Based on this, this research will focus on the utilization of papaya leaf extract, which has not been optimally utilized and is often discarded as waste. Therefore, this papaya leaf juice will be further utilized by adding PVA polymer using the electrospinning method. The resulting nanofibers will then be tested for their morphological characteristics, antibacterial activity against *Escherichia coli*, and their ability as a wound dressing in test animals. The development of nanofibers based on papaya leaf extract/PVA is expected to be an alternative modern wound dressing that is more hygienic, effective, and environmentally friendly compared to traditional methods.

METHODS

Tools and Materials

Tools. sieves, baking pans, knives, cutting boards, Erlenmeyer flasks, glass beakers, sieves, filter paper, aluminum foil, cling wrap, cotton wool, aluminum foil, electrospinning equipment, Erlenmeyer flasks, Petri dishes, hot plates, magnetic stirrers, incubator shakers, incubators, spiritus, and autoclaves.

Materials. papaya leaves, distilled water, PVA, 95% alcohol, NA and NB media, dan amoxilin.

Research Procedure

Research Stages

This research was conducted by initially preparing the main ingredients: papaya leaf extract and PVA solution. These two ingredients were then mixed to create 5% and 10% electrospinning solutions, which were used to fabricate nanofibers. The resulting nanofibers were then tested for antibacterial activity and wound dressing properties on experimental animals.

Research Procedure

Papaya Leaf Extraction

Papaya leaves were finely chopped, then distilled water was added and blended. The mixture was then collected in a glass beaker and left to stand for 24 hours. The liquid and solids were then separated, and the resulting solids were then dried. The dried product was called papaya leaf extract.

Electrospinning Solution Preparation

In this step, a 10% PVA solution was prepared by weighing 10 grams of PVA powder and adding 100 mL of distilled water while stirring for 1 hour at 85°C. After the PVA was completely dissolved, papaya leaf extract was slowly added to the prepared PVA solution and stirred for 30 minutes at 85°C. The papaya leaf extract added varied between 5 and 10% wt of the PVA weight.

Nanofiber Fabrication

The PVA/Electrospinning solution was cooled to room temperature, then transferred to a 10 mL plastic syringe with a flow rate of 1 mL/h. Electrospinning used a high-voltage DC supply with a voltage of 15 kV. The collector measured 8 x 8 cm, with a distance of 10 cm from the collector to the needle tip. Alligator clips from the high-voltage supply, with the positive (+) current lead, were clamped to the end of the spinneret, and the other, the negative (-) lead, was clamped to the collector. The electrospun nanofibers were collected on aluminum foil and stored.

Nanofiber Antibacterial Test

Escherichia coli ATCC 6538 bacteria were subcultured on Nutrient (NA) media using the continuous streak method, then incubated in an incubator for 24 hours at 37°C. The test bacteria were inoculated into 30 ml of Nutrient Broth (NB) media, then incubated in a shaker incubator at 37°C at 150 rpm overnight. The bacterial cell count was calculated using a McFarland 0.5 standard (1.5×10^8). Sterile Nutrient Agar (NA) media was poured into sterile Petri dishes (15-20 ml) and allowed to solidify. A 100 µL suspension of the test bacteria was taken and spread aseptically on the surface of the NA media. Sterile paper discs were immersed for 30 minutes in each nanofiber solution, a positive control of liquid amoxicillin, and sterile distilled water as a negative control. The soaked paper discs were taken using sterile tweezers and placed on the surface of the NA media. One petri dish consisted of 4 discs, namely discs for 10% nanofiber, 5% nanofiber, positive control, and negative control. The agar media was incubated in an incubator at 37°C for 12 to 24 hours. Observe and calculate the diameter of the inhibition zone formed around the paper disc after inoculation. The inhibition zone indicates the ability of nanofibers to inhibit the growth of *E. coli* ATCC 6538 bacteria.

Characterization

The 5% nanofiber characterization process was conducted using SEM to determine the morphology and success of nanofiber formation.

Nanofiber Testing on Test Animals

Preparation of Test Animals

The test animals used in this study were male mice (*Mus musculus*) aged 2-3 months, weighing 20 grams.

Wound Creation

Before wounding the mice, they were anesthetized by inhaling 10% ether. Then, the fur around the mice's backs was shaved to a diameter of 3 cm and cleaned with 70% alcohol. An incision 1 cm long and 2 mm deep was made on the mice's backs.

Nanofiber Attachment to Test Animals

Treatment was administered immediately after wounding by attaching the nanofibers to the wound area. Wound healing activity was measured by measuring the diameter of the wound surface 24 hours after nanofiber application.

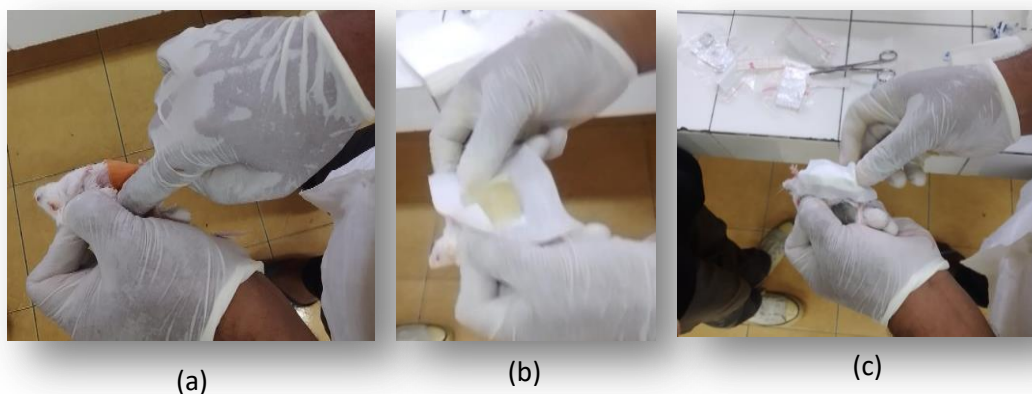


Figure 1. Wound Dressing Process (a) Store-Based Plaster (Hansaplast), (b) 10% Papaya Leaf Extract/PVA Mixture, and

RESULT AND DISCUSSION

Results and Discussion

Research Results

Papaya Leaf Extract/PVA Nanofiber Fabrication

Papaya leaf extract/PVA nanofibers were successfully fabricated using the electrospinning method at papaya leaf extract concentrations of 5% and 10%. The electrospinning process was carried out at a voltage of 15 kV, a flow rate of 1 mL/h, and a collector distance of 10 cm for 5 hours, resulting in papaya extract/PVA nanofibers (Figure 2).

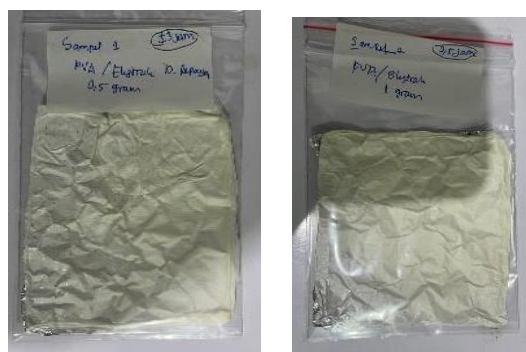


Figure 2. Results of the Electrospinning Process

Visually, it shows that nanofibers are formed continuously and relatively evenly on the collector surface. The resulting nanofibers were then analyzed using Scanning Electron Microscopy (SEM) to determine the morphology of the formed fibers. The SEM results on 5% papaya leaf extract/PVA nanofibers show the formation of nanofibers with a relatively

homogeneous and interconnected distribution. In addition, no significant amount of beads or beads were found on the fiber surface. The morphology of the SEM nanofibers can be seen in Figure 3.

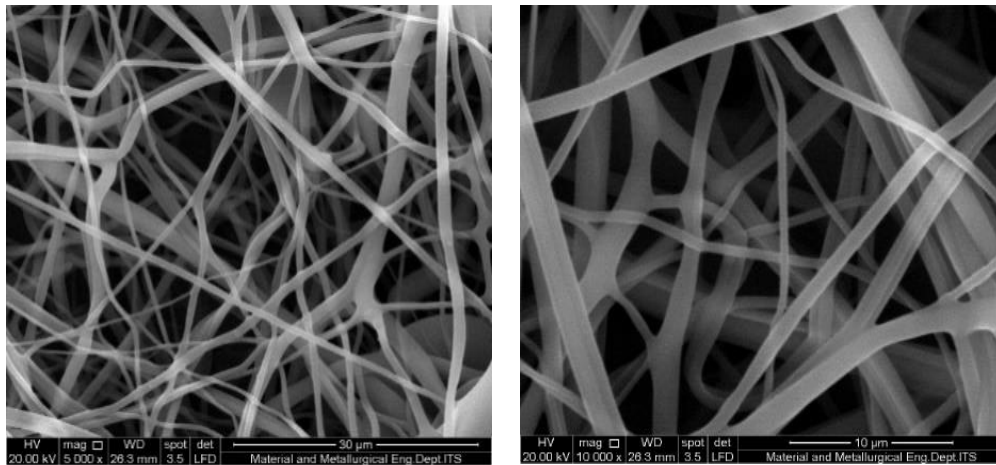


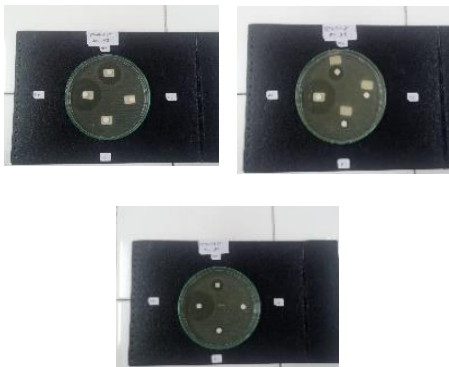
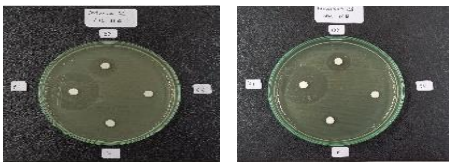
Figure 3. SEM Test Results for 5% Nanofiber (a) 5000x magnification and (b) 10000x magnification

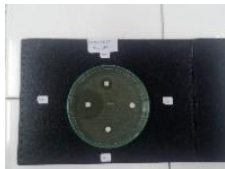
The homogeneous fiber distribution indicates that the electrospinning parameters used have been able to produce a stable polymer jet during the spinning process. According to Li dan Xia⁹, the intrinsic properties of the solution such as polymer type, viscosity, electrical conductivity, and operating conditions such as electric field, distance between spinneret and collector, and the surrounding environment have an important role in determining the morphology and diameter of the resulting nanofiber⁸.

Antibacterial Testing

Antibacterial testing was conducted using the disc diffusion method against *Escherichia coli* ATCC 6538. Testing was performed on 5% and 10% papaya leaf extract/PVA nanofibers, with amoxicillin as a positive control (K⁺) and sterile distilled water as a negative control (K⁻). The results are presented in Table 1.

Table 1. Antibacterial Test Results and Inhibition Zone Calculation

Time	Result	Inhibition Zone			
		K+	K-	5%	10%
7 Jam		28 mm	-	-	13,33 mm
24 Jam		29,56 mm	-	-	12,43 mm



The inhibition zone formed indicates that 10% papaya leaf extract/PVA nanofibers have antibacterial activity against *E. coli*. This antibacterial activity is influenced by the content of bioactive compounds in papaya leaves distributed in the nanofiber matrix. According to Kiran Peter et al¹⁰, papaya leaf extract has been proven effective against Gram-negative and Gram-positive bacteria tested, showing a broad spectrum of activity and analysis of phytochemical content capable of inhibiting bacterial growth.

Nanofiber Testing on Animals

Nanofiber Testing on Animals

Wound dressing testing was conducted on mice with a 1 cm incision. The treatments used consisted of conventional plaster (Hansaplast), a 10% papaya leaf extract/PVA solution, and 10% papaya leaf extract/PVA nanofibers (Figure 4).

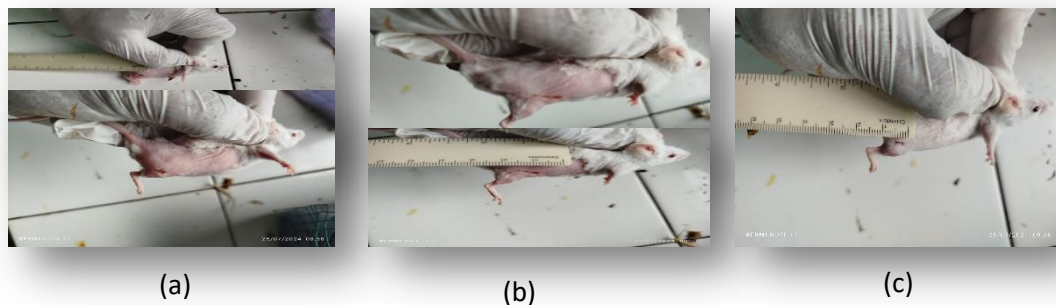


Figure 4. Wound Closure Process (a) Store Plaster (Hansaplast), (b) 10% Papaya Leaf Extract/PVA Solution, and (c) 10% Nanofiber

Observations over 9 days showed that the papaya leaf extract nanofiber/10% PVA provided the fastest wound closure compared to the other treatments. On day 9, the wound treated with the nanofiber had completely closed, with a wound length of 0 cm. The results of the wound closure observations can be seen in Table 2.

Table

2.

Wound	Variabel	Panjang			Closure
		Awal	Hari ke-1	Hari ke-9	
	Plester Hansaplast	1 cm	0,9 cm	0,2 cm	
	Larutan Ekstrak Daun Pepaya/PVA 10%	1 cm	0,7 cm	0,1 cm	
	Nanofiber 10%	1 cm	0,6 cm	0,0 cm	

Observation Results

Discussion

Morphology of Papaya Leaf Extract/PVA Nanofibers

The successful formation of nanofibers in this study demonstrates that the combination of papaya leaf extract and PVA can be successfully fabricated using the electrospinning method. PVA is a polymer widely used in biomaterial applications due to its biocompatibility, non-toxicity, and ability to form nanofibers with good stability^{6,11}.

During the electrospinning process, a high-voltage power supply (15 kV) is applied to the papaya leaf extract/PVA, creating a repulsion between charges on the drop surface at the end of the capillary that competes with the surface tension, which stabilizes the drop. When a critical condition is reached, the surface charge repulsion will dominate, the hemispherical surface of the liquid at the end elongates to form a cone shape known as a Taylor cone. The polymer jet will experience disruption, resulting in bending and continued spiral formation until the nanofibers are finally collected in the collector in solid form. Electrospinning parameters such as electric voltage, flow rate, and solution viscosity greatly influence the morphology of the resulting nanofibers¹².

SEM results show that the nanofibers have a relatively homogeneous fiber distribution and do not exhibit significant bead formation. The absence of bead formation indicates that the electrospinning parameters used are optimal. According to Bhardwaj and Kundu¹³, electrospinning is an effective method for producing nanofibers with a continuous structure and high surface area.

SEM results show that nanofibers have a relatively homogeneous fiber distribution and do not show significant bead formation. The absence of bead formation indicates that electrospinning parameters such as voltage, viscosity, and flow rate are appropriate. Huang et al⁸ explained that too low a solution viscosity can cause bead formation on nanofibers. In addition, Zargham et al⁵ stated that the stability of the flow rate greatly affects the fiber morphology and distribution of nanofiber deposition. According to Kenry and Lim¹⁴, the ECM-like structure of nanofibers can increase cell adhesion and proliferation, thereby accelerating skin tissue regeneration. According to Wang Q et al¹⁵ also reported that electrospun nanofibers have high porosity and good exudate absorption capabilities, making them very potential for use in biomedical applications.

In addition, the small pore size of nanofibers is able to inhibit the penetration of microorganisms from the external environment but still allows good oxygen exchange¹⁶. Xue et al¹⁷, explained that the high surface area of nanofibers allows the gradual release of active substances, thereby increasing the effectiveness of biomaterials in medical applications.

The results of this study are consistent with those of Ahlawat et al⁷, who reported that papaya leaf extract/PVA-gelatin-based nanofibers successfully demonstrated homogeneous fiber morphology and had the potential to be used as wound dressings. Research by Augustine et al¹⁸ also demonstrated that electrospun nanofibers have a high ability to support tissue regeneration because they are able to maintain an optimal wound environment.

Antibacterial Activity of Nanofibers

The antibacterial test used culture media (NA and NB) as a growth medium for *E. coli* bacteria, which served to assess the antibacterial activity of nanofibers as a wound dressing preparation using papaya leaf extract/PVA. The disc diffusion method was used in this antibacterial test, with positive (K^+) and negative (K^-) controls added at concentrations of 5 and 10%. The positive control used was amoxicillin for comparison, and the negative control used

was distilled water because distilled water does not contain toxins or substances that can inhibit or kill bacterial growth¹⁹.

Nanofiber papaya leaf extract/10% PVA showed antibacterial activity against *Escherichia coli* with the formation of inhibition zones of 13.33 mm and 12.43 mm. This antibacterial activity is influenced by the content of flavonoids, alkaloids, tannins, and saponins in papaya leaves which are able to damage bacterial cell membranes¹. Flavonoids are known to be able to inhibit nucleic acid synthesis and damage bacterial membrane permeability, while tannins can cause protein denaturation in bacterial cell walls²⁰. In addition, as a Gram-negative bacterium, *Escherichia coli* has an outer membrane of lipopolysaccharide (LPS) which functions as a permeability barrier to hydrophobic compounds including alkaloids [6]. The relatively thin peptidoglycan layer (5-10% of the cell wall), makes it less susceptible to direct damage by alkaloids with the mechanism of action of alkaloids, namely inhibition of peptidoglycan synthesis through interference with the transpeptidase enzyme. So it can be seen that by increasing the concentration from 5% to 10%, it shows that high doses can overcome the permeability barrier of *E. Coli*²¹.

The inhibition zone of nanofibers was still lower than the positive control amoxicillin because synthetic antibiotics have a more specific mechanism of action against *E. coli* bacteria. Nevertheless, papaya leaf extract nanofibers still show potential as a natural antibacterial for wound dressing applications. Ahmed et al²², explained that the antibacterial effectiveness of nanofibers is influenced by the concentration of the active ingredient and the ability to release bioactive substances from the polymer matrix. The gradual release of active substances (controlled release) is one of the main advantages of nanofibers compared to conventional wound dressings²³.

The results of this study support the research of Unnithan et al²⁴, which reported that biomaterial-based electrospun nanofibers have good antibacterial activity when combined with natural active ingredients. Research by Sonia and Sharma²⁵ also showed that polymer-based nanofibers have a more effective ability to deliver active substances than conventional membranes.

Nanofiber's Ability as a Wound Dressing

The results of animal tests showed that nanofibers containing papaya leaf extract/10% PVA provided the fastest wound healing process compared to other treatments. By day 9, the wounds treated with nanofibers had completely closed, with a healing rate reaching 100%.

The ability of nanofibers to accelerate wound healing is influenced by the combination of the nanofiber's physical structure and the bioactive content of papaya leaf extract. The nanofiber's structure, which resembles the ECM, supports fibroblast migration and the formation of new epithelial tissue. Furthermore, the flavonoids and papain enzyme in papaya leaves help reduce inflammation and accelerate skin tissue regeneration¹.

Moura et al²⁶, explained that modern wound dressings must be able to create a moist environment in the wound for optimal tissue regeneration. Nanofibers have the ability to maintain wound moisture while absorbing excess exudate, resulting in faster healing compared to conventional wound dressings.

Abrigo et al²⁷, stated that electrospun nanofibers can increase cell proliferation and accelerate wound healing because their fiber structure supports natural cell growth. The results of this study also showed that nanofibers provide better physical protection against microbial contamination than conventional plasters. Jiang Z et al²⁸, stated that nanofibers with high porosity can inhibit microbial growth while maintaining air exchange on the wound surface.

Overall, the results of this study indicate that papaya leaf extract/PVA nanofibers have good potential as a modern wound dressing because they can provide antibacterial activity, maintain wound moisture, and accelerate the wound healing process compared to conventional methods.

CONCLUSION

The conclusion drawn from this study is that the fabrication of papaya leaf extract/PVA nanofibers with concentrations of 5% and 10% has been successfully synthesized using the electrospinning method. Testing of nanofibers against *E. coli* bacteria showed an inhibition zone of 13.33 and 12.43 mm for nanofibers with a concentration of 10%.

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In Silico Evaluation of Ciplukan (*Physalis angulata* Linn.) Compounds as Potential Serotonin Reuptake Inhibitors for Antidepressant Development

Evaluasi In Silico Senyawa Ciplukan (*Physalis angulata* Linn.) Sebagai Inhibitor Reuptake Serotonin yang Potensial untuk Pengembangan Antidepresan

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Abstract

Depression is one of the psychiatric disorders that has become a global challenge. Patients with depressive disorders experience changes in serotonergic and noradrenergic functions in the central nervous system. Modulation of serotonin reuptake can be an effective approach in the development of antidepressant agents. Ciplukan (*Physalis angulata* Linn.) has anti-inflammatory and antioxidant properties, offering potential as a natural candidate for antidepressant development through serotonin reuptake inhibitors that can increase synaptic serotonin availability by inhibiting serotonin reuptake through hSERT. The aim of this research is to test the serotonin reuptake inhibitory activity in silico by molecular docking method to investigate the molecular interaction between active compounds in ciplukan extract with serotonin transporter. In this research, the software used is molegro virtual docker with human serotonin transporter (hSERT) target protein. The test ligands used are compounds of the alkaloid, glycoside, phenolic acid, and flavonoid groups. The comparison ligands used are SSRI drugs such as paroxetine, fluoxetine, escitalopram, and fluvoxamine. The results showed that Psychotrine, Catechin, and Eriodictyol, which are flavonoid compounds from ciplukan, have potential as antidepressants because they have the most negative rerank score, has the same significant amino acid bonds as the reference ligands, namely Asp 98 and Tyr 95 and has penetration in the blood brain barrier as shown by the results of the Lipinski analysis.

Abstrak

Depresi merupakan salah satu gangguan kejiwaan yang menjadi tantangan global. Penderita gangguan depresi mengalami perubahan fungsi serotonergik dan noradrenergik pada sistem saraf pusat. Modulasi reuptake serotonin dapat menjadi pendekatan yang efektif dalam pengembangan agen antidepresan. Ciplukan (*Physalis angulata* Linn.) mempunyai sifat antiinflamasi dan antioksidan sehingga berpotensi sebagai kandidat alami pengembangan antidepresan melalui inhibitor reuptake serotonin yang dapat meningkatkan ketersediaan serotonin sinaptik dengan menghambat reuptake serotonin melalui hSERT. Tujuan dari penelitian ini adalah untuk menguji aktivitas penghambatan reuptake serotonin secara in silico dengan metode molekuler docking untuk mengetahui interaksi molekuler antara senyawa aktif dalam ekstrak ciplukan dengan transporter serotonin. Pada penelitian ini perangkat lunak yang digunakan adalah molegro virtual docker dengan target protein human serotonin transporter (hSERT). Ligan uji yang digunakan adalah senyawa golongan alkaloid, glikosida, asam fenolik, dan flavonoid. Ligan pembanding yang digunakan adalah obat SSRI seperti paroxetine, fluoxetine, escitalopram, dan fluvoxamine. Hasil penelitian menunjukkan bahwa Psikotrin, Katekin, dan Eriodictyol yang merupakan senyawa flavonoid dari ciplukan berpotensi sebagai antidepresan karena mempunyai nilai rerank paling negatif, mempunyai ikatan asam amino signifikan yang sama dengan ligan acuan yaitu Asp 98 dan Tyr 95 serta mempunyai penetrasi pada sawar darah otak yang ditunjukkan dari hasil analisis Lipinski.

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Alfazarah A, Nurhayati A, Wijaya BM, Sari LA, Wulan ND, Muchlashi LA. In silico evaluation of ciplukan (*Physalis angulata* Linn.) compounds as potential serotonin reuptake inhibitors for antidepressant development. *J Riset Kefarm Indones*. 2026;8(2):168-181. doi:10.33759/jrki.v8i2.953

INTRODUCTION

Mental health problems, especially depressive disorders, are becoming an increasing global challenge. Depression is one of the leading causes of morbidity that has become a global problem. The prevalence of depression in the global population is known to reach 4.4%, with an estimated 322 million people living with the condition. Meanwhile, in Indonesia, the prevalence of depression reaches 3.7% and causes morbidity of 6.6% of the population⁽¹⁾. Depression is one of the psychiatric disorders characterized by a decrease in mood (hypotimia) and the emergence of negative feelings towards oneself. Depressive conditions are complex problems and can be caused by various triggering factors, both in terms of psychological, biological, genetic, and social. According to the *Diagnostic and Statistical Manual of Mental Disorders* edition V (DSM-V), a person is suspected of having depression if they experience five of the following ten symptoms, namely experiencing a decline in mood that continues for more than 2 weeks, increased emotional psychomotor activity in children and adolescents, loss of ability to feel happy and happy, weight loss or increase influenced by changes in appetite, sleep disturbances, easily feeling tired when doing light activities, decreased self-confidence, decreased ability to think and concentrate, and thoughts of self-harm or suicide. Depressive conditions need to be treated immediately, especially if they affect the patient's quality of life⁽²⁾.

Based on previous clinical studies conducted on depressed patients, changes in serotonergic function in the central nervous system were found. Serotonin (5-HT) is widely distributed in the nervous system and a decrease in its amount can cause depression, phobia. In addition to decreased serotonin levels, changes in serotonin (5-HT) receptors also play a role in the mechanism of depression⁽³⁾. Most of the 15 serotonin receptor subtypes are known to have a role in modulating depression and can be targeted by antidepressants, for example, 5-HT_{1A} and 5-HT_{1B}. In a study that quantified 5-HT_{1A} receptor levels showed an increase in the number of 5-HT_{1A} receptors and activation of 5-HT_(1A) receptors causes hyperpolarization which leads to decreased *firing* (neuronal activity) so that serotonin release decreases. In 5-HT_{1B} receptors where activation can reduce serotonin release through its action on the presynapse so that serotonin levels in the brain decrease⁽⁴⁾.

One of the herbal plants that offers potential for antidepressant development through the mechanism of *serotonin reuptake inhibitors* that can prevent serotonin degradation is ciplukan (*Physalis angulata* Linn.). Ciplukan is a wild herbal plant that grows in tropical and subtropical regions such as Indonesia which is still not widely utilized among the community⁽⁵⁾. Ciplukan contains various potential secondary metabolite compounds such as alkaloids, glycosides, phenolic acids, and flavonoids. These compounds show a variety of antidepressant activities. Based on research conducted by Kartika *et al.* (2021) showed that alkaloid compounds have activity as antidepressants with a mechanism of action that inhibits the reuptake of serotonin in the brain⁽⁶⁾. Other research related to plants that have the potential for antidepressants shows that the content of one of the secondary metabolites, namely glycosides, is thought to be responsible for the antidepressant effect but the mechanism of action is still unclear⁽⁷⁾. Phenolic compounds are widely used as antioxidants that can reduce oxidative stress so that they can protect cells in the brain from free radicals associated with depression⁽⁸⁾. Previous research on flavonoid compounds has shown activity as an antidepressant by increasing serotonin or 5-hydroxytryptamine (5-HT), norepinephrine (NE), and Brain-Derived Neurotrophic Factor (BDNF) levels, and reducing monoamine oxidase (MAO) activity⁽⁹⁾.

The management of depression can use pharmacological therapy in the form of antidepressant drugs. Selective serotonin reuptake inhibitors (SSRIs) are the first line of antidepressant drugs so they are often prescribed. The mechanism of action of SSRIs in general is by inhibiting the transport of serotonin into presynaptic neurons so that

serotonin levels in the brain increase. SSRI drugs include paroxetine, fluoxetine, escitalopram, and fluvoxamine. Antidepressant drugs are consumed for 6-9 months and some researchers even recommend 3-5 years and for life if necessary. However, long-term use may be associated with adverse effects, tolerability issues, or treatment discontinuation challenges⁽¹⁰⁾. Therefore, research on alternative treatment therapies for handling depression is urgent. This alternative therapy can be approached through wild herbal medicine in the form of ciplukan, a plant with anti-inflammatory and antioxidant properties, which has potential as an antidepressant from natural materials.

Based on the literature search regarding the biological activity of ciplukan, the author conducted in silico research with the molecular docking method which aims to investigate the molecular interactions between secondary metabolite compounds in ciplukan extract with the serotonin transporter. The molecular structure of active compounds was identified based on previous research. Furthermore, molecular simulation and docking were performed using specialized software to evaluate the strength of the interaction between the active compound and the target protein.

METHODS

Tools and materials

The research was conducted using hardware with Windows 11 specifications, 4GB RAM, 64-bit operating system and Intel(R) Core(TM) i3- 1005G1 CPU @ 1.20GHz 1.19 GHz processor. In addition, software such as Molegro Virtual Docker, ChemDraw Ultra 12.0, and ChemDraw 3D Pro 12.0 were used. In this research, the test ligand is a compound from Ciplukan (*Physalis angulata* Linn.) with comparison ligands such as Fluvoxamine, Fluoxetine, Escitalopram, and Paroxetine. The target protein was obtained from the Uniprot database (<https://www.uniprot.org/>) namely hSERT (PDB ID: 5173).

Ligand Preparation and Analysis

Test ligands and standard ligands were obtained from PubChem website (<https://pubchem.ncbi.nlm.nih.gov/>). Furthermore, the preparation was carried out with ChemDraw Ultra 12.0 application to identify the physicochemical properties of the compounds. Subsequently, ChemDraw 3D 12.0 to optimize ligand geometry and perform energy minimization to obtain stable molecular conformations. The optimized ligand structures were then saved in SYBYL2 format (*.mol2) for further molecular docking analysis using Molegro Virtual Docker.

Target Protein Preparation

The target protein structure of human serotonin transporter (hSERT) with PDB ID: 5173 was obtained through the Uniprot website (The website will be connected to the protein data bank website (<https://www.rcsb.org/>) to download the target protein. Protein preparation water molecules were removed from the protein structure, hydrogen atoms were added, and appropriate protonation states were assigned to obtain the receptor structure suitable for molecular docking analysis. The website will be connected to the protein data bank website (<https://www.rcsb.org/>) to download the target protein. Protein preparation water molecules were removed from the protein structure, hydrogen atoms were added, and appropriate protonation states were assigned to obtain the receptor structure suitable for molecular docking analysis.

Molecular Docking Method Validation

Validation is done by re-docking the test ligand with the target receptor. Re tethering is done to validate the PDB file downloaded on the website <https://www.rcsb.org/> and the software used as a tethering is Molegro

Virtual Docker (MVD) Ver. 5.0 as the *virtual screening docking* method studied, namely the compounds found in Ciplukan (*Physalis angulata* Linn.) The acceptance value of the re-docking is *Root Mean Square Deviation* (RMSD) < 2.0 Å. <https://www.rcsb.org/> and the software used as a tethering is Molegro Virtual Docker (MVD) Ver. 5.0 as the *virtual screening docking* method studied, namely the compounds found in Ciplukan (*Physalis angulata* Linn.) The acceptance value of the re-docking is *Root Mean Square Deviation* (RMSD) < 2.0 Å.

Docking Ligand against Target Protein

Docking is done by predicting the ligand's ability to bind (affinity) to the protein (receptor) using Molegro Virtual Docker software. Ligands were prepared through geometry optimization and energy minimization before docking simulations. Docking simulations were conducted using a predicted binding cavity with a volume of 213.504 Å³ and a surface area of 637.44 Å². The cavity center coordinates were x = 22.334, y = 125.798, and z = 178.100 Å. Ligands were docked flexibly into the active binding cavity to generate several possible binding conformations against the target protein. Docking simulations were performed with 10 independent runs for each ligand using the default RMSD threshold parameter in Molegro Virtual Docker. Ligand–protein interactions were evaluated using the MolDock scoring function and analyzed based on MolDock Score and Rerank Score values.

Visualization and Analysis of Docking Results

The success of this process can be seen based on the rerank score of each ligand. The rerank score value is a value that indicates the amount of bonding energy required for a compound to bind to a receptor, so that the rerank score can predict the activity of a compound on a particular receptor ⁽¹¹⁾. The smaller the rerank score value indicates a greater degree of compatibility between the ligand and the interacting receptor. In silico tests that are visualized and interpreted provide a representation of ligand bonds with receptors in the form of hydrogen bonds, steric interactions, and electronic interactions. Molegro Virtual Docker is a docking simulation software between protein and ligand in a fully integrated computational package. Molegro Virtual Docker has been applied to a variety of different proteins, with similar capabilities to other docking programs, such as AutoDock Vina and AutoDock4. Molegro Virtual Docker has four original scoring functions and four search algorithms. The integration between Molegro Virtual Docker and SAnDReS will increase the opportunity for detailed statistical analysis of docking results, which will improve the performance of Molegro Virtual Docker ⁽¹²⁾.

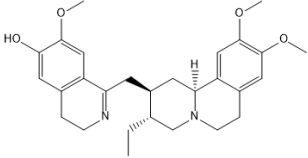
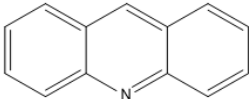
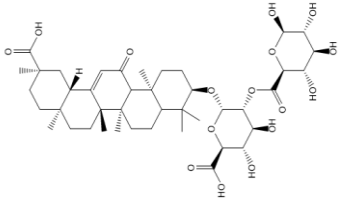
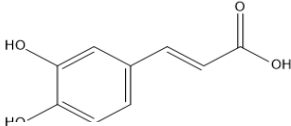
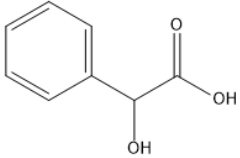
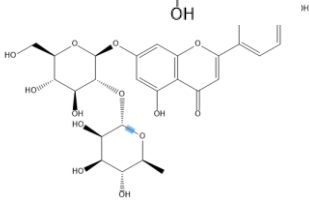
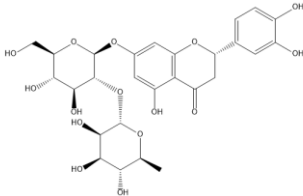
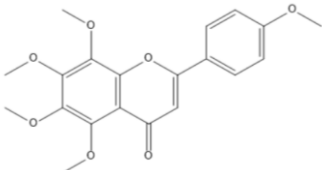
RESULT AND DISCUSSION

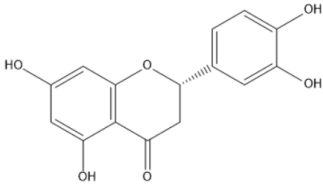
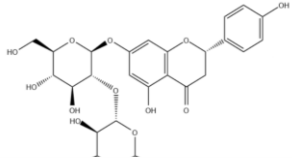
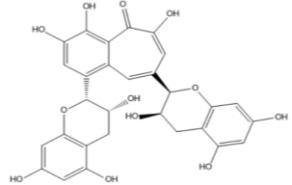
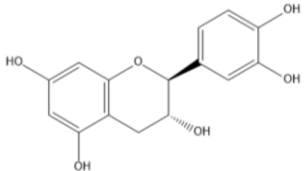
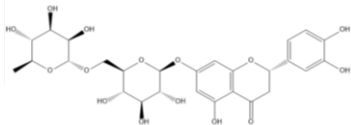
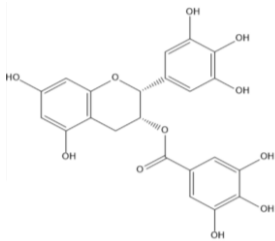
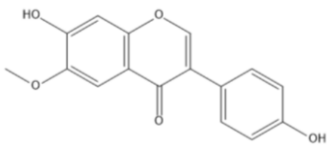
Ligand Preparation and Analysis

In this study, molecular binding of 16 active compounds found in Ciplukan (*Physalis angulata* Linn.) plants that have been studied previously in previous studies with test ligands against the hSERT (PDB ID : 5I73) was carried out. As in **Table 1**, the selection of metabolites from Ciplukan extract (*Physalis angulata* Linn.) was selected 16 compounds with the largest concentration taken from 4 identified secondary metabolites, namely 2 compounds in metabolites of the Alkaloid group from 12% alcohol isolate with filtration methods including Psychotrine and Acridine, 2 compounds in metabolites of the Phenolic Acid group from 70% acetone isolate with filtration methods including Captopril acid and E-strophantin acid, 2 compounds in metabolites of the glycoside group from 0,1 M with filtration method including Astringin acid and Mandelic acid and 10 compounds in Flavonoid metabolites from distilled water isolates with filtration method including Rhoifolin, Neoriocitrin, Tangeretin, Eriodictyol, Naringin, Theaflavins, Catechin,

Eriocitrin, Epigallocatechin gallate, and Glycitein where 16 compounds selected have previously been identified in previous studies ⁽¹³⁾.

Table 1. Secondary Metabolites of Ciplukan (*Physalis angulata* Linn.) ⁽¹³⁾.

Compound Structure	Compound Name	Concentration (% w/w)
	<i>Psychotrine</i>	6.353%
	<i>Acridine</i>	5.630%
	<i>Glycyrrhizin</i>	6.435%
	<i>Caffeic Acid</i>	4.362%
	<i>Mandelic Acid</i>	7.484%
	<i>Rhoifolin</i>	7.398%
	<i>Neoriocitrin</i>	6.200%
	<i>Tangeretin</i>	5.800%

Compound Structure	Compound Name	Concentration (% w/w)
	<i>Eriodictyol</i>	3.300%
	<i>Naringin</i>	3%
	<i>Theaflavins</i>	2.800%
	<i>Catechin</i>	2.700%
	<i>Eriocitrin</i>	2,500%
	<i>Epigallocatechin gallate</i>	2%
	<i>Glycitein</i>	0.003%

While the comparison ligand used is a *Selective Serotonin Reuptake Inhibitor* (SSRI) class drug, namely Citalopram, Fluvoxamine, Fluoxetine, Escitalopram, and Paroxetine. Test ligands and standard ligands were obtained from the PubChem website which were then prepared using the ChemDraw Ultra 12.0 application to obtain 2D structures as in **Table 1**. and determine the chemical physical properties of ligand compounds. Furthermore, using ChemDraw 3D 12.0 to minimize the energy of the ligand compound and get the total energy value and then stored in SYBYL2 format (*.mol2).

Target Protein Preparation

The target protein that binds to SSRI class drugs is the *human serotonin transporter* (hSERT) with PDB ID

5273⁽¹⁴⁾. The target protein was downloaded through the RCSB-PDB database according to the PDB Protein ID. Furthermore, the target protein was inserted into the Molegro Virtual Docker software without including the cofactor and water molecule. A cavity detection was performed on the target protein which is where the ligand binds.

Analysis of *docking* results

The *docking* process of test ligands and comparison ligands with proteins is performed using Molegro Virtual Docker software to predict the ability of ligands to bind (affinity) to proteins (receptors). The process resulted in a *Rerank Score* and ligand-protein interactions. While the Total Energy was obtained from ChemDraw 3D 12.0 in the ligand preparation process.

Total energy indicates the stability of the ligand structure. However, not all ligands that have low total energy can interact well with their receptors. Rerank Score (RS) describes the binding energy. Bond energy is the amount of energy required for the ligand to bind to the receptor. A lower rerank score indicates a higher affinity of the ligand for the protein⁽¹⁵⁾.

Table 2. Total energy value of ligand based on ChemDraw 3D 12.0 and docking result data of ligand with target protein using Molegro Virtual Docker.

Goals	Compound	Total Energy (kcal/mol)
Comparison Drug	<i>Paroxetine</i>	24.0987
	<i>Fluvoxamine</i>	24.0560
	<i>Escitalopram</i>	20.2398
	<i>Fluoxetine</i>	17.0929
Test Compound	<i>Epigallocatechin gallate</i>	4.3898
	<i>Naringin</i>	56.7073
	<i>Rhoifolin</i>	57.5105
	<i>Neoriocitrin</i>	49.8027
	<i>Psychotrine</i>	41.8010
	<i>Catechin</i>	-5.6718
	<i>Theaflavins</i>	41.1073
	<i>Eriocitrin</i>	19.9354
	<i>Glycitein</i>	33.1620
	<i>Tangeretin</i>	59.9918
	<i>Eriodictyol</i>	5.1657
	<i>Caffeic Acid</i>	-7.3263
	<i>Acridine</i>	1.8092
	<i>Mandelic Acid</i>	-4.0612
	<i>Glycyrrhizin</i>	128.2716

The comparison drug, fluoxetine, has the highest rerank score among the other comparison drugs, namely -

95.919 kcal/mol. However, it does not mean that fluoxetine is worse than other drugs because fluoxetine is a widely used SSRI class drug. There are eleven test ligands that have more negative RS than the comparator drug fluoxetine. The test ligands are compounds of Epigallocatechin gallate (-118.511), Naringin (-98,112), Neoriocitrin (-97,570), Theaflavins (-139.055), Eriodictyol (-100.485), Glycyrrhizin (-142.189), Psychotrine(-107.655), Rhoifolin (-137.997), Eriocitrin (-129.939), Catechin (-96.419), Tangeretin (-100.399). Among these compounds, Glycyrrhizin has the best RS value of -142.189 kcal/mol. The more negative rerank scores observed in these compounds may suggest favorable predicted interactions with hSERT. Comprehensive molecular docking results are detailed in **Table 3**.

Table 3. Amino acid residue analysis using Molegro Virtual Docker

Compound	Rerank Score (kcal/mol)	Hydrogen bond	Steric Interaction	Electrostatic Interaction
<i>Paroxetine</i>	-102,361	Ala 96, Ser 439	Asp 98, Ala 96, Tyr 95, Ile 172, Ser 439	-
<i>Fluvoxamine</i>	-107,822	Ala 96, Ser 336	Ala 96, Ser 336, Asp 98, Phe 335, Tyr 95	-
<i>Fluoxetine</i>	-95,919	-	Phe 335, Asp 98, Tyr 95	-
<i>Escitalopram</i>	-106,692	-	Tyr 95, Ala 96	Asp 98
<i>Epigallo catechin gallate</i>	-118,511	Ala 169, Asn 177, Tyr 95, Phe 335, Tyr 175, Ser 436	Ala 169, Asn 177, Ser 439, Tyr 95, Phe 335, Asp 98, Ser 438, Tyr 176, Ile 172, Tyr 175	-
<i>Naringin</i>	-98,112	Glu 495, Arg 104, Gln 332, Glu 493, The 497	Glu 494, Asp 328, Gln 332, Glu 493, Arg 104, Val 501, Thr 501, Thr 497, Tyr 175	-
<i>Neoriocitrin</i>	-97,570	Tyr 176, Glu 493, Trp 103, Asp 98, Phe 325, Tyr 95	Glu 493, Trp 103, Asp 98, Tyr 176, Gly 100, Gln 332, Ser 336, Asn 101, Phe 335, Tyr 95, Ser 438	-
<i>Theaflavins</i>	-139,055	Tyr 175, Pro 499, Glu 493, Gln 332	Tyr 175, Pro 499, Leu 502, Gly 498, Glu 493, Thr 497, Phe 556, Arg 104, Glu 494, Gln 332	-

Compound	Rerank Score (kcal/mol)	Hydrogen bond	Steric Interaction	Electrostatic Interaction
<i>Eriodictyol</i>	-100,485	Asn 177, Ala 169, Ser 439, Tyr 95, Ser 438, Tyr 176	Ser 439, Asn 177, Ala 169, Phe 341, Ala 96, Asp 98, Ser 438, Tyr 95, Tyr 176	-
<i>Glycyrrhizin</i>	-142,189	Arg 564, Ala 401, Tyr 107, Gln 562, Lys 490	Arg 104, Glu 494, Leu 563, Gln 562, Lys 490, Tyr 107, Lys 399, Gln 111, Arg 564, Ala 401, Ile 108	-
<i>Psychotrine</i>	-107,655	Ser 438	Tyr 95, Ser 438, Tyr 176, Asp 98, Phe 335, Gln 332	-
<i>Glycitein</i>	-90,757	Ala 96	Tyr 95, Phe 335, Ser 336, Asp 98, Ala 96	-
<i>Rhoifolin</i>	-137,997	Glu 493, Thr 497, Tyr 95, Asp 98, Ser 438	Arg 104, Gly 498, Thr 497, Phe 335, Glu 493, Tyr 95, Gly 338, Phe 341, Ile 172, Asp 98, Ser 438	-
<i>Caffeic Acid</i>	-74,146	Asp 98, Gln 332	Gln 332, Ser 336, Asn 101, Phe 335, Asp 98	-
<i>Eriocitrin</i>	-129,939	Asp 98, Glu 493, Tyr 175, Tyr 95, Ala 169	Asp 98, Phe 335, Ser 336, Ala 96, Arg 104, Glu 493, Thr 497, Tyr 175, Tyr 95, Ile 172, Ala 169	-
<i>Catechin</i>	-96,419	Asn 177, Ser 439, Ala 169, Asp 98	Asn 177, Ser 439, Ala 173, Ala 169, Asp 98, Ala 96, Tyr 95	-
<i>Tangeretin</i>	-100,399	-	Ser 336, Phe 335, Gln 332	-
<i>Mandelic Acid</i>	-68,965	Asn 351, Lys 85, Phe 347	Lys 85, Asn 351, Phe 88, Phe 347	-

Compound	Rerank Score (kcal/mol)	Hydrogen bond	Steric Interaction	Electrostatic Interaction
Acridine	-79,797	Arg 104	Ser 336, Gln 332, Arg 104	-
	Similarity of Interaction with amino acid Ala 96 through Hydrogen Bonds and Steric Interactions			
	Similarity of Interaction with amino acid Ser 439 through Hydrogen Bonds and Steric Interactions			
	Similarity of Interaction with amino acid Asp 98 through Steric Interactions			
	Similarity of Interaction with amino acid Ala 96 through Steric Interactions			
	Similarity of Interaction with amino acid Ile 172 through Steric Interactions			
	Similarity of Interaction with amino acid Phe 335 through Steric Interactions			
	Similarity of Interaction with amino acid Ser 336 through Steric Interactions			

Ligand interactions with amino acids in protein are crucial for the biological activity of a compound. From the analysis of ligand interactions with amino acids, it is known that of the 11 compounds with lower rerank scores than the reference drug, only 8 compounds showed interactions with hSERT similar to the natural ligand (reference drug). In hSERT inhibition, the amino acids Asp 98 and Tyr 95 are very significant for antidepressant activity. These amino acids bind to the protonated amine functional group in SSRIs. At physiological pH, this group is positively charged so it interacts with negatively charged amino acid residues in the hSERT protein. From the analysis of ligand interactions with amino acids, it is known that of the 11 compounds with lower rerank scores than the reference drug, 7 compounds showed interactions with the amino acids Asp 98 and Tyr 95. These compounds are epigallocatechin gallate, neoriotrin, eriodictyol, psychotrine, rhoifolin, eriocitrin, and catechin.

Based on the analysis of the results of the epigallocatechin gallate, neoriotrin, eriodictyol, psychotrine, rhoifolin, eriocitrin, dan catechin compounds which are flavonoid metabolite group compounds from Ciplukan, demonstrated favorable interactions with hSERT target protein. These compounds showed relatively negative rerank scores and interacted with several amino acid residues involved in the binding pocket, including Asp 98 dan Tyr 95 which are associated with hSERT. These may suggest a possible resemblance in binding mechanisms toward hSERT such as comparator drugs. However, molecular docking only predicts the possibility of ligand-target interaction and does not directly indicate clinical efficacy and biological activity. Therefore, these compounds may be considered as potential candidates as serotonin reuptake inhibitors with further investigation.

A molecule must reach its target in the body in sufficient amounts and remain in the body in its bioactive form for an adequate period. This analysis was conducted using SwissADME. Furthermore, the physicochemical properties of the compounds were predicted according to the Lipinski's Rule of Five, which serves as an indicator of a compound's ability to penetrate cell membranes through passive diffusion. Compounds that do not meet these criteria may exhibit impaired oral drug absorption. Based on the results of the Lipinski analysis on **Table 4**, eight

compounds were found to fulfill the criteria of Lipinski's Rule of Five. In the Lipinski analysis of 7 compounds that had lower rerank scores than the reference drug and interacted with the amino acids Asp 98 and Tyr 95, it was found that only 3 compounds had Lipinski values that met the requirements, including psychotrine, catechin, and eriodictyol.

Tabel 4. *Lipinski Rule of Five of Secondary Metabolite Compounds from *Physalis angulata* Linn.*

Compound	Molecule Weight	Lipophilicity (MLOGP)	H-Bond Acceptors	H-Bond Donors	Rotatable Bonds	Lipinski
	< 500	< 5	< 10	< 5	< 10	Yes
<i>Epigallocatechin gallate</i>	458.37	-0.44	11	8	4	No
<i>Naringin</i>	580.53	-2.77	14	8	6	No
<i>Rhoifolin</i>	578.52	-2.96	14	8	6	No
<i>Neoriocitrin</i>	596.53	-3.24	15	9	6	No
<i>Psychotrine</i>	464.60	2.77	6	1	6	Yes
<i>Catechin</i>	290.27	0.24	6	5	1	Yes
<i>Theaflavine</i>	564.49	-0.79	12	9	2	No
<i>Eriocitrin</i>	596.53	-3.24	15	9	6	No
<i>Glycitein</i>	284.26	0.77	5	2	2	Yes
<i>Tangeretin</i>	372.37	0.63	7	0	6	Yes
<i>Eriodictyol</i>	288.25	0.16	6	4	1	Yes
<i>Caffeic Acid</i>	180.16	0.70	4	3	2	Yes
<i>Acridine</i>	179.22	3.06	1	0	0	Yes
<i>Mandelic Acid</i>	152.15	0.78	3	2	2	Yes
<i>Glycyrrhizin</i>	822.93	0.02	16	8	7	No

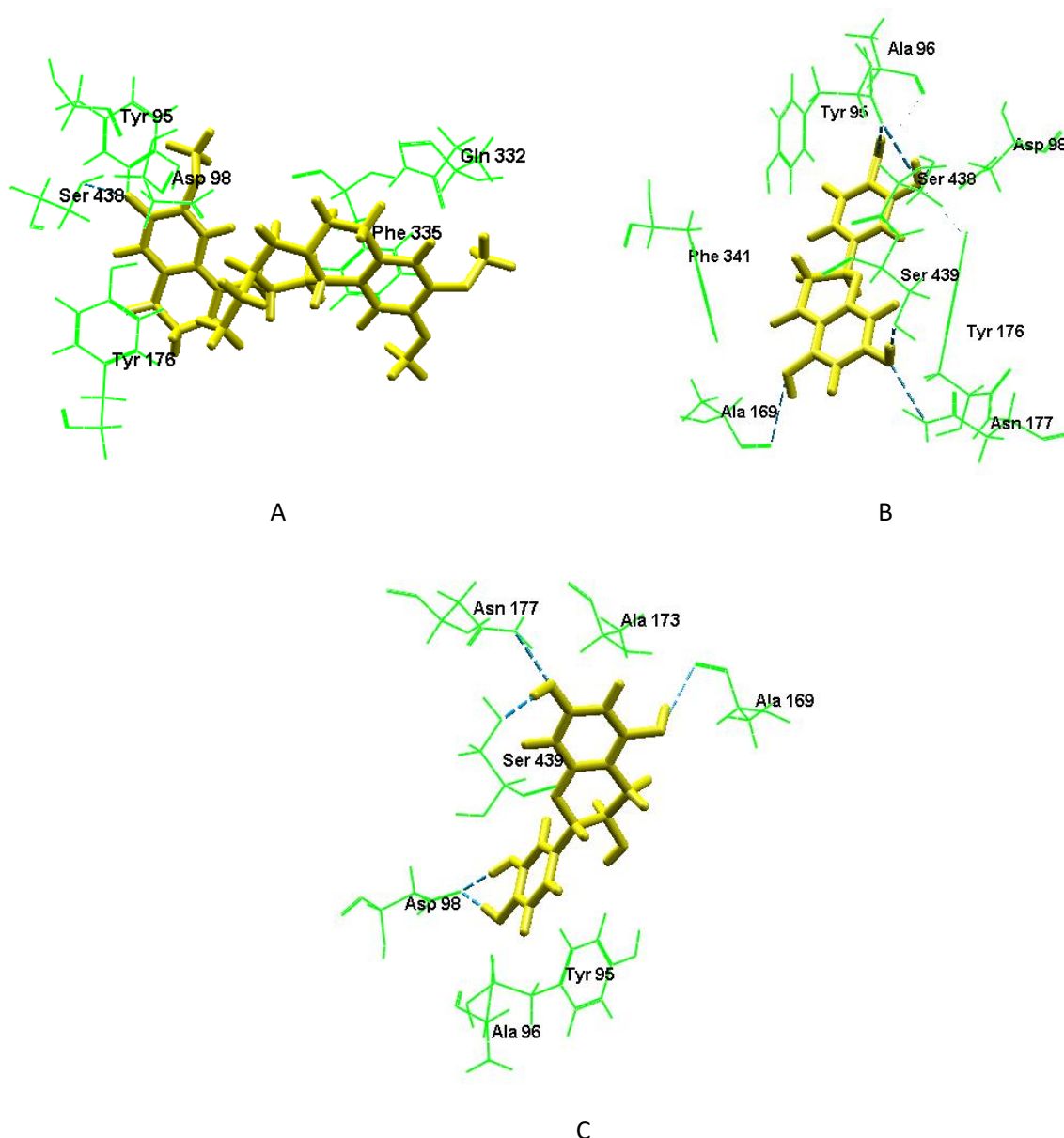


Figure 1. Visualization of test ligand binding (a) Psychotrine with hSERT; (b) Eriodictyol with hSERT; (c) Catechin with hSERT. Compound Psychotrine interacts with Asp 98 and Tyr 95 through Steric Interactions. Compound Eriodictyol interacts with Asp 98 and Tyr 95 through Steric Interactions. Compound Catechin interacts with Asp 98 and Tyr 95 through Steric Interactions.

Docking Validation Analysis

Validation of Docking Results can be determined from the RMSD value of natural ligand with target protein. The RMSD is used to determine the prediction of successful bonding of natural ligand compounds with target proteins and is an important parameter in the docking program. RMSD is said to be good if $\leq 2 \text{ \AA}$ where the greater the RMSD value, the greater the deviation and prediction error of natural ligand interaction with the target protein⁽¹⁸⁾. It is known in the results of **Table 4** that the RMSD of the natural ligand with the target protein is $\leq 2 \text{ \AA}$, which means that there is minimal deviation or prediction error between the interaction of the ligand and the target protein, so it can be concluded that the results can be validated properly.

Table 4. *Re-docking* Results of Natural Ligand with Target Protein

Target Protein	Protein Code	Natural Ligan	Binding Affinity	RMSD ($\leq 2 \text{ \AA}$)
hSERT	5I73	68P_701	-23,695	0.821121

LIMITATION

This study has several limitations that should be considered when interpreting the result. First, this study addresses a relatively novel research topic. Therefore, supporting literature is still limited. This limitation restricts comprehensive comparison with previous studies and may affect the depth of discussion. Further studies are required to strengthen the evidence and validate the findings of this research.

Second, this study was conducted using a computational (in silico) method with molecular docking without experimental validation using in vivo and in vitro methods. Therefore, the results of this study are predictive and may not fully represent the actual pharmacological effect under physiological conditions.

CONCLUSION

Of the 15 compounds identified in ciplukan extract and evaluated through molecular docking analysis, psychotrine, catechin, and eriodictyol demonstrated more negative rerank scores, has the same significant amino acid bonds as the reference ligands, namely Asp 98 and Tyr 95 and has penetration in the blood brain barrier as shown by the results of the Lipinski analysis. These findings suggest that the flavonoid compounds may exhibit favorable binding affinity toward the target receptor. However, the results of this study are predictive in nature and based solely on in silico molecular docking analysis. Therefore, further validation through in vitro and in vivo studies is required to confirm their potential antidepressant activity.

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AUTHOR CONTRIBUTION STATEMENT

Azki Alfazahrah: Conceptualization, Methodology, Supervision, Investigation, Data Curation, and Formal Analysis. Alifda Nurhayati : Data Curation, Investigation, Literature Review, and Writing – Original Draft. Bianti Meylia Wijaya : Conceptualization, Data Curation, Investigation, Formal Analysis, and Writing – Review & Editing. Larasati Atum Sari : Investigation, Literature Review, Data Curation, and Writing-Original Draft. Nabila Dinar Wulan : Data Curation, Investigation, Target Protein Analysis, and Control Ligand Analysis. All authors have read and approved the final version of the manuscript.

CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest.

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Determination of Caffeine Content in Ethanolic Extract of Robusta Coffee Beans (*Coffea canephora*) Using UV-Vis Spectrophotometry

Penentuan Kandungan Kafein dalam Ekstrak Etanol Biji Kopi Robusta (*Coffea canephora*) Menggunakan Spektrofotometri UV-Vis

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Abstract

Caffeine is one of the main bioactive compounds in robusta coffee beans (*Coffea canephora*) that has potential applications in pharmaceutical and cosmetic formulations, particularly for hair growth stimulation. This study aimed to determine the caffeine content in ethanolic extract of robusta coffee beans using UV-Visible spectrophotometry. The research was conducted using an experimental laboratory design. Robusta coffee bean extract was obtained by maceration using ethanol as the solvent. Qualitative identification of caffeine was followed by quantitative analysis using UV-Vis spectrophotometry at a maximum wavelength of 273 nm. A calibration curve was constructed using standard caffeine solutions, and the caffeine content of the extract was calculated based on the linear regression equation. The results showed that the calibration curve exhibited good linearity within the tested concentration range. Quantitative analysis revealed that the caffeine content of the ethanolic extract of robusta coffee beans was 221.58 mg/g extract, which is consistent with previously reported caffeine levels in *Coffea canephora*. These findings confirm that robusta coffee beans are a significant natural source of caffeine and support their potential utilization as raw material for pharmaceutical and cosmetic products. This study provides quantitative data that may serve as a scientific basis for further formulation development and quality control of caffeine-containing herbal products.

Abstrak

Kafein merupakan salah satu senyawa bioaktif utama dalam biji kopi robusta (*Coffea canephora*) yang memiliki potensi aplikasi dalam formulasi farmasi dan kosmetik, khususnya untuk stimulasi pertumbuhan rambut. Penelitian ini bertujuan untuk menentukan kandungan kafein dalam ekstrak etanol biji kopi robusta menggunakan spektrofotometri UV-Visible. Penelitian ini dilakukan dengan menggunakan desain laboratorium eksperimental. Ekstrak biji kopi robusta diperoleh dengan maserasi menggunakan etanol sebagai pelarut. Identifikasi kualitatif kafein diikuti dengan analisis kuantitatif menggunakan spektrofotometri UV-Vis pada panjang gelombang maksimum 273 nm. Kurva kalibrasi dibuat menggunakan larutan kafein standar, dan kandungan kafein ekstrak dihitung berdasarkan persamaan regresi linier. Hasil menunjukkan bahwa kurva kalibrasi menunjukkan linearitas yang baik dalam rentang konsentrasi yang diuji. Analisis kuantitatif mengungkapkan bahwa kandungan kafein ekstrak etanol biji kopi robusta adalah 221,58 mg/g ekstrak, yang konsisten dengan kadar kafein yang dilaporkan sebelumnya pada *Coffea canephora*. Temuan ini menegaskan bahwa biji kopi robusta merupakan sumber kafein alami yang signifikan dan mendukung potensi pemanfaatannya sebagai bahan baku untuk produk farmasi dan kosmetik. Studi ini memberikan data kuantitatif yang dapat berfungsi sebagai dasar ilmiah untuk pengembangan formulasi lebih lanjut dan pengendalian mutu produk herbal yang mengandung kafein.

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INTRODUCTION

Robusta coffee (*Coffea canephora*) is one of the most widely cultivated coffee species in Indonesia and plays an important role in the agricultural, pharmaceutical, and cosmetic industries. Compared to arabica coffee, robusta coffee is characterized by higher caffeine content, stronger flavor profile, and greater adaptability to tropical lowland

climates. Previous studies have reported that the caffeine content of robusta coffee beans ranges from 1.7–4%, which is considerably higher than that of arabica coffee.^{1,2} This characteristic makes robusta coffee a promising natural source of caffeine for pharmaceutical and cosmetic applications requiring standardized bioactive compounds. Caffeine is a naturally occurring methylxanthine alkaloid with various biological and pharmacological activities, including central nervous system stimulation, antioxidant activity, lipolytic effects, and stimulation of hair follicle growth. Due to these pharmacological properties, caffeine has been extensively utilized as an active ingredient in pharmaceutical, nutraceutical, and cosmetic formulations.^{3,4}

In recent years, increasing interest has been directed toward the use of naturally derived caffeine as an alternative to synthetic compounds, particularly in topical and hair care preparations. Several studies have demonstrated that caffeine can penetrate the skin barrier and exert biological effects on hair follicles by prolonging the anagen phase and inhibiting androgen-induced follicular miniaturization.^{5,6,7} These findings indicate that robusta coffee beans possess considerable potential as a natural raw material for the development of caffeine-based pharmaceutical and cosmetic products.

The effectiveness, safety, and quality consistency of caffeine-containing products are strongly influenced by the concentration of active compounds present in the raw materials. Therefore, accurate determination of caffeine content in coffee bean extracts is essential for quality control, dosage standardization, and formulation development. Various analytical techniques have been employed for caffeine determination, including high-performance liquid chromatography (HPLC), gas chromatography, and capillary electrophoresis.⁸ Although these methods provide high sensitivity and specificity, they generally require sophisticated instrumentation, complicated sample preparation procedures, and relatively high operational costs, which may limit their application in routine laboratory analysis, particularly in developing research facilities. Among various extraction solvents, ethanol is widely used for the extraction of plant bioactive compounds because of its relatively low toxicity, good extraction capability, ease of evaporation, and compatibility with pharmaceutical and cosmetic preparations.^{9,10} In addition, ethanolic extraction is considered more suitable for obtaining semipolar compounds such as caffeine while maintaining extract safety for further formulation development. However, despite the extensive cultivation of robusta coffee in Indonesia, studies evaluating caffeine content specifically in ethanolic extracts of robusta coffee beans remain limited.

UV-Visible spectrophotometry offers a practical alternative for routine caffeine analysis due to its simplicity, rapid analytical process, cost-effectiveness, and adequate sensitivity for quantitative determination.^{8,11,12} Caffeine exhibits characteristic absorbance in the ultraviolet region, enabling quantitative analysis using calibration curve-based measurements.⁴ Although numerous studies on caffeine determination have been reported, most investigations focus on brewed coffee beverages or utilize advanced chromatographic techniques such as HPLC. Limited studies have specifically investigated the determination of caffeine content in ethanolic extracts of robusta coffee beans using UV-Visible spectrophotometry, particularly for potential pharmaceutical and cosmetic applications. This condition indicates the existence of a research gap regarding the development of simple, accessible, and economical analytical methods for standardization of natural caffeine sources derived from robusta coffee. Therefore, this study aimed to determine the caffeine content in ethanolic extract of robusta coffee beans (*Coffea canephora*) using UV-Visible spectrophotometry. The findings of this study are expected to provide scientific data supporting the utilization of robusta coffee bean extract as a standardized natural source of caffeine and to contribute to the development of quality control methods for pharmaceutical and cosmetic formulations containing natural caffeine.

METHODS

Materials

Robusta coffee beans (*Coffea canephora*) were obtained from local coffee plantations in Indonesia and authenticated based on their morphological characteristics. The chemicals used in this study included ethanol 96% (analytical grade), caffeine standard, chloroform, distilled water, and sodium carbonate. All reagents used were of analytical grade.^{9,10,13}

Instruments

The instruments used in this study included a UV-Visible spectrophotometer, analytical balance, rotary evaporator, blender, glassware, volumetric flasks, micropipettes, Whatman filter paper, water bath, and measuring cylinders.^{9,10,13}

Preparation of Robusta Coffee Bean Powder

Fresh robusta coffee beans were cleaned to remove impurities and dried at room temperature. The dried coffee beans were then ground using a blender to obtain a fine powder. The powder was sieved to obtain uniform particle size and stored in an airtight container until further use.

Extraction of Robusta Coffee Beans

A total of 500 g of robusta coffee bean powder was extracted using the maceration method with 96% ethanol as solvent. The powder was soaked in ethanol with a ratio of 1:10 (w/v) and allowed to stand for 72 hours at room temperature with occasional stirring every 24 hours to maximize extraction efficiency. The filtrate obtained from maceration was filtered using Whatman filter paper. The residue was re-macerated twice using fresh solvent to ensure complete extraction of bioactive compounds. All filtrates were combined and concentrated using a rotary evaporator at 50°C under reduced pressure to obtain a thick ethanolic extract. The extract was then weighed to determine extraction yield and stored in a closed container prior to analysis.^{9,10} The extraction yield was calculated using the following equation:

$$\text{Yield (\%)} = \frac{\text{Weight of extract}}{\text{Weight of simplicia}} \times 100$$

Preparation of Standard Caffeine Solution

A stock solution of caffeine standard was prepared by dissolving 100 mg of pure caffeine in distilled water and diluting to 100 mL in a volumetric flask to obtain a concentration of 1000 ppm. Working standard solutions were prepared from the stock solution at concentrations of 2, 4, 6, 8, and 10 ppm by appropriate dilution using distilled water.

Determination of Maximum Wavelength

The maximum wavelength of caffeine was determined by scanning the standard caffeine solution at a concentration of 10 ppm using a UV-Visible spectrophotometer within the wavelength range of 200–400 nm. The wavelength showing maximum absorbance was selected as the analytical wavelength for quantitative determination.

Preparation of Calibration Curve

The absorbance of standard caffeine solutions at concentrations of 2, 4, 6, 8, and 10 ppm was measured at the determined maximum wavelength. A calibration curve was constructed by plotting concentration versus absorbance values, and the linear regression equation was calculated.^{8,11,12,13}

Determination of Caffeine Content in Ethanolic Extract

A total of 100 mg of ethanolic extract was dissolved in distilled water and transferred into a 100 mL volumetric flask. The solution was filtered to remove insoluble particles. The absorbance of the sample solution was measured using UV-Visible spectrophotometry at the determined maximum wavelength. The caffeine concentration in the extract was calculated using the regression equation obtained from the calibration curve.^{8,11,12,13} The caffeine content was calculated using the following equation:

$$\text{Caffeine content (\%)} = \frac{C \times V \times FP}{W} \times 100$$

where:

- C = concentration obtained from calibration curve (mg/mL)
- V = sample volume (mL)
- FP = dilution factor
- W = sample weight (mg)

Data Analysis

All measurements were performed in triplicate, and the results were expressed as mean $\pm$ standard deviation (SD). Linear regression analysis was used to evaluate the correlation between concentration and absorbance values of caffeine standard solutions.¹⁴

RESULT AND DISCUSSION

Determination of Maximum Wavelength and Calibration Curve

The determination of caffeine content in the ethanolic extract of robusta coffee beans (*Coffea canephora*) was carried out using UV-Visible spectrophotometry following method optimization and calibration procedures. Determination of the maximum wavelength (λ_{max}) is an important step in spectrophotometric analysis because it provides optimum sensitivity and analytical accuracy.¹³ The scanning results showed that caffeine exhibited maximum absorbance at 273 nm, which is consistent with previous studies reporting that caffeine absorbs strongly in the ultraviolet region at approximately 270–275 nm due to the presence of conjugated chromophore groups in its molecular structure.^{8,11,13}

The calibration curve was prepared using standard caffeine solutions at concentrations of 2, 4, 8, and 12 ppm. The absorbance values obtained for each concentration were 0.248, 0.362, 0.625, and 0.882, respectively. The increase in absorbance values was proportional to the increase in concentration, indicating compliance with Beer-Lambert's law within the tested concentration range.¹³ The linear regression equation obtained from the calibration curve was:

$$y = 0.064x + 0.111$$

with correlation coefficient:

$$R^2 = 0.992$$

The high correlation coefficient indicates good linearity and demonstrates that the analytical method possesses acceptable reliability for quantitative caffeine determination. Good linearity is an important parameter in analytical validation because it reflects the proportional relationship between analyte concentration and instrument response (8,14).

Table 1. Results of Caffeine Determination in Ethanolic Extract of Robusta Coffee Beans

Parameter	Value
Maximum wavelength (nm)	273
Standard concentration range (ppm)	2, 4, 8, 12
Absorbance of standards	0.248; 0.362; 0.625; 0.882
Sample concentration (ppm)	50
Sample absorbance	0.812
Calculated caffeine concentration (mg/L)	11.079
Caffeine content (mg/g extract)	221.58

Determination of caffeine Content in Ethanolic Extract

The ethanolic extract of robusta coffee beans was prepared at a concentration of 50 ppm and analyzed at the selected wavelength of 273 nm. The sample absorbance obtained was 0.812. Based on the regression equation from the calibration curve, the caffeine concentration in the sample solution was calculated to be 11.079 mg/L. Further calculation considering dilution factor and sample weight revealed that the caffeine content in the ethanolic extract reached 221.58 mg/g extract.

The relatively high caffeine content observed in this study is consistent with previous reports indicating that *Coffea canephora* generally contains higher caffeine concentrations than *Coffea arabica*.^{1,2} Robusta coffee is known to naturally synthesize higher levels of caffeine as part of its defense mechanism against pests and environmental stress.³ This characteristic contributes to the stronger bitterness and stimulant effects commonly associated with robusta coffee products.

Several factors may influence caffeine content in coffee extracts, including geographical origin, cultivation conditions, altitude, harvesting process, roasting conditions, extraction method, solvent polarity, and post-harvest treatment.^{1,3,8} Environmental factors such as soil composition, rainfall, and temperature may also affect alkaloid biosynthesis in coffee plants. Therefore, variations in caffeine content between studies are commonly reported in the literature.

The use of ethanol as extraction solvent in this study was considered appropriate because ethanol possesses semipolar properties that enable efficient extraction of alkaloid compounds such as caffeine. In addition, ethanol is relatively safe, environmentally friendly, easy to evaporate, and widely accepted in pharmaceutical and cosmetic preparations.^{9,10} The maceration method used in this study also allowed sufficient contact between solvent and plant material, thereby facilitating extraction of bioactive compounds from robusta coffee beans.⁹

From an analytical perspective, the findings demonstrate that UV-Visible spectrophotometry provides a practical and reliable approach for caffeine determination in coffee extracts. Compared with chromatographic techniques such as HPLC or gas chromatography, UV-Vis spectrophotometry offers advantages including simpler sample preparation, shorter analysis time, lower operational costs, and broader accessibility in routine laboratory settings.^{8,11,12} Although chromatographic methods provide higher specificity and sensitivity, UV-Vis spectrophotometry remains highly useful for preliminary quantitative analysis and quality control applications.

The quantified caffeine content obtained in this study also indicates the potential utilization of robusta coffee bean extract as a natural active ingredient in pharmaceutical and cosmetic formulations. Previous studies reported that caffeine can stimulate hair follicle proliferation, prolong the anagen phase, and inhibit androgen-induced follicular miniaturization, thereby supporting its application in hair growth-promoting formulations such as hair tonics, serums,

and emulgels.⁴⁻⁷ Therefore, the quantitative data generated in this study may serve as a scientific basis for dosage standardization and formulation development of caffeine-containing topical products derived from natural sources.

Overall, the results demonstrate that UV-Visible spectrophotometry is a suitable, reliable, and economical analytical method for the determination of caffeine content in ethanolic extracts of robusta coffee beans. The findings of this study contribute valuable scientific data supporting the standardization and utilization of *Coffea canephora* as a natural source of caffeine for pharmaceutical and cosmetic applications.

LIMITATION

Limitation This study was limited to the use of UV-Visible spectrophotometry, which may be less specific compared to chromatographic techniques. Future studies are recommended to employ more advanced analytical methods for validation and comparison.

CONCLUSION

This study successfully determined the caffeine content in ethanolic extract of robusta coffee beans (*Coffea canephora*) using UV-Visible spectrophotometry. Caffeine showed maximum absorbance at 273 nm with good linearity of the calibration curve. The caffeine content obtained was 221.58 mg/g extract, indicating that robusta coffee is a potential natural source of caffeine. The results also demonstrated that ethanolic extraction combined with UV-Visible spectrophotometry is a simple, reliable, and cost-effective method for caffeine determination. These findings provide scientific support for the standardization and further development of robusta coffee extract in pharmaceutical and cosmetic applications.

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AUTHOR CONTRIBUTION STATEMENT

DM: Conceptualization, Methodology, Investigation, Writing—Original Draft Preparation; **VP:** Supervision, Writing—Review and Editing.

CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest.

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Formulation and Physicochemical Characterization of Guava (*Psidium guajava* L.) Leaf Extract-Loaded Chitosan Nanoparticles

Formulasi dan Karakterisasi Fisikokimia Nanopartikel Kitosan yang Mengandung Ekstrak Daun Jambu Biji (*Psidium guajava* L.)

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Abstract

Guava (*Psidium guajava* L.) leaf extract is rich in secondary metabolites; however, its utilization as a therapeutic agent is frequently hindered by poor solubility and low bioavailability. The development of submicron-scale drug delivery systems, specifically chitosan nanoparticles, presents an approach to overcome these limitations. This study aimed to formulate and characterize chitosan nanoparticles loaded with guava leaf extract to fulfill the physicochemical stability parameters of a nanosuspension preparation. The extraction process was conducted using the maceration method with 96% ethanol. The nanoparticles were synthesized via the ionic gelation method using a 1% chitosan polymer and sodium tripolyphosphate (NaTPP) as the cross-linking agent. The characterization of the nanoparticle system included physical stability, particle size, polydispersity index (Pdl), zeta potential, and morphological observation using a Transmission Electron Microscope (TEM). The freeze-dried powder was subsequently formulated into a nanosuspension, and its physical quality was evaluated. The results indicated a corrected extract yield of 26.65%. The selected nanoparticle formula (F4) exhibited an average particle size of 923.6 nm, a Pdl of 0.606, and a zeta potential of +21.6 mV, demonstrating a spherical morphology but with a relatively broad particle size distribution. The evaluation of the nanosuspension over four weeks demonstrated a physically stable preparation with a pH range of 4.18–4.21, a specific gravity up to 1.0795 g/mL, and a 100% redispersion capability. In conclusion, the guava leaf extract was successfully prepared as a preliminary submicron particle formulation, demonstrating its potential to be further optimized and developed into a physicochemically stable nanosuspension.

Abstrak

Ekstrak daun jambu biji (*Psidium guajava* L.) kaya akan metabolit sekunder; namun, pemanfaatannya sebagai agen terapeutik seringkali terhambat oleh kelarutan yang buruk dan bioavailabilitas yang rendah. Pengembangan sistem penghantaran obat skala submikron, khususnya nanopartikel kitosan, menghadirkan pendekatan untuk mengatasi keterbatasan ini. Studi ini bertujuan untuk memformulasikan dan mengkarakterisasi nanopartikel kitosan yang dimuat dengan ekstrak daun jambu biji untuk memenuhi parameter stabilitas fisikokimia dari sediaan nanosuspensi. Proses ekstraksi dilakukan menggunakan metode maserasi dengan etanol 96%. Nanopartikel disintesis melalui metode gelasi ionik menggunakan polimer kitosan 1% dan natrium tripolifosfat (NaTPP) sebagai agen pengikat silang. Karakterisasi sistem nanopartikel meliputi stabilitas fisik, ukuran partikel, indeks polidispersitas (Pdl), potensial zeta, dan pengamatan morfologi menggunakan Mikroskop Elektron Transmisi (TEM). Bubuk yang dikeringkan beku kemudian diformulasikan menjadi nanosuspensi, dan kualitas fisiknya dievaluasi. Hasil penelitian menunjukkan perolehan ekstrak terkoreksi sebesar 26,65%. Formula nanopartikel yang dipilih (F4) menunjukkan ukuran partikel rata-rata 923,6 nm, Pdl 0,606, dan potensial zeta +21,6 mV, menunjukkan morfologi bulat tetapi dengan distribusi ukuran partikel yang relatif lebar. Evaluasi nanosuspensi selama empat minggu menunjukkan preparasi yang stabil secara fisik dengan rentang pH 4,18–4,21, berat jenis hingga 1,0795 g/mL, dan kemampuan redispersi 100%. Kesimpulannya, ekstrak daun jambu biji berhasil disiapkan sebagai formulasi partikel submikron awal, menunjukkan

potensinya untuk dioptimalkan dan dikembangkan lebih lanjut menjadi nanosuspensi yang stabil secara fisikokimia.

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INTRODUCTION

The development of traditional therapies is currently growing, particularly those based on plants, in line with the increasing "back to nature" trend in society¹. One tropical plant from the Myrtaceae family that shows potential for development as a therapeutic agent is guava (*Psidium guajava* L.)². Various empirical studies have demonstrated that guava leaves possess a broad spectrum of pharmacological properties, including antibacterial, antioxidant, antidiabetic, and anti-inflammatory activities^{3–5}. This pharmacological potential is closely related to its secondary metabolite content, such as steroids, triterpenoids, saponins, tannins, and specifically polyphenolic and flavonoid compounds^{6,7}.

Despite having demonstrated pharmacological efficacy, the utilization of natural extracts is frequently hindered by issues related to their fundamental biopharmaceutical properties. Specifically for guava leaf extract, its primary therapeutic constituents, such as complex tannins and specific flavonoids like quercetin exhibit low aqueous solubility, poor lipid membrane permeability, and susceptibility to presystemic degradation via environmental oxidation and gastric hydrolysis. Consequently, this specific phytochemical profile can lead to variable absorption and limited bioavailability when applied in biological systems using conventional dosage forms.

To overcome these limitations and optimize the therapeutic effects of guava leaf extract, the development of submicron-scale drug delivery systems ($< 1 \mu\text{m}$), such as nanoparticles, serves as a viable solution⁸. The use of nanocarrier technology offers various advantages, including the ability to enhance solubility, improve bioavailability, modify targeted delivery routes, and protect sensitive active substances from environmental and enzymatic degradation⁹.

One type of nanoparticle carrier polymer that is frequently utilized for drug delivery is chitosan. Chitosan is a natural carbohydrate-derived polymer produced through the partial deacetylation of chitin. Its utilization is favored due to its general biocompatibility, low toxicity, and capability to form stable electrostatic interactions between the positively charged amine groups of chitosan and the negative charges of polyanionic agents¹⁰. This interaction facilitates the formation of nanoparticle systems via the ionic gelation method, a relatively simple technique that avoids toxic organic solvents and is capable of protecting the encapsulated bioactive compounds.

While chitosan nanoparticles have been explored for delivering isolated, single-molecule active compounds, their application for encapsulating multi-component whole plant extracts presents a specific biophysical challenge. The diverse phytochemical matrix of crude extracts can disrupt polymer cross-linking, which may lead to particle aggregation. The novelty of this study lies in optimizing the ionic gelation parameters to encapsulate the multi-component matrix of guava (*Psidium guajava* L.) leaf extract, and translating this into a physically stable liquid nanosuspension—an approach that remains limited in current literature. Therefore, this study aimed to formulate guava leaf extract into a chitosan nanoparticle system using the ionic gelation method and to perform a physicochemical

characterization. The evaluated parameters included particle size, polydispersity index, zeta potential, particle morphology, and the physical stability of the nanosuspension to ensure that this delivery system meets formulation quality requirements.

METHODS

This research was an experimental laboratory study focusing on the formulation design and physicochemical evaluation of a nanotechnological preparation. The formulation design utilized a single-factor optimization approach focused on the cross-linking agent. Specifically, four nanoparticle formulas (F1–F4) were developed by varying the volume of the 0.4% sodium tripolyphosphate (NaTPP) solution (1, 2, 3, and 4 mL) while maintaining a constant concentration and volume of the chitosan polymer. The basis for selecting these specific NaTPP volume variations was to determine the optimal stoichiometric ratio between the positively charged amine groups of chitosan and the negatively charged phosphate groups of NaTPP. This optimization is necessary to achieve the correct degree of cross-linking required to form stable submicron particles and to prevent irreversible particle aggregation. Furthermore, to ensure the reliability and reproducibility of the data, all formulation procedures and subsequent physicochemical evaluations were conducted in triplicate. All stages of formulation design and preparation characterization were carried out at the Semi-Solid Laboratory, Faculty of Pharmacy, Universitas Pancasila, Jakarta, from August to December 2024.

Materials and Instruments

The primary instrument used in the extraction stage was a rotary evaporator to concentrate the solvent. For nanoparticle characterization, high-precision instruments utilized included a Malvern Particle Size Analyzer (PSA) to measure particle size, and a Transmission Electron Microscope (TEM) to observe particle morphology. Other supporting equipment consisted of an analytical balance, sieves, an oven, a blender, glassware (Pyrex), a magnetic stirrer, a digital pH meter, a refrigerator, a pycnometer, and a freeze dryer.

The main raw material used in this study was fresh guava (*Psidium guajava* L.) leaves collected from a plantation area in Bekasi, West Java. The solvent used for the extraction process was 96% ethanol. The materials for formulating the nanoparticle system and nanosuspension included chitosan polymer, sodium tripolyphosphate (NaTPP) as a cross-linking agent, 10% dimethyl sulfoxide (DMSO), propylene glycol, Pulvis Gummi Arabici (PGA), sodium carboxymethyl cellulose (Na-CMC), 70% sorbitol, peppermint oil, benzoic acid, glacial acetic acid, and phosphate buffer at pH 6.

Plant Determination and Preparation

The guava leaves were initially determined at the UPT Laboratorium Herbal Materia Medica Batu to verify the exact plant species. The fresh leaves were cleaned and dried to reduce moisture content and prevent the growth of microbes and fungi that could degrade the quality of the active compounds¹¹. The dried leaves (simplicia) were then ground and sieved using a 4/18 mesh sieve to obtain an optimal surface area, allowing for easy wetting by the solvent, in accordance with pharmacopoeial standards^{12,13}.

Extraction and Characterization

The The extraction of the dried powder was performed using the maceration method with 96% ethanol as the solvent. The extraction was carried out at room temperature ($\pm 25^\circ\text{C}$) with an initial simplicia-to-solvent ratio of 1:10. The initial maceration process was conducted for 5 days, with occasional daily stirring to facilitate the diffusion of active compounds. The process was followed by one remaceration cycle using a simplicia-to-solvent ratio of 1:5 to ensure maximum extraction. The resulting macerate was filtered, and the collected filtrate was then concentrated using a rotary evaporator at 50°C . Subsequently, it was further concentrated over a water bath until a thick extract was formed. Based on the extraction of 1,000 g of dried powder, 266.48 g of thick extract was produced. This corresponds to a calculated extract yield of 26.65% and a DER-native (Drug Extract Ratio) of 3.75. The quality of this thick extract was subsequently evaluated through organoleptic examination, yield determination, moisture content, total ash content, residual solvent, and phytochemical screening. These parameters were measured to ensure the extract met the standard quality parameters for natural materials ^{14–16}.

Preparation and Evaluation of Nanoparticles

The nanoparticle system was prepared using the ionic gelation method, which relies on the electrostatic interaction between the positively charged chitosan polymer and NaTPP acting as a polyanionic cross-linking agent ^{9,10}. The thick extract was dissolved in a mixed solvent (10% DMSO, propylene glycol, 96% ethanol, and distilled water). A 1% chitosan solution was added, followed by the dropwise addition of a 0.4% NaTPP solution under continuous magnetic stirring (200 rpm) until a stable, homogeneous turbid colloidal suspension was formed.

The evaluation of the nanoparticle preparation included determining the particle size, which ideally falls within the submicron range ¹⁷, and measuring the zeta potential to predict the electrostatic repulsive forces between particles that prevent aggregation ¹⁸. The nanoparticle morphology was confirmed using TEM. The characterized liquid nanoparticles were subsequently dried into a powder using the freeze-drying method ¹⁹.

Formulation and Evaluation of Nanosuspension

The nanoparticle powder was formulated into a liquid nanosuspension preparation. The powder was dissolved in propylene glycol and dispersed into a suspension base consisting of a combination of suspending agents, PGA (5%) and Na-CMC (1%), which had been previously swelled with hot water. Into this mixture, 70% sorbitol, benzoic acid, peppermint oil, and phosphate buffer (pH 6) were added. The use of phosphate buffer aimed to maintain the pH range of the preparation following the addition of the benzoic acid preservative ²⁰. The physical quality evaluation of the nanosuspension was conducted over a four-week storage period under controlled room temperature conditions ($\pm 25^\circ\text{C}$). The evaluated parameters included organoleptic examination, pH measurement, specific gravity measurement for aqueous preparations ²¹, viscosity analysis to monitor rheological stability, as well as sedimentation volume and redispersion tests.

Data Analysis

The data obtained from the extract characterization, nanoparticle evaluation, and nanosuspension physical quality tests were analyzed using descriptive qualitative and quantitative approaches. It must be noted that all quantitative measurements—including particle size, polydispersity index (Pdl), zeta potential, pH, and specific gravity—were conducted on the best formula as single observational measurements ($n = 1$) without technical replications. Due to this single-replication study design, the resulting data are strictly expressed as absolute single values rather than as a mean $\pm$ standard deviation (SD). All acquired data were presented in the form of tables, figures, or narratives to be

compared with established standard parameters and literature regarding the physicochemical quality of pharmaceutical preparations.

RESULT AND DISCUSSION

Plant Determination and Standardization

The initial phase of the study involved plant determination at the Herbal Materia Medica Batu Laboratory Unit, which confirmed that the specimen used was indeed guava (*Psidium guajava* L.) from the Myrtaceae family. Fresh guava leaves were dried for five days to reduce moisture content and prevent the growth of microbes and fungi, which could potentially degrade the active compounds ¹¹. The dried leaves were then ground and sieved using a 4/18 mesh sieve. This process ensured an optimal surface area for the powder, allowing for efficient solvent wetting during extraction, in accordance with the standards set by the Indonesian Materia Medica ^{12,13}.

Extraction and Characterization of Guava Leaf Extract

Table 1. Phytochemical Screening Results of Guava Leaf Extract

No	Compound	Result
1	Alkaloids	+
2	Flavonoids	+
3	Saponins	+
4	Tannins	+
5	Quinones	-
6	Triterpenoids	+
7	Steroids	+
8	Coumarins	-
9	Essential Oils	-
10	Phenolics	+
11	Glycosides	+

Table 1 presents the phytochemical screening results of the guava leaf extract. The extraction was performed using the maceration method with 96% ethanol. Extraction from 1 kg of dried powder yielded 266.48 g of thick extract, representing a yield of 22.36% and a DER-native value of 4.85. Phytochemical screening identified the presence of secondary metabolites, including alkaloids, flavonoids, saponins, tannins, triterpenoids, steroids, phenolics, and glycosides. These findings align with previous studies indicating that guava leaves are rich in polyphenols and flavonoids, which serve as potential anti-inflammatory agents ⁵.

Quality characterization of the extract demonstrated a corrected yield of 26.65% and a corresponding DER-native of 3.75. Furthermore, the evaluation showed a moisture content of 7.65% (meeting the <10% requirement), a total ash content of 4.23%, and a residual ethanol content of 0.67%. Organoleptically, the extract appeared as a thick, dark greenish-brown mass with a characteristic odor and an astringent taste. These combined results confirm that the preparation fulfills the established standard quality parameters for thick guava leaf extract ^{14–16}.

Formulation and Characterization of Nanoparticles

Table 2. Nanoparticle Formulation

Ingredients	F1	F2	F3	F4
Guava Leaf Extract	300 mg	300 mg	300 mg	300 mg

Dimethyl Sulfoxide (DMSO) 10%	20 mL	20 mL	20 mL	20 mL
Propylene Glycol	20 mL	20 mL	20 mL	20 mL
Ethanol 96%	20 mL	20 mL	20 mL	20 mL
Chitosan 1%	20 mL	20 mL	20 mL	20 mL
Sodium Tripolyphosphate (NaTPP) 0.4%	1 mL	2 mL	3 mL	4 mL
Distilled Water	20 mL	20 mL	20 mL	20 mL

Table 2 outlines the nanoparticle formulations. The nanoparticles were synthesized using the ionic gelation method, which relies on the electrostatic interaction between positively charged chitosan and polyanionic sodium tripolyphosphate (NaTPP) acting as a cross-linker⁹. As an initial screening tool, a preliminary 5-day physical stability evaluation was conducted by observing changes in color, turbidity, and macroscopic sediment formation. Among the tested variations, Formula 4 (F4) demonstrated the most stable visual characteristics with no observable macroscopic degradation. Due to resource constraints in characterization, F1, F2, and F3 were excluded from advanced testing, and F4 was subsequently selected as the primary representative candidate for further comprehensive instrumental characterization.

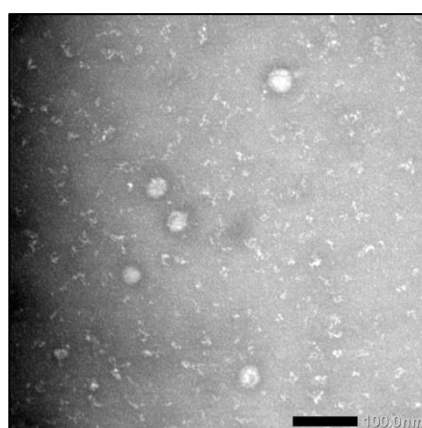


Figure 1. Morphology of Guava Leaf Extract Nanoparticles under Transmission Electron Microscope (TEM)

Figure 1 illustrates the morphology of the guava leaf extract nanoparticles. Particle size characterization using a Particle Size Analyzer revealed a mean particle size of 923.6 nm for formulation F4. While this value falls within the acceptable submicron limit for nanoparticle systems (<1000 nm), it is situated at the upper boundary of this classification⁶. Furthermore, the formulation exhibited a Polydispersity Index (Pdl) of 0.606, which indicates a broad particle size distribution and a polydisperse system, rather than a strictly homogeneous dispersion^{17,22}. Zeta potential analysis showed a value of +21.6 mV. This indicates a moderate positive surface charge that provides partial electrostatic repulsion; however, this moderate charge alone may not be strong enough to guarantee long-term colloidal stability against aggregation without the presence of additional steric stabilizers^{18,23}. TEM observations confirmed that the particles were generally spherical. The liquid nanoparticle preparation was subsequently dried into a powder using the freeze-drying technique¹⁹.

Physical Quality Evaluation of Nanosuspension

Table 3. Physical Stability Evaluation of the Nanosuspension Over Four Weeks

Evaluation Parameter	Week 1	Week 2	Week 3	Week 4	Observation (Over 4 Weeks)	Remarks / Reference
Organoleptic	Yellowish-brown color, minty aromatic odor	Yellowish-brown color, minty aromatic odor	Yellowish-brown color, minty aromatic odor	Yellowish-brown color, minty aromatic odor	Yellowish-brown color, minty aromatic odor	Physically stable
pH	4.21	4.20	4.20	4.18	4.18 – 4.21	Maintained by phosphate buffer
Specific gravity (g/mL)	1.0754	1.0765	1.0768	1.0795	> 1.00 g/mL	Meets aqueous vehicle criteria
Viscosity (cP)	96	91	86	82	82 – 96	Structural relaxation of polymers
Sedimentation ratio	0.05	0.05	0.10	0.10	100%	Particles settle slowly/stable
Redispersion (%)	100	100	100	100	100%	Particles redisperse perfectly upon shaking

Table 3 summarizes the physical evaluation results of the nanosuspension. The dried nanoparticle powder was formulated into a liquid nanosuspension using suspending agents consisting of a combination of Pulvis Gummi Arabici (PGA) and Sodium Carboxymethyl Cellulose (Na-CMC) to maintain dispersion stability. Physical stability testing over a 4-week period indicated that the nanosuspension exhibited a very good and stable profile overall.

Organoleptically, the preparation underwent no significant changes, maintaining the characteristic yellowish-brown color of the guava leaf extract and a minty aromatic odor from the addition of peppermint oil. The pH remained constant between 4.18 and 4.21 during storage. This acidity is primarily influenced by the benzoic acid preservative; however, the stability was successfully maintained by the phosphate buffer in the formula, preventing pH fluctuations that could degrade the active ingredients ²⁰.

The specific gravity was consistently measured above 1.00 g/mL, reaching 1.0795 g/mL by the fourth week. These results indicate that the formulation meets the criteria for specific gravity in aqueous-based suspensions ²¹. Values greater than 1.00 g/mL also indicate that the particles were successfully suspended by the added agents.

Critical parameters for suspensions include the rate of sedimentation and ease of redispersion. The sedimentation volume and redispersion tests yielded optimal results of 100%. This proves that the particles did not form

"caking" (hard sediment) but rather formed loose floccules that could be perfectly redispersed into a homogeneous suspension with light shaking.

LIMITATION

Despite the formulation and physicochemical characterization of the guava (*Psidium guajava* L.) leaf extract chitosan nanoparticles, this study acknowledges several important limitations. First, the botanical extract and the resulting nanoparticles were not chemically standardized based on specific active marker compounds (such as quercetin or specific tannins). Consequently, critical formulation parameters, specifically the entrapment efficiency and drug loading capacity, were not quantitatively determined. Second, while the formulated particles fell within the submicron range, the particle size distribution remained relatively broad, indicating a polydisperse system that requires further optimization to achieve strict homogeneity. Third, the physical stability evaluation of the nanosuspension was strictly conducted over a short-term period of four weeks at room temperature. The absence of accelerated stability testing under varying extreme temperature and humidity conditions means that the long-term thermodynamic stability of the colloidal system is not fully established. Fourth, the methodological scope of this study was confined to the physical and morphological parameters of the delivery system. The *in vitro* release profile, which is crucial for understanding the release kinetics of the entrapped bioactive compounds from the chitosan polymeric matrix, was not investigated. Furthermore, as the primary objective centered on pharmaceutical formulation, the study lacks *in vivo* biological assessments. Thus, the actual pharmacological efficacy, cellular uptake, and potential toxicity profiles of the formulated nanosuspension remain unverified. To address these constraints, future research should focus on chemical standardization to quantify encapsulation efficiency, refine the formulation to narrow the size distribution, and incorporate comprehensive accelerated stability studies. Additionally, *in vitro* drug release kinetics and *in vivo* pharmacological and toxicological evaluations are highly recommended to ascertain the biological efficacy, safety, and translational potential of the developed nanosuspension.

CONCLUSION

Based on the research findings and physical evaluations conducted, it can be concluded that guava (*Psidium guajava* L.) leaf extract was successfully prepared as a preliminary submicron particle formulation utilizing the ionic gelation method. The selected formula (F4) exhibited physical characteristics approaching the upper limits of a submicron colloidal system, with a mean particle size of 923.6 nm, a polydispersity index of 0.606, and a moderate zeta potential of +21.6 mV. While the particles possessed a generally spherical morphology, the broad size distribution indicates that the formulation requires further optimization to be considered a strictly homogeneous and mature nanoparticle system.

Furthermore, the development of these submicron particles into a liquid nanosuspension produced a formulation that maintained physical integrity over a short-term 4-week storage period at room temperature. The preparation fulfilled basic physical quality parameters, including organoleptic consistency, a stable acidic pH (4.18–4.21), suitable specific gravity (>1.00 g/mL), alongside adequate sedimentation volume and complete redispersion (100%) without caking. Overall, this study demonstrates the physical feasibility of using chitosan as a structural carrier for guava leaf extract. However, conclusions regarding its full effectiveness as a pharmaceutical delivery system remain

limited, as critical parameters—such as entrapment efficiency, active compound release profiles, in vivo bioactivity, and long-term toxicity were not evaluated and must be addressed in future studies.

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AUTHOR CONTRIBUTION STATEMENT

WP: Investigation, Data curation, Writing - Original draft preparation; **DR:** Conceptualization, Methodology, Supervision, Writing - Review & Editing; **YF:** Conceptualization, Validation, Supervision, Writing - Review & Editing.

CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest regarding the research, authorship, and/or publication of this article.

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Comparison of Ethyl Acetate and Ethanol Solvents on Total Flavonoid Content from (*Gnetum gnemon L.*) Using UV-Vis Spectrophotometry

Perbandingan Pelarut Etil Asetat dan Etanol terhadap Kandungan Flavonoid Total dari (*Gnetum gnemon L.*) Menggunakan Spektrofotometri UV-Vis

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Abstract

Melinjo peel (*Gnetum gnemon L.*) is an agricultural by-product that is generally underutilized, despite its high content of flavonoid compounds with potential antioxidant activity. This study aimed to compare the effectiveness of ethanol and ethyl acetate as extraction solvents for flavonoids from melinjo peel (*Gnetum gnemon* pericarp) based on differences in solvent polarity. Extraction was carried out using the maceration method, followed by quantitative analysis using UV-Vis spectrophotometry. Sample preparation involved drying and pulverizing the melinjo peel, extraction with ethanol and ethyl acetate at a material-to-solvent ratio of 1:10, phytochemical screening, thin-layer chromatography analysis, preparation of a quercetin calibration curve, and determination of total flavonoid content. Total flavonoid content was determined through the formation of a complex with aluminum chloride, and absorbance was measured at a wavelength of approximately 430 nm. Statistical analysis was conducted to evaluate differences between the two solvents. The results demonstrated that the ethanol extract produced a higher total flavonoid content (51.86 mg quercetin equivalent per gram of extract) compared to the ethyl acetate extract (46.05 mg quercetin equivalent per gram). The higher extraction efficiency of ethanol is attributed to its greater polarity, which enhances the solubilization of polar flavonoid compounds. In conclusion, ethanol is a more effective solvent than ethyl acetate for extracting flavonoids from melinjo peel, and these findings support the potential utilization of melinjo peel as a natural source of bioactive compounds for applications in the food, pharmaceutical, and cosmetic industries.

Abstrak

Kulit melon (*Gnetum gnemon L.*) merupakan produk sampingan pertanian yang umumnya kurang dimanfaatkan, meskipun memiliki kandungan senyawa flavonoid yang tinggi dengan potensi aktivitas antioksidan. Studi ini bertujuan untuk membandingkan efektivitas etanol dan etil asetat sebagai pelarut ekstraksi flavonoid dari kulit melon (*perikarp Gnetum gnemon*) berdasarkan perbedaan polaritas pelarut. Ekstraksi dilakukan menggunakan metode maserasi, diikuti dengan analisis kuantitatif menggunakan spektrofotometri UV-Vis. Preparasi sampel meliputi pengeringan dan penghancuran kulit melon, ekstraksi dengan etanol dan etil asetat dengan rasio bahan terhadap pelarut 1:10, skrining fitokimia, analisis kromatografi lapis tipis, pembuatan kurva kalibrasi kuersetin, dan penentuan kandungan flavonoid total. Kandungan flavonoid total ditentukan melalui pembentukan kompleks dengan aluminium klorida, dan absorbansi diukur pada panjang gelombang sekitar 430 nm. Analisis statistik dilakukan untuk mengevaluasi perbedaan antara kedua pelarut tersebut. Hasil penelitian menunjukkan bahwa ekstrak etanol menghasilkan kandungan flavonoid total yang lebih tinggi (51,86 mg setara kuersetin per gram ekstrak) dibandingkan dengan ekstrak etil asetat (46,05 mg setara kuersetin per gram). Efisiensi ekstraksi etanol yang lebih tinggi disebabkan oleh polaritasnya yang lebih besar, yang meningkatkan pelarutan senyawa flavonoid polar. Kesimpulannya, etanol merupakan pelarut yang lebih efektif daripada etil asetat untuk mengekstrak flavonoid dari kulit melinjo, dan temuan ini mendukung potensi pemanfaatan kulit melinjo sebagai sumber alami senyawa bioaktif untuk aplikasi di industri makanan, farmasi, dan kosmetik.

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INTRODUCTION

Gnetum gnemon L., commonly known as melinjo, belongs to the family Gnetaceae and thrives in tropical regions, particularly in Indonesia¹. While its seeds are widely used in traditional products like crackers (emping), the pericarp, typically discarded as agricultural waste harbors bioactive compounds with antioxidant and antibacterial potential, notably flavonoids. These polyphenols exhibit robust biological activities, including radical scavenging to mitigate oxidative stress, a key precursor to degenerative diseases^{2,3}. Flavonoids neutralize free radicals, activate endogenous antioxidant enzymes, and prevent cellular damage. They also exert anti-inflammatory effects by inhibiting pro-inflammatory signaling pathways, such as NF- κ B, while reducing cytokines (e.g., TNF- α , IL-6), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS)⁴. Adequate dietary intake correlates with reduced risks of cardiovascular disease, type 2 diabetes, dementia, and cancer angiogenesis, underscoring their relevance in public health⁵.

The pursuit of natural antioxidants for food, cosmetics, and pharmaceuticals necessitates efficient extraction methods from local sources like melinjo pericarp. Solvent selection is paramount, governed by the "like dissolves like" principle, where polarity dictates solubility^{3,5}. Flavonoids span polar to semi-polar structures, making solvent polarity a critical determinant of extraction yield. Ethanol, a polar protic solvent, effectively dissolves polar phenolics and is favored for its safety, availability, and food-grade status, especially in aqueous mixtures (30-70% v/v). Ethyl acetate, semi-polar, excels in fractionating intermediate-polarity flavonoids, yielding purer fractions despite potentially lower crude extracts. Comparing these solvents elucidates polarity's impact on total flavonoid extraction efficiency from melinjo pericarp platform and strictly^{6,7}.

This study compares ethanol and ethyl acetate for maceration-based extraction of total flavonoids from *G. gnemon* L. pericarp, quantified via UV-Vis spectrophotometry at 430 nm post- AlCl_3 complexation⁸. Maceration, a simple, cost-effective room-temperature soaking technique, ensures equilibrium extraction despite longer durations. Melinjo, a gymnosperm originating from Indo-Malaya and Melanesia, reaches 25-30 m in height with a lifespan exceeding a century. National production exceeded 2.4 million quintals in 2024^{7,9}. Its oval leaves, monopodial stems (10-20 cm diameter), and multilayered seeds support versatile uses: leaves and seeds as vegetables, bark for ropes and composites, wood for paper, and traditional remedies for vision issues or bites¹⁰.

Phytochemical screening confirms flavonoids alongside stilbenoids (e.g., resveratrol, gnetin), tannins, saponins, alkaloids, and polyphenols, conferring antioxidant and cytotoxic properties. Thin-layer chromatography (TLC) separates compounds based on adsorption and solubility, using silica gel stationary phases and solvent mixtures, enhanced by high-performance variants (HPTLC) or densitometry for quantification^{11,12}.

UV-Vis spectrophotometry provides accurate, sensitive quantification of bioactive concentrations via linear regression (high R^2), though selectivity limitations necessitate derivatization or prior fractionation. Validation per international guidelines ensures reliability¹³. Preliminary assays, Shinoda for flavonoids (pink/orange with Mg/HCl), Dragendorff/Mayer/Wagner for alkaloids (orange/red precipitates), foam test for saponins, and FeCl_3 for tannins, verify metabolite presence¹⁴. By valorizing melinjo pericarp waste, this research advances sustainable sourcing of natural actives, informing pharmaceutical and food industries amid rising demand for plant-based health products.

However, studies comparing the effectiveness of ethanol and ethyl acetate solvents in extracting total flavonoids from melinjo pericarp (*Gnetum gnemon* L.) are still limited. Solvent selection greatly affects the flavonoid

content obtained and its potential as a natural antioxidant source. In addition, melinjo peel is often considered agricultural waste despite containing bioactive compounds with potential applications in pharmaceutical, food, and cosmetic fields. Therefore, this study aims to compare the effect of ethanol and ethyl acetate solvents on the total flavonoid content of melinjo pericarp extract using UV-Vis spectrophotometry to determine the more effective solvent. The results of this study are expected to provide scientific information regarding optimal flavonoid extraction and support the utilization of melinjo peel as a valuable natural resource.

METHODS

This study employed a pre-experimental design. A single dependent variable was selected, though other confounding variables may influence it. The research utilized a Randomized Block Design (RBD), a straightforward approach to assess the impact of independent variables (solvents: ethanol vs. ethyl acetate) on the dependent variable (total flavonoid content from *Gnetum gnemon* L. pericarp), as measured by UV-Vis spectrophotometry.

Materials

Materials. *Gnetum gnemon* L. pericarp, ethyl acetate (EtOAc), 96% ethanol, quercetin standard.

Research Procedure

Sample Preparation

Gnetum gnemon L. pericarp was thoroughly washed, oven dried at 40°C to low moisture content, and pulverized into fine powder using a blender. The powder was stored in airtight containers prior to extraction.

Extraction Process

Fifty grams of pericarp powder were macerated separately with 500 mL ethyl acetate and 96% ethanol (10:1 v/w ratio) for 2 × 24 h with intermittent agitation. Extracts were filtered and concentrated to viscous residue using a water bath at 50°C.

Phytochemical Screening

Qualitative analysis identified secondary metabolites (alkaloids, flavonoids, saponins, tannins) in *G. gnemon* L. pericarp extracts. Alkaloids were tested with Dragendorff, Mayer, and Wagner reagents (positive: orange brown precipitates). Flavonoids via Shinoda test (Mg filings + conc. HCl; positive: red-orange color)¹⁵. Saponins by foam test (stable foam ≥1 cm in distilled water). Tannins with 1% FeCl₃ (positive: dark green-black or deep blue color)¹⁶.

Thin Layer Chromatography (TLC)

TLC analysis detected flavonoids in extracts on silica gel GF₂₅₄ plates (1 cm spot separation). Mobile phase: n-butanol: acetic acid:water (4:1:5 v/v/v). Plates were developed, air-dried, and visualized under UV light (254/366 nm) for flavonoid fluorescence, followed by 5% AlCl₃ spray (yellow/olive-green spots confirmatory). R_f values calculated relative to the solvent front and compared with the quercetin standard¹⁷.

Total Flavonoid Content Assay

One mL extract was mixed with 1 mL 10% AlCl₃ and 1 mL 1 M sodium acetate, diluted to 10 mL with distilled water, and incubated for 30 min. Absorbance measured at $\lambda_{\text{max}} \approx 430$ nm using Uv-Vis spectrophotometer against a quercetin standard curve¹⁸.

Quercetin Standard Curve Preparation

Quercetin stock solution (10–50 ppm) was prepared, and absorbance was measured at λ_{max} . Linear regression ($y = bx + a$, $r^2 > 0.99$) plotted concentration vs. absorbance for total flavonoid quantification (mg QE/g extract)¹¹.

Data Analysis

UV-Vis spectrophotometric absorbance data were processed using the linear regression equation derived from the quercetin standard curve. Total flavonoid content from each solvent was calculated as mg quercetin equivalents per gram extract (mg QE/g). One-way ANOVA ($\alpha = 0.05$) was subsequently applied to determine significant differences between solvents on total flavonoid yield.

RESULT AND DISCUSSION

Extract Preparation

Extraction of *Gnetum gnemon* L. pericarp was performed using maceration with ethanol and ethyl acetate solvents. This method was selected for its simplicity and avoidance of heating, minimizing degradation of heat-sensitive secondary metabolites, particularly flavonoids, while facilitating optimal diffusion from plant matrix to the solvent.

Table 1. Extract Yield from *G. gnemon* L. Pericarp

Crude material weight	Extract weight	Yield (%)
560 g	85 g	15,17%

Ethanol extraction yielded higher extractive value (15.17%) compared to ethyl acetate. Ethanol's polar to semi polar nature effectively solubilizes polar secondary metabolites (flavonoids, tannins, saponins), whereas semi polar ethyl acetate preferentially extracts intermediate polarity compounds, resulting in lower overall yield. Ethanol extract yield data from 560 g crude material (85 g extract) is presented in Table 1.

Phytochemical Screening

Qualitative phytochemical analysis of ethanol extract confirmed flavonoids, alkaloids, saponins, and tannins. Flavonoid test (Shinoda) produced orange-red coloration via flavilium ion formation from carbonyl reduction. Alkaloids yielded characteristic precipitates with Mayer, Dragendorff, and Wagner reagents. Saponins formed stable foam (>1 cm) in distilled water tannins produced dark green-black color with FeCl_3 due to phenolic-iron complexation¹⁹. Complete screening results are shown in Table 2.

Table 2. Phytochemical Screening Results

Compound	Reagent	Test Result	Conclusion
Alkaloid	Dragendorff	Orange precipitate	+
	Mayer	White precipitate	+
	Wagner	Brown precipitate	+
Flavonoid	Mg + conc. HCl (Shinoda)	Reddish-brown color	+
Saponin	Vigorous shaking in water	Stable foam (>1 cm, HCl-labile)	+
Tannin	FeCl_3 1%	Dark green-black color	+

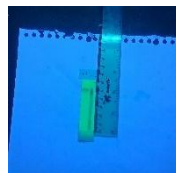
Thin Layer Chromatography (TLC)

TLC analysis revealed spots migrating 5.3 cm while the eluent front advanced 6.0 cm, yielding an R_f value of 0.88. This relatively high R_f indicates that flavonoids in the *Gnetum gnemon* L. pericarp extract exhibit lower polarity

relative to the stationary phase (silica gel), enabling greater migration with the mobile phase^{18,20}. This observation aligns with characteristics of flavonoid aglycones, which are typically less polar than their glycosylated counterparts²¹.

The quercetin marker exhibited a migration distance of 5.0 cm (Rf 0.83). The close similarity in Rf values between sample spots and quercetin marker supports the identification of flavonoids in the extract. Visual documentation under both visible light and ultraviolet irradiation (254/366 nm) further confirmed characteristic flavonoid spots, which typically exhibit distinctive fluorescence at specific wavelengths. Results are summarized in Table 3.

Table 3. Thin Layer Chromatography (TLC) Analysis

Compound	Spot Distance (cm)	Eluent Front (cm)	Rf Value	Marker Distance (cm)	Documentation
Flavonoid	5.3	6	0,88	5	 

The TLC results showed Rf values of 0.88 for the sample and 0.83 for quercetin, indicating the presence of flavonoid compounds, likely quercetin or related flavonols, in *Gnetum gnemon* L. pericarp extract. These findings are consistent with previous studies reporting similar Rf values using the same mobile phase system. Phytochemical screening also confirmed the presence of flavonoids, alkaloids, saponins, and tannins, in agreement with earlier research on melinjo peel extracts. In addition, the positive Shinoda test supported the presence of flavonoids through the characteristic red-orange coloration formed during the reaction.

Total Flavonoid Content Analysis

Total Flavonoid Content Analysis of Ethanol Extract from *G. gnemon* L. Pericarp

Total flavonoid content in the ethanol and ethyl acetate extract of *Gnetum gnemon* L. pericarp was determined using UV-Vis spectrophotometry based on flavonoid- AlCl_3 complex formation²³. This method is widely adopted for flavonoid analysis due to its high sensitivity and reproducibility. Absorbance measurements were recorded at the maximum wavelength of approximately 430 nm^{22,23}.

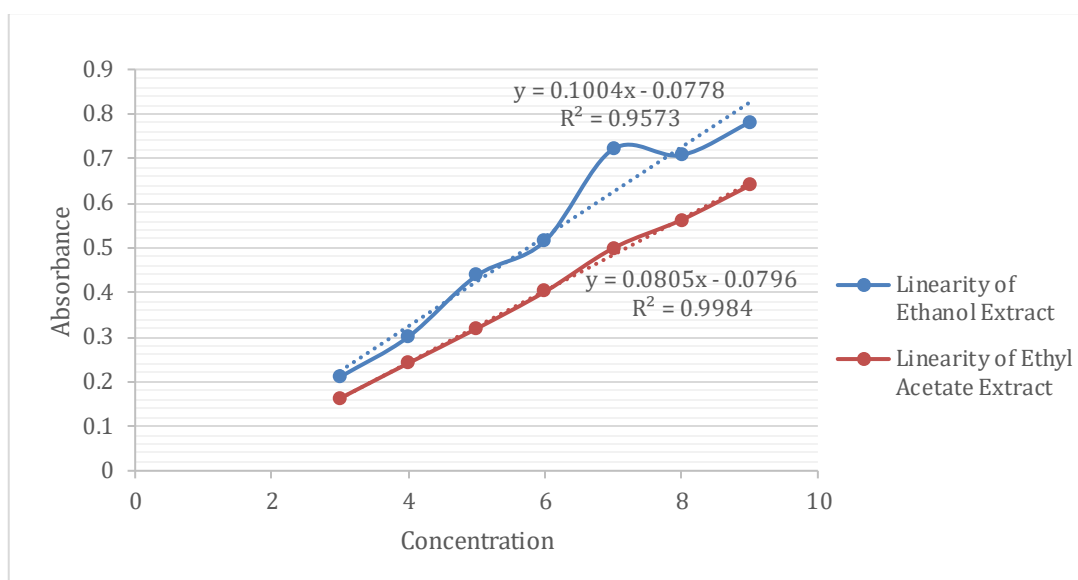


Figure 1. Quercetin Standard Calibration Curve

The calibration curve for the ethanol extract is depicted in Figure 1, with a linear regression equation of $y=0.1004x-0.0778$. and the calibration curve for the ethyl acetate extract is shown in Figure 1, with a linear regression equation of $y=0.0805x-0.0796$. The coefficient of determination (r^2) approaching one confirms excellent linearity, validating the curves for flavonoid quantification in the samples.

Table 4. Comparison of Total Flavonoid Content of *Gnetum gnemon* L. Pericarp Extracts Using Ethanol and Ethyl Acetate Solvents

Solvent	Replication 1	Replication 2	Replication 3	Mean Absorbance	Total Flavonoid Content (mg QE/g)
Ethanol	5,1725	5,0558	5,1622	5,1302	51,86
Ethyl Acetate	3,9124	3,7651	3,8626	3,8647	46,05

Calculation of total flavonoid content of ethanol extract of melinjo skin

Replication 1= 5,1725, Replication 2= 5,0558, Replication 3= 5,1622

$$\text{Mean Absorbance:} = \frac{5,1725 + 5,0558 + 5,1622}{3} = 5,1302$$

$$\begin{aligned} \text{Quercetin Equivalent Concentration:} &= \frac{y+0,778}{0,1004} \\ &= \frac{5,1302 + 0,778}{0,1004} = \frac{5,208}{0,1004} = 51,86 \mu\text{g/ml} \end{aligned}$$

$$\begin{aligned} \text{Concentration Conversion} &= \frac{c \times v}{m} \\ &= \frac{51,86 \times 50}{50} \\ &= 51,86 \text{ mgQE/g} \end{aligned}$$

Calculation of total flavonoid content of ethyl acetate extract of melinjo skin

Replication 1 = 3,9124, replication 2 = 3,7651, replication 3 = 3,8626

$$\text{Mean Absorbance} = \frac{3,9124 + 3,7651 + 3,8626}{3} = 3,8647$$

$$\begin{aligned}
 \text{Quercetin Equivalent Concentration} &= \frac{y+0,778}{0,1004} \\
 &= \frac{5,1302+0,778}{0,1004} = \frac{3,8467+0,778}{0,1004} = 4,6247/0,1004 \\
 &= 46,05 \mu\text{g/mL}
 \end{aligned}$$

$$\begin{aligned}
 \text{Concentration Conversion} &= \frac{c \times v}{m} \\
 &= \frac{46,05 \times 50}{50} \\
 &= 46,05 \text{ mgQE/g}
 \end{aligned}$$

Based on Table 4, the ethanol extract yielded a significantly higher total flavonoid content (51.86 mg QE/g) compared to the ethyl acetate extract (46.05 mg QE/g), representing a difference of 5.81 mg QE/g (approximately 10.8%). This outcome aligns with the fundamental principle of “like dissolves like,” whereby a polar solvent such as ethanol preferentially dissolves polar compounds, including the majority of flavonoid glycosides and hydroxylated aglycones present in plant matrices. These findings are consistent with a study by Nawaz et al, on *Phaseolus vulgaris* seeds, which demonstrated that ethanol yielded significantly greater total phenolic and flavonoid content than less polar solvents, attributed to ethanol’s capacity to disrupt hydrogen bonds and penetrate the plant cell matrix more effectively⁵. Similarly, Palaiogiannis et al reported that successive solvent extraction of *Cistus creticus* leaves yielded higher polyphenol fractions with more polar solvents, corroborating polarity-dependent extraction efficiency¹³.

The lower flavonoid yield from ethyl acetate (46.05 mg QE/g) reflects its semi-polar nature, which selectively extracts intermediate-polarity flavonoids such as flavonoid aglycones rather than the more abundant polar glycosylated forms. This is consistent with the findings of Parhusip et al, who reported that ethyl acetate extracts of red melinjo (*Gnetum gnemon* L.) peel exhibited distinct phytochemical profiles dominated by semi-polar constituents, including stilbenoid compounds such as resveratrol⁷. Kozhantayeva et al similarly observed that ethyl acetate fractions of *Chamaenerion latifolium* produced lower total flavonoid content compared to ethanol fractions, though with greater selectivity for specific aglycone subclasses¹⁰. These comparative findings suggest that while ethyl acetate may be suitable for targeted isolation of specific flavonoid classes, ethanol remains superior for total flavonoid recovery.

The total flavonoid content obtained in this study (51.86 mg QE/g for ethanol extract) is comparable to values reported in the literature for other plant pericarps and natural sources. Aminah et al reported total flavonoid content of avocado peel ethanol extract ranging from 10.29 to 27.32 mg QE/g, suggesting that *G. gnemon* L. pericarp is a considerably richer source of flavonoid²¹. Furthermore, Hayat et al determined total flavonoid content in *Marrubium vulgare* at 34.5 mg QE/g, again lower than the values found in this study, reinforcing the potential of melinjo pericarp as a high-yield natural flavonoid source²⁴. The semi-polar fraction retains significant bioactivity⁷. Together, these comparisons position melinjo pericarp as a promising and competitive raw material for natural bioactive compound development, with ethanol extraction as the preferred method for maximum flavonoid recovery.

The reliability of the UV-Vis spectrophotometric method employed in this study is substantiated by the high linearity of the quercetin calibration curves (r^2 approaching 1.0) for both ethanol and ethyl acetate extracts. Shraim et al conducted a critical evaluation of the AlCl₃ colorimetric assay and affirmed its wide applicability for total flavonoid determination in plant extracts, while also noting the importance of appropriate standard selection and interference control³. The use of quercetin as a reference standard in this study is well-established in the literature; Ramadhani et

al and Haresmita and Pradani both validated quercetin-based calibration curves for flavonoid quantification in natural products, demonstrating good reproducibility and accuracy^{22,23}. The consistent absorbance values across three replicates in this study further validate the precision of the analytical approach. These methodological strengths support the credibility and reproducibility of the findings reported here.

CONCLUSION

This study demonstrated that ethanol is a more effective solvent than ethyl acetate for extracting total flavonoids from melinjo peel (*Gnetum gnemon* L.) using the maceration method, yielding 51.86 mg QE/g compared to 46.05 mg QE/g, a difference attributable to ethanol's superior polarity and capacity to solubilize polar flavonoid glycosides. Phytochemical screening confirmed the presence of flavonoids, alkaloids, saponins, and tannins, while TLC analysis corroborated flavonoid identity through close R_f correspondence with quercetin. UV-Vis spectrophotometry with AlCl₃ complexation proved to be a reliable and reproducible quantification method, supported by high-linearity calibration curves. These findings establish melinjo pericarp as a high-yield natural source of bioactive flavonoids with potential applications in the food, pharmaceutical, and cosmetic industries.

Future research is recommended to explore fractionation of the ethanol extract to isolate and characterize individual flavonoid compounds, as well as to evaluate their specific biological activities such as antioxidant, anti-inflammatory, and antimicrobial potency. Studies employing advanced extraction techniques such as ultrasound-assisted extraction, deep eutectic solvents, or supercritical fluid extraction may further optimize flavonoid recovery. Additionally, in vivo bioavailability studies and toxicological assessments are warranted prior to the development of melinjo peel-based formulations for commercial application.

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Molecular Docking Investigation of *Caffeic Acid Phenethyl Ester* (CAPE) Interaction with IL-6 and TNF- α for Anti-Inflammatory Potential

Investigasi Docking Molekuler Interaksi *Caffeic Acid Phenethyl Ester* (CAPE) dengan IL-6 dan TNF- α untuk Potensi Anti-inflamasi

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Abstract

Inflammation is a physiological response to tissue injury mediated by pro-inflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- α). Although conventional anti-inflammatory drugs are effective, their long-term use is associated with adverse effects, prompting interest in safer natural alternatives. *Caffeic acid phenethyl ester* (CAPE), a major bioactive constituent of propolis, has been reported to exhibit anti-inflammatory properties. This study aimed to investigate the interaction of CAPE with IL-6 and TNF- α using molecular docking to evaluate binding affinity and molecular interactions. Docking simulations were performed to predict the binding behavior of CAPE and compare it with diclofenac sodium as a reference compound. CAPE exhibited binding affinities of -5.1 kcal/mol against TNF- α and -5.0 kcal/mol against IL-6, whereas diclofenac sodium showed slightly stronger binding affinities of -5.5 kcal/mol and -5.2 kcal/mol, respectively. In addition, CAPE shared several interacting residues with the reference compound, suggesting a similar binding orientation within the active sites of both proteins. These findings indicate that CAPE possesses favorable binding characteristics toward TNF- α and IL-6. However, the obtained binding affinities represent preliminary predictions of ligand-protein interactions and require further validation through molecular dynamics simulations and experimental studies. This study provides molecular insights into the anti-inflammatory potential of propolis-derived compounds and supports their further exploration as natural lead compounds for anti-inflammatory drug development.

Abstrak

Peradangan adalah respons fisiologis terhadap cedera jaringan yang dimediasi oleh sitokin pro-inflamasi, termasuk interleukin-6 (IL-6) dan faktor nekrosis tumor-alfa (TNF- α). Meskipun obat anti-inflamasi konvensional efektif, penggunaan jangka panjangnya dikaitkan dengan efek samping, sehingga mendorong minat pada alternatif alami yang lebih aman. *Caffeic acid phenethyl ester* (CAPE) konstituen bioaktif utama propolis, telah dilaporkan menunjukkan sifat anti-inflamasi. Studi ini bertujuan untuk menyelidiki interaksi CAPE dengan IL-6 dan TNF- α menggunakan penambatan molekuler untuk mengevaluasi afinitas pengikatan dan interaksi molekuler. Simulasi penambatan dilakukan untuk memprediksi perilaku pengikatan CAPE dan membandingkannya dengan natrium diklofenak sebagai senyawa referensi. CAPE menunjukkan afinitas pengikatan sebesar -5,1 kkal/mol terhadap TNF- α dan -5,0 kkal/mol terhadap IL-6, sedangkan natrium diklofenak menunjukkan afinitas pengikatan yang sedikit lebih kuat, yaitu -5,5 kkal/mol dan -5,2 kkal/mol. Selain itu, CAPE memiliki beberapa residu yang berinteraksi dengan senyawa referensi, menunjukkan orientasi pengikatan yang serupa di dalam situs aktif kedua protein tersebut. Temuan ini menunjukkan bahwa CAPE memiliki karakteristik pengikatan yang menguntungkan terhadap TNF- α dan IL-6. Namun, afinitas pengikatan yang diperoleh merupakan prediksi awal interaksi ligan-protein dan memerlukan validasi lebih lanjut melalui simulasi dinamika molekuler dan studi eksperimental. Studi ini memberikan wawasan molekuler tentang potensi antiinflamasi senyawa turunan propolis dan mendukung eksplorasi lebih lanjut sebagai senyawa utama alami untuk pengembangan obat antiinflamasi.

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INTRODUCTION

Inflammation is a fundamental protective mechanism that the body initiates in response to tissue damage, preventing further injury and supporting recovery¹. This process begins with cellular disruption, leading to the release of phospholipids such as arachidonic acid, which is subsequently converted into inflammatory mediators including leukotrienes, prostaglandins, and thromboxanes by cyclooxygenase and lipoxygenase enzymes. Among these mediators, pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- α) play pivotal roles in orchestrating immune responses and regulating both acute and chronic inflammation^{2,3}.

IL-6 activates inflammatory signaling pathways, induces the synthesis of acute-phase proteins, and supports T-cell proliferation and B-cell differentiation. TNF- α serves as an early mediator by promoting the production of additional pro-inflammatory cytokines and triggering endothelial activation, which increases vascular permeability. Dysregulated levels of these cytokines have been linked to increased disease severity and adverse clinical outcomes, highlighting the importance of precise modulation to prevent excessive inflammation^{4,5}.

Conventional anti-inflammatory drugs, such as nonsteroidal anti-inflammatory agents, are effective but are often associated with gastrointestinal, renal, and cardiovascular side effects. This limitation has driven growing interest in natural alternatives that offer comparable efficacy with lower risk profiles. Propolis, a resinous substance produced by bees, contains bioactive compounds such as flavonoids and caffeic acid phenethyl ester (CAPE)⁶⁻⁸; Idacahyati et al., 2020). CAPE has been shown to inhibit prostaglandin synthesis, enhance immune cell phagocytosis, and promote tissue repair⁹. Its ability to inhibit cyclooxygenase-2 (COX-2), a key enzyme in inflammatory pathways, further supports its potential as a natural anti-inflammatory agent¹⁰.

Despite these promising findings, the precise molecular interactions of CAPE with key inflammatory cytokines, particularly IL-6 and TNF- α , remain poorly characterized. Understanding these interactions is critical for elucidating the mechanisms underlying CAPE's anti-inflammatory effects and for optimizing its therapeutic application. Addressing this gap is particularly urgent given the increasing prevalence of inflammatory disorders and the need for safer, more effective treatments.

Although CAPE has been extensively reported to exhibit anti-inflammatory effects through inhibition of NF- κ B activation, suppression of COX-2 expression, and regulation of oxidative stress pathways, most previous studies have mainly focused on cellular mechanisms and downstream inflammatory mediators. Several studies have demonstrated the anti-inflammatory activity of CAPE in various disease models. However, information regarding its direct molecular interactions with major pro-inflammatory cytokines, particularly IL-6 and TNF- α , remains limited. Furthermore, comparative studies evaluating CAPE against conventional anti-inflammatory drugs at the molecular level are still scarce. Therefore, elucidating the binding behavior of CAPE toward these cytokines is important to better understand its mechanism of action and support the development of propolis-derived anti-inflammatory agents.

To the best of our knowledge, this study is among the few investigations that simultaneously evaluate the interaction of CAPE with both IL-6 and TNF- α using molecular docking and compare its binding characteristics with diclofenac sodium as a reference compound. This approach provides molecular insights into the anti-inflammatory potential of CAPE and may support the development of safer natural therapeutic agents. Therefore, this study aims to evaluate the anti-inflammatory potential of caffeic acid phenethyl ester by investigating its molecular interactions

with IL-6 and TNF- α using docking simulations. The research seeks to provide mechanistic insights into CAPE's activity, contributing to the development of novel, safe, and effective herbal-based therapies for managing inflammation.

METHODS

This study employed an *in silico* molecular docking approach to investigate the interactions of caffeic acid phenethyl ester (CAPE) with target proteins interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α). The workflow included ligand and protein retrieval, structural preparation, docking simulations, and interaction analysis.

Tools and Materials

Tools. The study utilized a combination of hardware, software, and online servers. Hardware included a Lenovo laptop (model 1A02S74R) with Intel Core i5-1035G1 CPU @ 1.00 GHz, 4 GB RAM, and Windows 11 Pro 64-bit. Software and online resources included AutoDockTools 1.5.7 (Morris et al., 2009), BIOVIA Discovery Studio Visualizer 2021, PyRx 0.9.9, PyMOL, PubChem, RCSB Protein Data Bank, and PLIP (Protein-Ligand Interaction Profiler)¹¹.

Materials. The 3D structure of caffeic acid phenethyl ester (CAPE) was obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) as shown in Figure 1. The target protein structures were obtained from the Protein Data Bank (PDB), including IL-6 (PDB ID: 1ALU) and TNF- α (PDB ID: 6OP0). PDB ID 1ALU was selected because it represents the crystal structure of human IL-6 with sufficient resolution and has been previously used in molecular docking studies involving inflammatory targets. Similarly, PDB ID 6OP0 was chosen because it corresponds to human TNF- α complexed with a co-crystallized ligand, allowing identification and validation of the active binding site through redocking analysis^{12,13}.

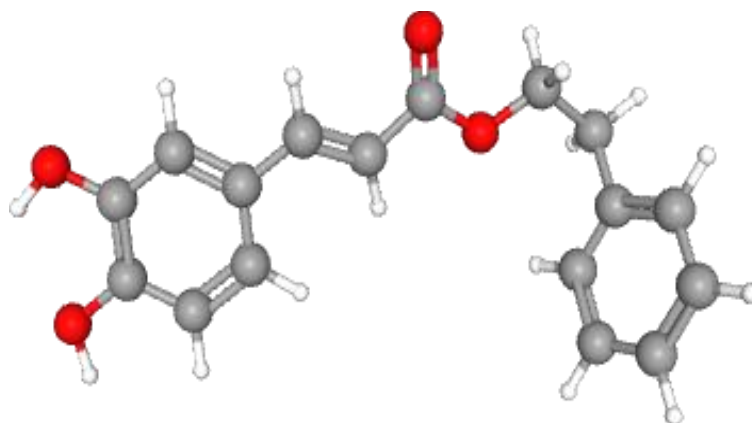


Figure 1. Caffeic Acid Phenethyl Ester (CAPE) in 3D struture

Research Procedure

Step I: Retrieval and Preparation of Protein Structures

The IL-6 and TNF- α protein structures were retrieved from the PDB database. Protein preparation involved removing water molecules, ions, and irrelevant ligands using AutoDockTools 1.5.7 to focus on the active binding domains for molecular docking. Chain selection was performed to isolate the biologically relevant protein chains used for docking analysis. The co-crystallized ligands were retained during the validation process and subsequently removed before ligand docking. Polar hydrogen atoms and Gasteiger charges were added, and the prepared receptors were saved in PDBQT format.

Step II: Validation of Docking Method

Docking validation was conducted by redocking the native ligand into the prepared target protein. The method was considered valid if the root-mean-square deviation (RMSD) was ≤ 2 Å, ensuring reliable docking parameters for subsequent ligand testing.

Step III: Retrieval and Preparation of CAPE Structure

The 3D structure of CAPE was obtained from PubChem and visualized using BIOVIA Discovery Studio Visualizer 2021. The ligand was prepared for docking, including optimization and conversion to compatible file formats. Ligand geometry optimization and energy minimization were performed prior to docking to obtain a stable conformation suitable for interaction analysis. The optimized ligand structure was converted into PDBQT format using AutoDockTools 1.5.7.

Step IV: Molecular Docking Simulation

Molecular docking simulations were performed using AutoDock Vina version 1.5.7 implemented in PyRx version 0.9.9. Grid box parameters were defined according to the binding pocket of the co-crystallized ligand identified during redocking validation. For TNF- α (6OP0), the grid center coordinates were $x = -13.917$, $y = -1.482$, and $z = 19.021$, with dimensions of $15 \times 15 \times 15$ Å. For IL-6 (1ALU), the grid center coordinates were $x = -7.677$, $y = -12.743$, and $z = 0.048$, with dimensions of $12 \times 12 \times 12$ Å. Docking calculations were performed with an exhaustiveness value of 8, generating nine binding poses for each ligand. The docking pose exhibiting the lowest binding energy and an orientation consistent with the active binding site was selected as the best pose for further analysis. Visualization and interaction analyses were carried out using PyMOL, Discovery Studio Visualizer 2021, and the PLIP webserver.

Step V: Analysis of Molecular Docking Interactions

Binding affinities for each docking pose were analyzed using AutoDock Vina 1.5.7, with the lowest energy conformations considered the best. Poses were clustered in PyRx based on structural similarity. Protein-ligand interactions, including hydrogen bonds, hydrophobic interactions, π - π stacking, and electrostatic interactions, were identified. Further quantitative analysis was conducted using the PLIP webserver, reporting all non-covalent interactions, distances, interaction energies, and 3D visualizations.

Data Analysis

The best docking pose was selected based on the lowest binding affinity value and its consistency with the active site residues identified during redocking validation. Docking results were evaluated primarily through binding affinity scores and interaction types. Conformational analysis, energy calculations, and quantitative interaction data from PLIP provided detailed insights into the molecular interactions between CAPE and the target proteins.

RESULT AND DISCUSSION

Target Protein Preparation

Target protein preparation was conducted to obtain optimized receptor structures suitable for molecular docking simulations. Proper protein preparation is a critical step in molecular docking studies because inaccuracies in receptor structures may significantly affect binding affinity predictions and ligand–receptor interaction profiles¹⁴. In this study, the target proteins analyzed were TNF- α (PDB ID: 6OP0) and IL-6 (PDB ID: 1ALU). Caffeic acid phenethyl ester (CAPE) was used as the test compound, while diclofenac sodium was employed as a reference compound because of its well-established anti-inflammatory activity and widespread clinical use. Although diclofenac primarily

exerts its therapeutic effects through cyclooxygenase (COX) inhibition rather than direct inhibition of IL-6 or TNF- α , it was included as a comparator to provide a benchmark for evaluating the relative binding behavior of CAPE toward these inflammatory targets.

Macromolecular chain separation was performed as the initial step in receptor preparation to isolate the biologically relevant protein chains. The separated receptor structures were saved in .pdb format and subsequently optimized using AutoDockTools. During optimization, water molecules were removed and hydrogen atoms were added to each amino acid residue. Removal of crystallographic water molecules is necessary because they may introduce artificial hydrogen bonding and reduce docking accuracy if not properly treated¹⁵.

Hydrogen atoms were added to ensure accurate modeling of hydrogen bonding interactions and proper protonation states during docking simulations. Furthermore, Gasteiger charges were assigned to maintain electrostatic stability and improve docking reliability. Electrostatic interactions play an essential role in ligand binding and significantly influence docking scoring functions¹⁶. The optimized receptor structures (Figure 2) were then saved in .pdbqt format and used for docking validation and molecular docking simulations. The systematic preparation of TNF- α and IL-6 receptors ensured that the docking protocol provided a reliable representation of ligand–protein interactions and supported accurate prediction of CAPE binding behavior toward inflammatory target proteins.

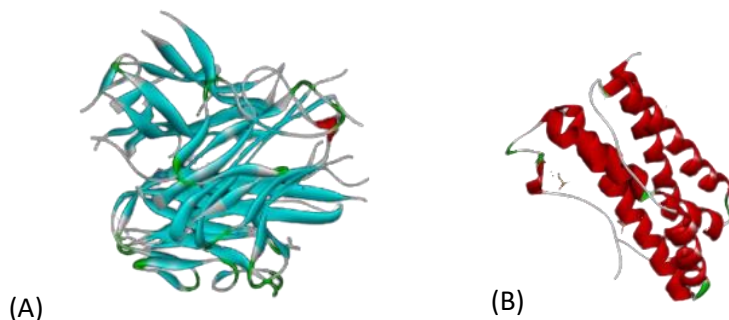


Figure 2. Prepared structures of IL-6 (A) and TNF- α (B) target proteins used for molecular docking analysis.

Docking Method Validation

Validation of the molecular docking method was conducted to ensure the accuracy and reliability of the docking protocol prior to evaluating the binding affinity of the test compounds toward the inflammatory target proteins. The validation process used the root mean square deviation (RMSD) parameter, which measures the structural deviation between the crystallographic conformation of the native ligand and the redocked pose. RMSD is widely accepted as an indicator of docking accuracy because it reflects how well the docking protocol can reproduce the experimentally observed ligand–protein interactions¹⁷.

A docking protocol is generally considered reliable when the RMSD value is less than 2 Å, indicating that the predicted ligand orientation closely matches the experimental crystallographic structure¹⁸. In this study, validation was performed by redocking the native ligands into the active sites of the inflammatory target proteins TNF- α (PDB ID: 6OP0) and IL-6 (PDB ID: 1ALU). The native ligand used for TNF- α was A7A [(R)-{1-[(2,5-dimethylphenyl)methyl]-6-(1-methyl-1H-pyrazole-4-yl)-1H-benzimidazol-2-yl}(pyridin-4-yl)methanol], while the native ligand for IL-6 was TLA (L(+)-tartaric acid). The redocking results produced RMSD values of 0.943 Å for TNF- α and 1.666 Å for IL-6, both of which fall within the acceptable validation range.

Method validation was carried out by redocking the native ligands into their respective binding sites¹⁹. The docking results were analyzed using Discovery Studio 2021 and visualized with PyMOL. The RMSD values obtained were 0.943 Å for TNF- α and 1.666 Å for IL-6, both of which meet the accepted validation criteria. These results confirm that the docking parameters used in this study were reliable and suitable for docking simulations of the test compound. The docking validation results are presented in Table 1.

Table 1. Molecular docking validation results for TNF- α and IL-6 based on root mean square deviation (RMSD) values.

Ligand	Protein Target	Grid Center	Grid Box Dimensions (Å)	RMSD (Å)
A7A / R)-{1-[(2,5-dimethylphenyl)methyl]-6-(1-methyl-1H-pyrazole-4-yl)-1H-benzimidazol-2-yl}(pyridin-4-yl)methanol	TNF- α : 6OP0	x = -13.917 y = -1.482 z = 19.021	x = 15 y = 15 z = 15	0.943
TLA / L(+)-TARTARIC ACID	IL-6 : 1ALU	x = -7.677 y = -12.743 z = 0.048	x = 12 y = 12 z = 12	1.666

These RMSD values indicate that the docking parameters used in this study were capable of accurately reproducing the binding poses of the native ligands within the active sites of TNF- α and IL-6. Therefore, the docking protocol can be considered valid and suitable for predicting the binding interactions and affinity of the tested compounds. The validated docking method increases the reliability of subsequent molecular docking simulations and supports the interpretation of ligand–protein interaction results

Ligand Preparation

Ligand preparation is an important step in molecular docking studies because the accuracy of docking results depends on the quality of the ligand structure used in the simulation. In this study, the three-dimensional structure of caffeic acid phenethyl ester (CAPE) was obtained from the PubChem Compound database (CID: 5281787). PubChem is a widely used public chemical database that provides reliable structural and physicochemical information for bioactive compounds, including natural products and phytochemicals, making it suitable as a source of ligand structures in computational drug discovery studies²⁰.

The structure was downloaded in .sdf format and converted using AutoDock tools to generate a .pdb file for docking analysis. The CAPE structure was downloaded in Structure Data File (.sdf) format and subsequently converted into Protein Data Bank (.pdb) format using AutoDock Tools to ensure compatibility with the docking software. Proper ligand preparation, including file format conversion and structural optimization, is essential to obtain accurate predictions of ligand–protein interactions and binding affinity²¹.

After conversion, the ligand structure was visualized using Discovery Studio Visualizer to verify the molecular geometry and structural integrity prior to docking simulations. Visualization helps ensure that the ligand structure is correctly prepared and free from structural errors that could affect docking results. A well-prepared ligand structure improves docking reliability and enhances the accuracy of predicted binding interactions with the target proteins²².

Molecular Docking Analysis

The molecular docking analysis provided binding affinity values and interaction profiles between ligands and the target proteins TNF- α and IL-6. Binding affinity values reflect the strength and stability of ligand with receptor complexes and are commonly used as preliminary indicators of biological activity in structure-based drug design. More negative binding energy values indicate stronger molecular interactions and greater stability of the ligand–receptor complex, suggesting a higher probability of biological activity²³.

All tested ligands, including caffeic acid phenethyl ester (CAPE), diclofenac sodium, and native ligands, showed negative binding affinity values, indicating that the interactions with TNF- α and IL-6 receptors occurred spontaneously under simulated conditions. Docking analysis against TNF- α demonstrated that diclofenac sodium exhibited the strongest binding affinity with a value of -5.5 kcal/mol, whereas CAPE showed a binding affinity of -5.1 kcal/mol. Similarly, docking analysis against IL-6 showed that diclofenac sodium produced a binding affinity value of -5.2 kcal/mol, while CAPE demonstrated a slightly lower binding affinity of -5.0 kcal/mol. The comparison of binding affinity values between CAPE and diclofenac sodium is presented in Table 2.

Table 2. Comparison of Binding Affinities of CAPE and Diclofenac Sodium Against Target

Protein Target	Compound	Binding Affinity (kcal/mol)
TNF- α	Diclofenac sodium	-5.5
	Caffeic Acid Phenethyl Ester	-5.1
IL-6	Diclofenac sodium	-5.2
	Caffeic Acid Phenethyl Ester	-5.0

Although diclofenac sodium exhibited slightly lower binding energies than CAPE, the differences between both compounds were less than 1 kcal/mol. Such differences are generally considered relatively small and suggest comparable interaction stability. According to Ferreira et al. (2024), compounds exhibiting differences below 1 kcal/mol may possess similar binding behavior within the receptor cavity²⁴.

Although CAPE exhibited binding affinity values comparable to those of diclofenac sodium, the values obtained (approximately -5 kcal/mol) should be interpreted as preliminary predictions of ligand–protein interactions rather than direct evidence of anti-inflammatory activity. Molecular docking provides only an estimation of binding propensity and does not necessarily reflect biological efficacy. Therefore, these findings indicate the potential ability of CAPE to interact with TNF- α and IL-6, which should be further confirmed through molecular dynamics simulations and experimental studies. CAPE contains aromatic rings and polar functional groups that enable both hydrophobic and electrostatic interactions, which are known to contribute significantly to ligand stabilization within protein binding sites. Previous studies have reported that CAPE exhibits anti-inflammatory activity through modulation of inflammatory mediators and inhibition of pro-inflammatory signaling pathways, supporting the relevance of the docking results obtained in this study⁹.

Visualization of Molecular Docking Interactions

Visualization of molecular docking interactions revealed distinct interaction patterns between caffeic acid phenethyl ester (CAPE), diclofenac sodium, and the inflammatory target proteins TNF- α and IL-6 (Figure 3). In the TNF- α receptor, diclofenac sodium formed hydrogen bonds with LEU120C and SER60C, accompanied by hydrophobic interactions involving LEU57C, TYR59C, and TYR151C (Figure 3B). Similarly, CAPE interacted with TNF- α through key residues including LEU120C and TYR59C, suggesting that CAPE occupies a binding region comparable to that of the reference compound (Figure 3A).

For the IL-6 receptor, no hydrogen bond interactions were observed for either CAPE or diclofenac sodium. However, both compounds established hydrophobic interactions with residues LEU178A, ARG179A, and ARG182A, which are considered important contributors to ligand stabilization within the binding cavity (Figure 3C and 3D). The presence of shared interacting residues indicates that CAPE exhibits a binding orientation similar to that of diclofenac sodium and may potentially modulate inflammatory pathways through comparable molecular interactions.

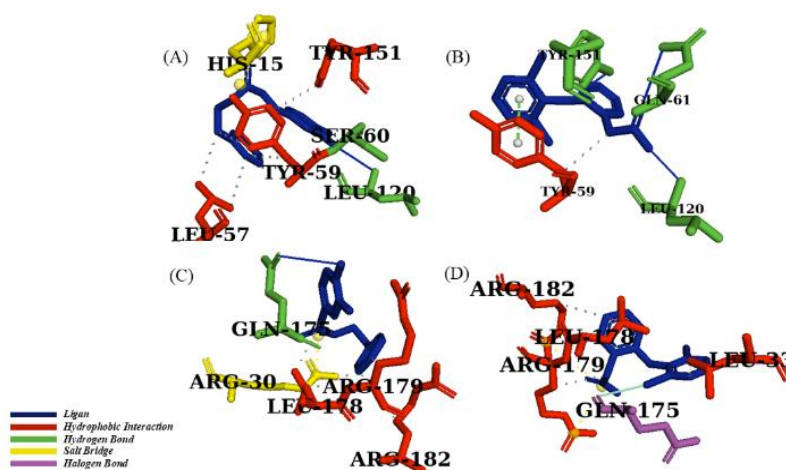


Figure 3. Molecular interaction profiles of caffeic acid phenethyl ester (CAPE) and diclofenac sodium with inflammatory target proteins. (A) CAPE–TNF- α , (B) Diclofenac sodium–TNF- α , (C) CAPE–IL-6, and (D) Diclofenac sodium–IL-6. Hydrogen bonds and hydrophobic interactions are shown to illustrate the binding patterns within the active sites of the target proteins.

These interactions indicate stable accommodation of the ligand within the TNF- α binding pocket. CAPE exhibited a comparable interaction pattern, particularly with residues LEU120C and TYR59C, suggesting that CAPE occupies a similar binding region within the active site. The presence of shared interacting residues indicates structural compatibility between CAPE and the TNF- α binding pocket and suggests the possibility of competitive binding within this region. Similar molecular docking studies have demonstrated that phytochemical compounds are capable of interacting with TNF- α through comparable amino acid residues, supporting their potential as anti-inflammatory agents²⁵.

In the IL-6 receptor, hydrogen bond interactions were not observed for CAPE or the reference ligand. However, hydrophobic interactions were detected involving residues LEU33A, LEU178A, ARG179A, and ARG182A. CAPE shared several interacting residues with the reference ligand, particularly LEU178A, ARG179A, and ARG182A, indicating a similar binding orientation within the IL-6 interaction cavity. Hydrophobic interactions play an important role in stabilizing ligand–protein complexes and may significantly contribute to binding stability even in the absence of hydrogen bonding interactions. Molecular docking studies have shown that non-covalent interactions such as hydrophobic contacts are essential determinants of ligand stability within protein binding sites²⁶.

The interaction patterns observed in both TNF- α and IL-6 receptors indicate that CAPE forms stable non-covalent interactions dominated primarily by hydrophobic contacts. Although the binding affinity values of CAPE were slightly lower than those of diclofenac sodium, the similarity of interacting residues suggests that CAPE interacts with

key regions involved in inflammatory signaling. IL-6 and TNF- α are well-recognized inflammatory mediators frequently targeted in molecular docking studies for anti-inflammatory drug discovery, and their inhibition is associated with modulation of inflammatory pathways¹³.

From a structural perspective, the aromatic rings and ester functional groups of CAPE enable hydrophobic and π -related interactions within the binding pockets, contributing to stabilization of ligand–receptor complexes. These findings suggest that CAPE demonstrates a consistent interaction profile toward both TNF- α and IL-6, supporting its potential as a natural anti-inflammatory candidate. Overall, the docking results provide a molecular-level explanation for the anti-inflammatory activity of CAPE and indicate that this compound may serve as a promising lead compound for further anti-inflammatory drug development.

LIMITATION

This study was limited to an *in silico* molecular docking approach to evaluate the interaction of caffeic acid phenethyl ester (CAPE) with IL-6 and TNF- α target proteins. Nevertheless, molecular docking provides an important preliminary insight into ligand–receptor interactions and binding affinity prediction. Further studies involving molecular dynamics simulations and experimental approaches (*in vitro* or *in vivo*) are recommended to confirm and extend the findings of this study.

CONCLUSION

This study demonstrated that caffeic acid phenethyl ester (CAPE) is capable of interacting with the inflammatory target proteins TNF- α and IL-6, exhibiting binding affinity values comparable to those of diclofenac sodium and sharing several key interacting residues within the binding sites. These findings suggest that CAPE possesses favorable binding characteristics and may contribute to the modulation of inflammatory pathways. However, the binding affinity values obtained from molecular docking represent preliminary predictions of ligand with protein interactions and should not be considered direct evidence of anti-inflammatory activity. Therefore, CAPE may serve as a promising natural lead compound for anti-inflammatory drug development, although further molecular dynamics simulations and experimental studies are necessary to validate its biological efficacy and therapeutic potential.

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AUTHOR CONTRIBUTION STATEMENT

KAACS: Conceptualization, Methodology, Software; **AN:** Data curation, Writing Original draft preparation; **AN:** Visualization, Investigation; **PMK:** Supervision; **KAACS:** Software, Validation; **PMK:** Writing- Reviewing and Editing.

CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest.

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Antibacterial Activity Test of Combined Extracts of Guava Leaves (*Psidium guajava* L.) and Celery Stalks (*Apium graveolens* L. var. *dulce*) Against *Staphylococcus aureus* and *Escherichia coli*

Uji Aktivitas Antibakteri Ekstrak Gabungan Daun Jambu Biji (*Psidium guajava* L.) dan Batang Seledri (*Apium graveolens* L. var. *dulce*) Terhadap *Staphylococcus aureus* dan *Escherichia coli*

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Abstract

Antibiotic resistance in pathogenic bacteria such as *Staphylococcus aureus* and *Escherichia coli* has become a critical global health concern. Flavonoids, tannins, and saponins in guava leaves (*Psidium guajava* L.) and celery stalks (*Apium graveolens* L. var. *dulce*) suggest their potential as natural antibacterial agents, but studies on their combined effectiveness remain limited. This study aimed to evaluate the synergistic effect, optimal concentration, and antibacterial activity of combined guava leaf and celery stalk extracts against gram-positive and gram-negative bacteria. This laboratory-based experimental study used the maceration method with 96% ethanol as the solvent. Antibacterial activity was assessed through the disc diffusion method against *S. aureus* and *E. coli*. The study tested 40% single extracts and combinations in ratios of 1:1, 1:2, and 2:1. The findings showed that the combination did not improve antibacterial activity compared with the most active single extract under the disc diffusion assay. The highest antibacterial activity against *S. aureus* was observed in the 2:1 combination, producing an inhibition zone of 11.06 mm, although still lower than the 40% guava leaf extract (11.75 mm). In *E. coli*, all treatments demonstrated weak activity, with the 1:1 combination yielding the highest inhibition zone of 3.74 mm. Statistical analysis using ANOVA and Games-Howell indicated a significant difference for *S. aureus*, while the Kruskal-Wallis test showed no significant difference for *E. coli* ($p > 0.05$). The combination of guava leaf and celery stalk extracts did not exhibit a synergistic effect. The 2:1 combination was the most effective against *S. aureus*, while the 1:1 combination showed limited activity against *E. coli*.

Abstrak

Resistensi antibiotik pada bakteri patogen seperti *Staphylococcus aureus* dan *Escherichia coli* telah menjadi masalah kesehatan global yang kritis. Flavonoid, tanin, dan saponin dalam daun jambu biji (*Psidium guajava* L.) dan batang seledri (*Apium graveolens* L. var. *dulce*) menunjukkan potensi mereka sebagai agen antibakteri alami, tetapi studi tentang efektivitas gabungan mereka masih terbatas. Studi ini bertujuan untuk mengevaluasi efek sinergis, konsentrasi optimal, dan aktivitas antibakteri dari ekstrak daun jambu biji dan batang seledri gabungan terhadap bakteri gram positif dan gram negatif. Studi eksperimental berbasis laboratorium ini menggunakan metode maserasi dengan etanol 96% sebagai pelarut. Aktivitas antibakteri dinilai melalui metode difusi cakram terhadap *Staphylococcus aureus* dan *Escherichia coli*. Studi ini menguji 40% ekstrak tunggal dan kombinasi dalam rasio 1:1, 1:2, dan 2:1. Hasil penelitian menunjukkan bahwa kombinasi tersebut tidak meningkatkan aktivitas antibakteri dibandingkan dengan ekstrak tunggal yang paling aktif dalam uji difusi cakram. Aktivitas antibakteri tertinggi terhadap *Staphylococcus aureus* diamati pada kombinasi 2:1, menghasilkan zona inhibisi sebesar 11,06 mm, meskipun masih lebih rendah daripada ekstrak daun jambu biji 40% (11,75 mm). Pada *E. coli*, semua perlakuan menunjukkan aktivitas yang lemah, dengan kombinasi 1:1 menghasilkan zona inhibisi tertinggi sebesar 3,74 mm. Analisis statistik menggunakan ANOVA dan Games-Howell menunjukkan perbedaan yang signifikan untuk *Staphylococcus aureus*, sedangkan uji Kruskal-Wallis menunjukkan tidak ada perbedaan yang signifikan untuk *E. coli* ($p > 0,05$). Kombinasi ekstrak daun jambu biji dan batang seledri tidak menunjukkan efek sinergis. Kombinasi 2:1 adalah yang paling efektif terhadap *S. aureus*,

sedangkan kombinasi 1:1 menunjukkan aktivitas terbatas terhadap *E. coli*.

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INTRODUCTION

Bacterial infections are one of the main factors causing disease worldwide. In 2019, there were 7.7 million deaths due to bacterial infections caused by 33 different types of bacteria.¹ Antibiotics are used for the treatment of bacterial infections by inhibiting and killing bacteria. However, inappropriate use of antibiotics can lead to bacterial resistance.²

S. aureus is a major cause of death in 135 countries. In 2019, more than 1 million deaths occurred, with 299,000 cases associated with fatal bloodstream infections.¹ Based on a 2022 survey by the *World Health Organization* (WHO) on the distribution of priority pathogens in 70 hospitals across 15 provinces in Indonesia, the percentage of *Methicillin-Resistant S. aureus* (MRSA) in Papua was 100%, higher than in DKI Jakarta at 30%.³ A total of 290,000 deaths occurred due to fatal peritoneal and intra-abdominal infections caused by *E. coli*. Deaths due to *E. coli* occurred in 37 cities across various countries.¹ The percentage of *E. coli* resistance to cephalosporin antibiotics was highest in Sumatra at 75%, followed by DKI Jakarta at 68%. Meanwhile, *E. coli* resistance to carbapenem antibiotics in DKI Jakarta was 13%.³

Secondary metabolite compounds contained in guava leaf (*Psidium guajava* L.) and celery stalk (*Apium graveolens* L. var. *dulce*) are known to contain flavonoids, saponins, and tannins, which are effective as antibacterial agents.^{4,5} Based on research, *Psidium guajava* L. leaf extract effectively inhibits the growth of *S. aureus* at a concentration of 15% with an inhibition zone of 6.75 mm.⁶ Meanwhile, against *E. coli*, a concentration of 50% produces an inhibition zone of 10.42 mm with strong effectiveness.⁷ *Apium graveolens* L. var. *dulce* has the potential to inhibit the growth of *S. aureus* at a concentration of 20% with an inhibition zone of 2.8 mm.⁵ The maceration method was chosen because it is effective in extracting secondary metabolite compounds such as flavonoids, tannins, alkaloids, steroids, and terpenoids.⁸

Guava leaf (*Psidium guajava* L.) and celery stalk (*Apium graveolens* L. var. *dulce*) were selected due to their well-documented antibacterial properties, particularly flavonoids, saponins, and tannins, which work through distinct mechanisms: flavonoids disrupt cell membrane integrity, tannins precipitate bacterial proteins, and saponins increase membrane permeability.⁹ Combining the two extracts is expected to produce a synergistic antibacterial effect by simultaneously targeting multiple bacterial mechanisms.¹⁶ *S. aureus* and *E. coli* were chosen as bacterial models because they represent Gram-positive and Gram-negative bacteria with structurally different cell walls, making them suitable for a comprehensive evaluation of antibacterial activity. The combination ratios of 1:1, 1:2, and 2:1 were selected to systematically determine the most effective proportion of each extract and to identify the optimal ratio for synergistic activity.¹⁶ To date, no study has investigated the antibacterial activity of the combined extract of *Psidium guajava* L. leaf and *Apium graveolens* L. var. *dulce* against both *S. aureus* and *E. coli*, representing a research gap that this study aims to address.

METHODS

The study was conducted using an experimental laboratory method. It aims to determine the synergistic effect, potential concentration, and antibacterial effectiveness of the combination of guava leaf extract (*Psidium guajava* L.) and celery stalk (*Apium graveolens* L. var. *dulce*) against Gram-positive and Gram-negative bacteria. The sample extraction method used the maceration principle with 96% ethanol pro analysis as the solvent. The antibacterial activity test was carried out using the disc diffusion method. The study was conducted at the Microbiology and Phytochemistry Laboratory of STIKes IKIFA from December 2024 to August 2025.

Tools : Analytical balance (OHAUS), evaporating dish (Pyrex), beaker glass (Pyrex), 60-mesh sieve, grinder, rotary evaporator (IKA RV 8), maceration vessel, glass funnel (Pyrex), stirring rod, test tubes (Pyrex), measuring cylinder (Iwaki), beaker glass (Pyrex), test tube rack, Bunsen burner, Petri dish (Norma), Erlenmeyer flask (Pyrex), hot plate (Maspion), autoclave (Hirayama), incubator, oven (Shimademy), forceps, vortex mixer (Dragon Lab), micropipette (Dragon Lab) and tips, vernier caliper, spatula, inoculating loop, laminar air flow cabinet, microscope slide.

Materials: *Psidium guajava* L. leaves, *Apium graveolens* L. var. *dulce*, 96% ethanol pro analysis, distilled water, Nutrient Agar (NA) medium (Millipore), filter paper, aluminum foil, HVS paper, magnesium powder (Mg), concentrated HCl, 10% FeCl₃ solution, ciprofloxacin 500 mg tablets (Hexapharm Jaya), 0.9% NaCl, disc paper, 0.5 McFarland solution, sterile cotton swab, Eosin Methylene Blue agar (Millipore), Gram staining kit (Indoreagen).

Research Procedure

1. Plant collection

A total of 2 kg of dried *Psidium guajava* L. leaves were obtained from UPT Laboratorium Herbal Materia Medica Batu, and 4 kg of fresh *Apium graveolens* L. var. *dulce* were obtained from a plantation area in Lembang, Bandung.

2. Plant Determination

Plant determination was carried out at Yayasan Generasi Biologi Indonesia for *Apium graveolens* L. var. *dulce* and at UPT Laboratorium Herbal Materia Medica Batu for *Psidium guajava* L. leaves.

3. Sample preparation

Fresh simplicia were wet-sorted, washed, cut, and oven-dried at 50°C. The dried simplicia were dry-sorted, ground, and sieved through a 60-mesh sieve. The resulting powder was stored in a tightly closed container protected from light.

4. Extract preparation⁹

A total of 200 g of *Psidium guajava* L. leaf simplicia powder and 227 g of *Apium graveolens* L. var. *dulce* simplicia powder were weighed and macerated with 96% ethanol (4000 mL and 4540 mL, respectively) at a 1:10 (w/v) ratio. The samples were soaked for 6 hours with occasional stirring and then left to stand for 18 hours. The extraction was repeated using half of the initial solvent volume. All macerates were collected and filtered, and the filtrates were evaporated under reduced pressure using a rotary evaporator to obtain a thick extract. The extracts were stored in brown containers, and the extraction yield was calculated.

$$\text{Extract yield} = \frac{\text{Weight of thick extract}}{\text{Weight of dry powder}} \times 100\%$$

5. Extract evaluation test

- a. Organoleptic test⁹

Psidium guajava L. extract showed a thick consistency, dark brown color, characteristic odor, and astringent taste, while *Apium graveolens* L. var. *dulce* extract showed a thick consistency, dark green color, and characteristic odor and taste.

b. Phytochemical screening

1) Flavonoid Shinoda test⁴

Mix 1 mL of diluted extract with 0.5 g magnesium powder, then add 2 to 3 drops of concentrated hydrochloric acid. A positive result is indicated by a red or yellow color.

2) Flavonoid alkaline reagent test¹⁰

Add a few drops of sodium hydroxide solution to 1 mL of extract. A positive result is indicated by a yellow color.

3) Tannin test⁴

Add 2 to 4 drops of 10 percent ferric chloride solution to 1 mL of diluted extract. A positive result is indicated by a bluish black or green color.

4) Saponin test¹¹

Mix 1 g of extract with 10 mL of hot water, allow to cool, and shake for 10 minutes. A positive result is indicated by stable foam with a height of 1 to 10 cm that persists for at least 10 minutes and remains after the addition of 2N hydrochloric acid.

c. Total flavonoid content⁹

Dissolve approximately 0.2 g of extract in 25 mL of ethanol, stir for 30 minutes, filter, and adjust to volume. Prepare standard solutions in ethanol at concentrations of 100, 75, 50, and 25 µg per mL. Mix 0.5 mL of sample or standard with ethanol, aluminum chloride, sodium acetate, and water, then incubate for 30 minutes at room temperature. Measure absorbance at the maximum wavelength using a blank without aluminum chloride, construct a calibration curve, and calculate the flavonoid content

6. Bacterial identification

Gram staining was carried out by inoculating bacterial culture onto a glass slide containing physiological NaCl, followed by heat fixation and sequential staining with crystal violet, Lugol's iodine, alcohol decolorization, and safranin as a counterstain; Gram-positive bacteria were identified by their purple coloration and cocci morphology, whereas Gram-negative bacteria appeared pink to red with rod-shaped morphology when observed under a microscope.¹² Identification of *Escherichia coli* was performed by streaking a bacterial isolate onto Eosin Methylene Blue (EMB) agar and incubating at 35°C for 24 hours, with positive results indicated by the formation of characteristic metallic green colonies.¹³

7. Antibiotic and extract sensitivity test⁵

Antibacterial activity was evaluated using the disc diffusion method by preparing ciprofloxacin solutions (2 and 4 µg/mL) and plant extracts of guava leaves and celery stalks at concentrations of 10%, 20%, 30%, 40%, and 50%. Nutrient Agar (NA) was poured into Petri dishes and allowed to solidify, followed by inoculation of bacterial suspension using a sterile cotton swab. Sterile paper discs (6 mm diameter) were impregnated with 20 µL of each test solution, placed on the inoculated media, and experiments were conducted in triplicate for each concentration. Plates were incubated at 37°C for 24 hours, after which the inhibition zones were observed and

measured. All used materials were decontaminated by immersion in disinfectant solution for approximately 30 ± 5 minutes, followed by boiling for 30 ± 5 minutes.

8. Antibacterial activity test⁵

Antibacterial activity of guava leaf (*Psidium guajava* L.) extract (40%; F1), celery stalk (*Apium graveolens* L. var. *dulce*) extract (40%; F2), and their combinations at ratios of 1:1 (F3), 1:2 (F4), and 2:1 (F5) was evaluated using the disc diffusion method. Bacterial suspensions were spread onto solidified Nutrient Agar (NA), followed by placement of 6 mm sterile discs impregnated with 20 μ L of each test solution, including ciprofloxacin (4 μ g/mL) as a positive control and 96% ethanol as a negative control. All treatments were performed in triplicate and incubated at 37°C for 24 h, after which inhibition zones were measured. All materials were decontaminated by immersion in disinfectant and subsequent boiling (30 ± 5 min each).

Data Analysis

Data analysis was performed by measuring the Diameter of Inhibition Zone (DIZ) using a caliper (mm), calculated as¹⁴:

$$\frac{(a-c)+(b-c)}{2}$$

Description:

a = vertical diameter

b = horizontal diameter

c = paper disc diameter

The data were further analyzed using one-way ANOVA with Statistical Product and Service Solutions (SPSS) version 26.0 for Windows.

RESULT AND DISCUSSION

1. Plant Determination

Fresh celery stalks was collected from a plantation in Lembang, Bandung and identified as *Apium graveolens* subsp. *dulce* (Mill.) Schübl. & G.Martens Dried guava leaf was obtained from UPT Laboratorium Herbal Materia Medica Batu and identified as *Psidium guajava* L.

2. Extract Preparation

The maceration method was employed to preserve thermolabile antibacterial constituents, including flavonoids, tannins, and saponins.¹⁵ The extraction yield of celery stem was 14.27%, while that of guava leaf extract was 13.29%. The yields complied with the Indonesian Herbal Pharmacopoeia (FHI) 2nd edition requirement of not less than 12.3%.⁹ However, specific standards for celery stem yield remain unavailable. Yield may be influenced by factors including plant variety, harvest age, geographical conditions, extraction parameters, solvent type, agitation intensity, extraction time, and temperature.⁸

3. Extract evaluation test

Guava leaf extract was a viscous, dark brown extract with a characteristic odor. In contrast, celery stem extract was dark green, exhibited a characteristic celery odor, and was viscous in form. The organoleptic properties of guava leaf extract complied with the Indonesian Herbal Pharmacopoeia (FHI) 2nd edition.⁹ However,

specific standards for the organoleptic evaluation of celery stem have not been reported. This evaluation was conducted as a preliminary identification based on sensory characteristics perceived by the senses.

Table 1. Results of Phytochemical Screening

No.	Simplicia	Identification	Result	Remark
1	Guava Leaf	Flavonoid (shinoda)	Yellow	Positive (+)
2		Flavonoid (alkaline reagent)	Orange	Positive (+)
3		Tannin	Greenish-black	Positive (+)
4		Saponin	Foam height 5 cm within ≥ 10 minutes and persists after addition of 2N HCl	Positive (+)
5	Celery Stalk	Flavonoid (shinoda)	Yellow	Positive (+)
6		Flavonoid (alkaline reagent)	Yellow	Positive (+)
7		Tannin	Orange	Negative (-)
8		Saponin	Foam height 1 cm within ≥ 10 minutes and persists after addition of 2N HCl	Positive (+)

In Table 1. Phytochemical screening was performed to confirm antibacterial secondary metabolites, namely flavonoids, tannins, and saponins, using specific reagents. Guava leaf extract was positive for all three compounds, whereas celery stem extract contained flavonoids and saponins only. Flavonoids were identified using Shinoda (concentrated HCl + Mg) and NaOH reagents. The Shinoda reaction produces flavilium salts, indicated by an orange color due to formation of a flavilium cation.¹⁶ The NaOH test confirmed flavonoids through formation of yellow–brown acetophenone derivatives following C-ring cleavage.¹⁶ Flavonoids contribute to antibacterial activity by disrupting bacterial cell membrane integrity and inhibiting nucleic acid synthesis, thereby impairing bacterial growth and replication.¹⁷ Tannins reacted with FeCl_3 to form blue-black or dark green complexes via interaction with phenolic hydroxyl groups and Fe^{3+} .¹⁶ Tannins precipitate bacterial proteins and inhibit bacterial enzymes, leading to disruption of metabolic process essential for bacterial survival.⁹ Saponins showed positive results by stable foam formation due to their amphiphilic properties and micelle formation.⁵ This amphiphilic nature enables saponins to interact with and increase bacterial membrane permeability, causing leakage of intracellular contents and ultimately bacterial cell death.⁹

Total flavonoid content was determined to quantify antibacterial bioactive compounds and was analyzed at Agricultural Post-Harvest Assembly and Modernization Agency. Guava leaf extract contained 600.05 mg/100 g, while celery stalks extract contained 1851.83 mg/100 g. Differences may reflect variation in flavonoid types, as the method does not distinguish individual compounds. Guava leaves are rich in quercetin, which exhibits antibacterial activity by disrupting cell permeability, inhibiting nucleic acid synthesis, and reducing enzyme activity.^{9,17} In contrast, celery stem predominantly contains apigenin, which shows antibacterial effects through

membrane damage and intracellular disruption.^{9,14} Despite its lower total flavonoid content, guava leaf extract demonstrated stronger antibacterial activity, likely due to the presence of quercetin.¹⁷

4. Bacterial identification

Bacterial identification was performed to confirm the species used in this study. Gram staining is based on the ability of bacterial cell walls to retain or release the crystal violet–iodine (CV–I) complex during alcohol decolorization.¹² Gram-positive bacteria such as *S. aureus* possess a thick peptidoglycan layer that retains the CV–I complex, resulting in a purple color.¹² In contrast, Gram-negative bacteria such as *E. coli* have a thin peptidoglycan layer and an outer membrane, allowing alcohol to extract the CV–I complex, after which cells take up safranin and appear red.¹² Microscopic observation showed *S. aureus* as purple cocci in clusters, consistent with reported characteristics.¹² *E. coli* appeared as red rod-shaped cells, also in agreement with the literature.¹²

To confirm *E. coli* identification, a biochemical test was performed using selective differential agar. This medium inhibits Gram-positive bacteria and differentiates Gram-negative bacteria based on metabolic activity. After incubation on EMB agar, *E. coli* produced colonies with a characteristic metallic green sheen.¹³ This feature is attributed to strong lactose fermentation, leading to acid production and precipitation of eosin Y and methylene blue, forming the metallic green complex on colony surfaces.^{12,13}

5. Antimicrobial Susceptibility Testing and Antibacterial Activity

Table 2a. Antibacterial Activity of Single Extracts at Various Concentrations

Bacteria	Treatment	Concentration	Inhibition Zone Diameter (mm) Mean $\pm$ SD	n	Activity Category	Sig.
<i>S. aureus</i>	Ciprofloxacin	2 μ g/mL	8.07 $\pm$ 0.180	3	Moderate	a
		4 μ g/mL	9.43 $\pm$ 1.038	3	Moderate	a
		10%	11.53 $\pm$ 1.153	3	Strong	a
		20%	13.23 $\pm$ 1.565	3	Strong	ab
	Guava Leaf	30%	12.47 $\pm$ 1.056	3	Strong	ab
		40%	13.23 $\pm$ 1.276	3	Strong	b
		50%	13.56 $\pm$ 1.165	3	Strong	b
	Celery Stalk	10%	2.67 $\pm$ 0.227	3	Weak	a
		20%	2.73 $\pm$ 0.455	3	Weak	a
		30%	3.76 $\pm$ 0.322	3	Weak	a
		40%	5.27 $\pm$ 0.719	3	Moderate	b
		50%	5.87 $\pm$ 0.681	3	Moderate	b
<i>E. coli</i>	Ciprofloxacin	2 μ g/mL	9.28 $\pm$ 1.678	3	Moderate	a
		4 μ g/mL	10.07 $\pm$ 0.840	3	Strong	a
	Guava Leaf	10%	0.99 $\pm$ 0.191	3	Weak	a
		20%	1.82 $\pm$ 0.250	3	Weak	a

Bacteria	Treatment	Concentration	Inhibition Zone Diameter (mm) Mean $\pm$ SD	n	Activity Category	Sig.
	Celery Stalk	30%	3.75 $\pm$ 0.844	3	Weak	b
		40%	4.21 $\pm$ 0.514	3	Weak	b
		50%	4.09 $\pm$ 0.399	3	Weak	b
		10%	0 $\pm$ 0	3	—	a
		20%	1.19 $\pm$ 0.382	3	Weak	ab
		30%	0.86 $\pm$ 0.889	3	Weak	ab
		40%	1.73 $\pm$ 0.201	3	Weak	b
		50%	1.94 $\pm$ 0.326	3	Weak	b

Note: Different letters within each treatment group indicate significant differences ($p < 0.05$). Ciprofloxacin: one-way ANOVA, $p = 0.506$ (*E. coli*), $p = 0.088$ (*S. aureus*). Guava leaf vs. *S. aureus*: one-way ANOVA, $p = 0.345$ (ns). Guava leaf vs. *E. coli*: one-way ANOVA, $p = 0.000$, Post-hoc Tukey. Celery stalk vs. *S. aureus*: one-way ANOVA, $p = 0.000$, Post-hoc Tukey. Celery stalk vs. *E. coli*: Kruskal-Wallis, $p = 0.039$. ns = not significant.

Table 2b. Antibacterial Activity of 40% Single Extracts and Their Combination

Bacteria	Treatment	Ratio/Concentration	Inhibition Zone Diameter (mm) Mean $\pm$ SD	n	Activity Category	Sig.
S. aureus	Guava Leaf	40%	11.75 $\pm$ 0.368	3	Strong	a
	Celery Stalk	40%	4.58 $\pm$ 0.438	3	Weak	b
	Combination	1:1	10.26 $\pm$ 0.463	3	Strong	a
		1:2	8.09 $\pm$ 2.124	3	Moderate	ab
		2:1	11.06 $\pm$ 1.094	3	Strong	a
	Control (+)	4 μ g/mL	9.95–13.74	3	Moderate– Strong	—
	Control (–)	—	0 $\pm$ 0	3	—	—
E. coli	Guava Leaf	40%	3.25 $\pm$ 0.498	3	Weak	a
	Celery Stalk	40%	2.47 $\pm$ 0.788	3	Weak	a
	Combination	1:1	3.74 $\pm$ 1.769	3	Weak	a
		1:2	3.28 $\pm$ 0.958	3	Weak	a
		2:1	2.43 $\pm$ 0.672	3	Weak	a
	Control (+)	4 μ g/mL	13.28–17.45	3	Strong	—
	Control (–)	—	0 $\pm$ 0	3	—	—

Note: Different letters within each bacterial group indicate significant differences ($p < 0.05$). *S. aureus*: one-way ANOVA, $p = 0.000$, Post-hoc Games-Howell (data not homogeneous, Levene $p = 0.033$). *E. coli*: Kruskal-Wallis, $p = 0.335$ (no significant difference among treatments). Variation in positive control values reflects independent experimental runs conducted on different days. ns = not significant.

Following bacterial identification, antibiotic sensitivity and extract activity were evaluated using inhibition zone diameter as the primary parameter. Ciprofloxacin was used as a positive control and exhibited antibacterial activity against both bacteria, with the highest inhibition observed against *E. coli* at 4 µg/mL, measuring 10.07 mm and categorized as strong, compared to *S. aureus*, measuring 9.43 mm and categorized as moderate.

Guava leaf extract demonstrated strong antibacterial activity against *S. aureus*, with the highest inhibition at 50% concentration, measuring 13.56 mm, followed closely by 40% at 13.23 mm. In contrast, activity against *E. coli* was markedly lower, with the highest inhibition observed at 40%, measuring 4.21 mm and categorized as weak. The lower activity against *E. coli* compared to *S. aureus* is likely attributable to structural differences in their cell walls. As a Gram-negative bacterium, *E. coli* possesses an outer membrane composed of lipopolysaccharides (LPS) that acts as an additional permeability barrier, limiting the penetration of hydrophilic antibacterial compounds such as tannins and saponins into the cell.¹² This outer membrane reduces the accessibility of bioactive compounds to their intracellular targets, resulting in smaller inhibition zones compared to *S. aureus*, which lacks this outer membrane and is therefore more susceptible to tested extracts.

Celery stalk extract showed moderate activity against *S. aureus* at higher concentrations, with the greatest inhibition at 50%, measuring 5.87 mm, and at 40%, measuring 5.27 mm. However, its activity against *E. coli* was weak to negligible, with the highest inhibition at 50%, measuring 1.94 mm.

Overall, both extracts exhibited increased antibacterial activity with increasing concentration. However, the difference between 40% and 50% concentrations was minimal, indicating a plateau effect.¹² Therefore, 40% concentration was considered the most optimal and efficient for antibacterial activity testing.

Antibacterial testing of 40% guava leaf extract, 40% celery stem extract, and their combinations showed that against *S. aureus*, guava leaf extract alone produced the highest inhibition of 11.75 mm, while celery stalk showed the lowest at 4.58 mm. No combination exceeded the activity of guava leaf extract, although the 2:1 ratio showed the closest effect at 11.06 mm.

Against *E. coli*, all treatments exhibited weak activity. The highest inhibition was observed in the 1:1 combination at 3.74 mm, slightly higher than guava leaf extract at 3.25 mm and celery stalk extract at 2.47 mm. Overall, combinations did not markedly improve antibacterial activity compared to single extracts. Differences in inhibition zone results between single extracts and their combinations may be influenced by several factors, including extract stability and homogeneity, variations in bacterial suspension density, and agar medium thickness.¹⁸

LIMITATION

This study is subject to several limitations. The antibacterial evaluation relied on the disc diffusion method, which primarily reflects inhibition zone formation and may not accurately quantify the minimum effective concentration of the extracts. The absence of MIC and MBC determination limits the interpretation of antibacterial potency. In addition, only a single extract concentration was selected for combination testing, which may not fully capture the potential interaction profile between the two extracts. The study was also restricted to two representative bacterial strains, thereby limiting broader applicability. Future studies should incorporate quantitative

antibacterial assays, evaluate a wider range of extract concentrations and ratios, and include additional microbial strains to better characterize the antibacterial potential and interaction effects.

CONCLUSION

The combination of *Psidium guava* L. leaf extract and *Apium graveolens* L. var. *dulce* stalk extract did not enhance antibacterial activity compared with the most active single extract. Against *S. aureus*, the 2:1 combination showed the highest inhibition among the combinations, with an inhibition zone of 11.06 mm, but it remained lower than 40% guava leaf extract. Against *E. coli*, all combination showed weak antibacterial activity, with the 1:1 combination producing the highest inhibition zone of 3.74 mm.

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AUTHOR CONTRIBUTION STATEMENT

DIS: Conceptualization, Methodology, Validation, Investigation, Resources, Writing-Original draft, Writing-review & editing ; **PES:** Software, Formal analysis, Data curation, Visualization ; **IAH:** Project administration, Funding acquisition, Supervision.

CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest

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