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Research Article

Active Compound Identification and Antimicrobial Activity Analysis of Green Betel Leaves (*Piper betle* L.) Essential Oil Against *Staphylococcus epidermidis* and *Candida albicans*

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Abstract

Long-term and excessive use of drugs in the therapeutic management of microbial infections leads to increased antimicrobial resistance. It is necessary to select therapeutic management using natural ingredients, as well as combine some natural ingredients with conventional drugs, to prevent antimicrobial resistance. Green betel leaves have a high potential as a drug, especially as an antimicrobial agent. The study identified the active compound in the green betel leaf essential oil and determined its antimicrobial activity against *Staphylococcus epidermidis* and *Candida albicans*. The bioactive components in the oil were analyzed using gas chromatography-mass spectrometry (GC-MS). The antimicrobial and antifungal properties of green betel leaf essential oil were determined using the Kirby-Bauer disc diffusion method. Based on the GC-MS analysis, green betel leaf essential oil contains chavicol acetate, chavibetol, eugenol acetate, α-selinene, and β-selinene. The antibacterial activity assay showed that green betel leaf essential oil could inhibit the growth of *S. epidermidis* at a concentration of 12,5%, while the antifungal activity showed that the zone inhibition of *C albicans* was formed in the concentration of 12,5%. The zone inhibition diameter of antifungal activity tests was higher than in the antibacterial activity assay. The combination of essential oils with antibiotics and antifungal agents did not show increased activity, suggesting that the combination was not synergistic.

Keywords: antibacterial; antifungal; essential oil; green betel

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1 Introduction

Essential oils have a higher potential for antimicrobial activity than the ethanolic extract due to their purity [1]. Not all plants can be extracted for their essential oils. Green betel is an aromatic plant from which essential oil can be extracted. An analysis of its bioactive compounds reported that green betel leaves contain essential oils with phenolic and terpenoid compounds. Those compounds have antimicrobial activity, especially as antifungals and antibacterials [2].

Betel plants can grow and develop properly throughout Indonesia and in Asian countries such as Malaysia, Thailand, the Philippines, India, Sri Lanka, and Bangladesh. Betel plants can grow well in almost all climates and soil types, especially when planted in open areas that get full sunlight, and they can also grow in shaded areas with sunlight intensity of around 50%. The planting location of the betel plant does not require a large area. It can be planted on a house terrace, wall, fence, polybag, pot, or even in a recycled container [3]. Therefore, the potential of betel in Indonesia can be optimized, including being used as a good antimicrobial agent. Thus, research using green betel leaves is a consideration in the development of alternative antimicrobials. In this study, green betel leaves were used against *Staphylococcus epidermidis*, which causes acne vulgaris, and *Candida albicans*, which causes oral candidiasis.

Acne vulgaris is a disease that typically appears during adolescence and into adulthood. It is characterized by the appearance of blackheads, papules, pustules, nodules, and cysts in several parts of the human body, such as on the face or back [4]. According to the Indonesian cosmetic dermatology study, it showed that in 2006, the number of acne vulgaris sufferers was 60%, in 2007, the number of acne vulgaris sufferers was 80%, and in 2009, the number of acne vulgaris sufferers was 90% [5]. The high number of acne cases has an impact on reducing the standard of living, decreasing self-confidence, and hampering the social activities of sufferers. Several factors can affect the onset of acne, including heredity, race, food, climate, hygiene, and stress or psychological factors [6]. In addition, acne can also arise due to bacterial infections *caused by bacteria such as Propionibacterium acnes* and *Staphylococcus epidermidis* [7].

Management of acne vulgaris can be done through topical or systemic drug therapy. Topical medications such as Benzoyl peroxide (BPO), topical clindamycin, or topical retinoids can be an initial therapeutic option for acne treatment. However, topical antibiotics such as topical clindamycin are not recommended as primary monotherapy due to the risk of resistance. Systemic antibiotics are recommended in patients with moderate to severe acne, as well as inflammatory acne that has been resistant to topical treatment. The use of monocycline and doxycycline antibiotics is now more effective when compared to tetracycline-class antibiotics. However, the use of these options should be careful and guided by the severity of the patient's acne [6].

The other infectious disease that usually occurs in humans is oral candidiasis. Oral candidiasis is a lesion caused by fungal infection of the oropharyngeal cavity. *Candida albicans* is the most common Candida species, accounting for the majority of oral candidiasis. Oral candidiasis is quite common in Indonesia, with 7,098 cases reported and 24,482 cases reported among HIV/AIDS patients [8]. The pharmacological treatment for C. albicans fungal infection is antifungal. Some common antifungals include nystatin, amphotericin B, clotrimazole, ketoconazole, fluconazole, and itraconazole. However, there is an increase in antifungal resistance, particularly among Candida fungus species [9]. Fluconazole and nystatin, which are the most effective treatments in oral candidiasis, have developed drug resistance for various Candida species due to overuse of these drugs, so this resistance has become a serious medical problem [10]. Previous research has described processes underlying *C. albicans* resistance associated with biofilms, which are comparable to those in bacterial biofilms [11].

Long-term and excessive use of drugs in the therapeutic management of acne vulgaris and oral candidiasis leads to increased antimicrobial resistance [12]. Therefore, it is necessary to select therapeutic management options that use natural ingredients, as well as combinations of natural ingredients and

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conventional drugs, to prevent antimicrobial resistance. This study seeks to see the ability of green betel leaves essential oil to inhibit the growth of microbes that cause diseases.

2 Method

2.1 Extraction of essential oil

A total of 30 kg of fresh green leaves were extracted using a steam-distillation system for 6 hours. The distillate was collected in a separating funnel. The oily phase was removed, dried over anhydrous sodium sulfate, and stored in a dark glass bottle at 4 °C [13].

2.2 Chemical compound identification

Green betel leaf essential oil was submitted for GC–MS analysis in the Integrated Laboratory for Research and Testing, Universitas Gadjah Mada, Indonesia. The analyses were performed using a Shimadzu GC2010 system equipped with an HP5-MS fused silica capillary column (30 m × 0.25 mm × 0.25 μm). The chromatographic conditions were as follows: carrier gas, helium at a flow rate of 1.0 mL min^{-1}; injector temperature, 280 °C; MS interface temperature, 280 °C; ion source temperature, 260 °C; and ionization energy, 70 eV. The oil samples, 0.2 mL, were dissolved in 0.3 mL of purified ethanol, and 1 μL of the solution was injected for analysis. The isolated compounds were identified by their retention time and determined in reference to a series of *n*-alkanes and verified by a comparison of mass spectral data with the literature, and eventually by comparing their mass spectra with the GC–MS spectral library.

2.3 Preparation of Microbial Suspension

Candida albicans and *Staphylococcus epidermidis* were prepared using the swab technique based on [14] with modifications. One loop from a single colony of *C. albicans* was inoculated aseptically into Sabouraud Dextrose Broth (SDB) medium and incubated for 16 hours at 37 °C to obtain freshly grown strains. Furthermore, *S. epidermidis* was inoculated using the same method, using Nutrient Broth (NB) as the culture medium. An amount of 100 μL overnight culture was transferred to 5 mL fresh media and incubated until the fungal concentration reached equivalent to 0.5 McFarland (approximately 1.5 ×108 CFU/mL) and used for antifungal susceptibility test [15].

2.4 Antimicrobial activity test

The antimicrobial susceptibility test was conducted using the agar diffusion method, following the National Committee for Clinical Laboratory Standards (CLSI) 2009 procedures. Green betel leaf essential oil was prepared at concentrations of 50%, 25%, 12.5%, 6.25%, 3.125%, 1.5625%, and 0.78125%, and diluted in 1% DMSO. Chloramphenicol, as a positive antibiotic control, was prepared at a concentration of 20 mg/mL. Meanwhile, nystatin, as a positive antifungal control, was prepared at a concentration of 100,000 IU/mL. DMSO was used as the negative control. The samples were dripped as much as 20 μL on the disc paper and then placed on the agar media containing *C. albicans* or *S. epidermidis* culture. Then, it was incubated for 16 hours at 37 °C. The zone of inhibition was measured using a caliper on three diagonal sides, and the average was calculated.

3 Result and Discussion

3.1 Extraction of Essential Oil

The oil obtained from the 30 kg of green betel leaves used in this study was 13 mL. The essential oil produced from the steam-water distillation of green betel leaves required the addition of anhydrous sodium sulfate to remove any remaining water in the oil.

3.2 Chemical compound identification

GC-MS analysis showed that 68 chemical compounds were found in green betel leaves essential oil, as shown in Figure 1. A thermal gradient gas chromatography system was used in this study. The temperature was set from a low temperature to an increasingly high temperature over time, with a maximum temperature of 280 °C [16]. The lower the boiling point of the substance, the faster it passes through the column (shorter retention time). Thus, the shorter retention time indicated the higher

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polarity [17]. Therefore, the phenolic group has a shorter retention time than the terpenoid group because the phenolic group is more polar than the terpenoid group [18].

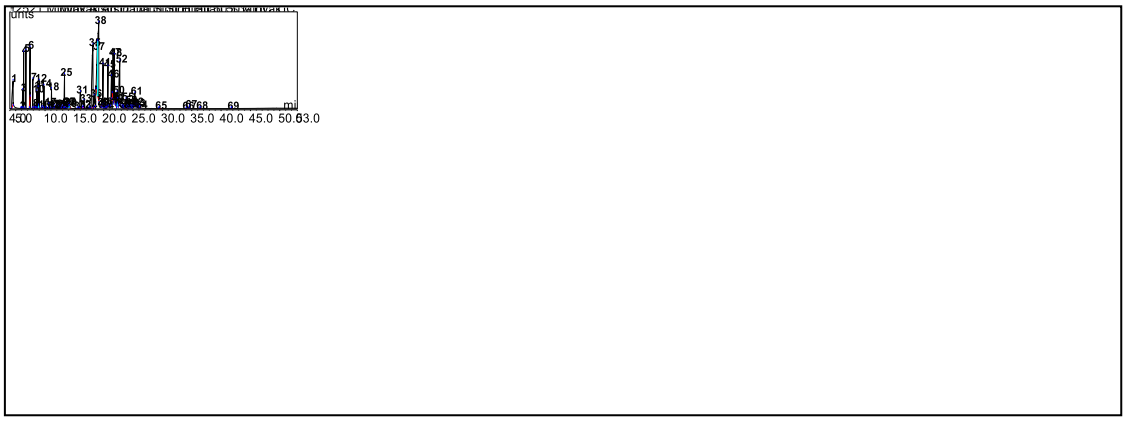

Figure 1 Chromatogram of Green Betel Leaves Essential Oil

Among the chemical compounds detected in the extract, there are five compounds that have the highest concentration. These compounds were chavicol acetate (13.28%), chavibetol (13.25%), eugenol acetate (6.68%), α-selinene (4.11%), and β-selinene (3.88%). Chavicol acetate, chavibetol, and eugenol acetate are categorized as phenolic compounds, while α-selinene and β-selinene are categorized as terpenoid compounds, as shown in Table 1. According to Azahar (2020), compounds that have antibacterial activity include various combinations of phytoconstituents such as phenols, sterols, flavonoids, polyphenols, tannins, and hydroxychavicol. Based on the GC-MS analysis, the highest compounds in green betel leaf essential oil, such as chavicol acetate, chavibetol, and eugenol acetate, are bioactive constituents of phenolic chemical compounds that possess a strong antimicrobial activity[19].

Table 1 Five Highest Compounds of Green Betel Leaves Essential Oil

Compound Approximation	Retention Time (minutes)	Percent Area	SI Hit #1	Molecular Weight
Chavicol acetate	18,16	13.28%	888	176
Chavibetol	19,15	13.25%	908	164
Eugenol acetate	22,73	6.68%	920	206
α-selinene	21,82	4.11%,	931	204
β-selinene	21,60	3.88%	942	204

Phenolic compounds and their derivatives work by denaturing bacterial cell proteins [20]. Phenols that react with bacterial cells through the adsorption process cause the formation of hydrogen bonds. At low levels, the interaction between phenol and bacterial cells forms a protein-phenol complex via weak bonds, which then decomposes, allowing phenol to enter bacterial cells and cause protein denaturation. Then, the interaction between phenol and bacterial cells at high levels causes protein coagulation and rupture of cell membranes [21].

The terpenoid group detected by GC-MS in this study comprises aromatic sesquiterpenes (valencene), which are known to exhibit antibacterial activity. Research in 2023 conducted by Oliveira et al., mentioned that in *Staphylococcus aureus* bacteria, valencene activity as an antibacterial is determined by acting as an inhibitor of efflux pumps in the bacterial strain [22]. Compounds with Efflux Pump Inhibitor (EPI) properties will help restore the antimicrobial activity of agents that have previously lost efficacy against microbes during biofilm formation [23], [24].

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The phenolic group detected in this study has an antifungal mechanism that has been described previously. These active components will bind to ergosterol in the fungal cell, and these bonds will inhibit ergosterol biosynthesis and cause the formation of fungal cell pores. The state of fungal cells with pores will lead to disruption of the integrity of the fungal membrane and will further damage the membrane. When the membrane is damaged, there will be cell leakage and loss of membrane potential. In the end, there will be a disruption of cellular function that causes the fungal cell to die [25], [26], [27]. In addition, one of its bioactive components, eugenol acetate, can inhibit biofilm formation on fungal cells; the presence of biofilms on fungal cells increases their virulence [28], [29].

3.3 Antimicrobial activity test

The results of the antimicrobial and antifungal activity test of green betel leaf essential oil (Piper betle L.) on the growth of *S. epidermidis* and *C. albicans* using the Kirby-Bauer disc paper diffusion method on Sabouraud dextrose agar (SDA) media can be seen in Figure 2. The figure shows that the inhibition zone formed in the single antifungal test against *C. albicans* is greater than in the single antibacterial test against *S. epidermidis*.

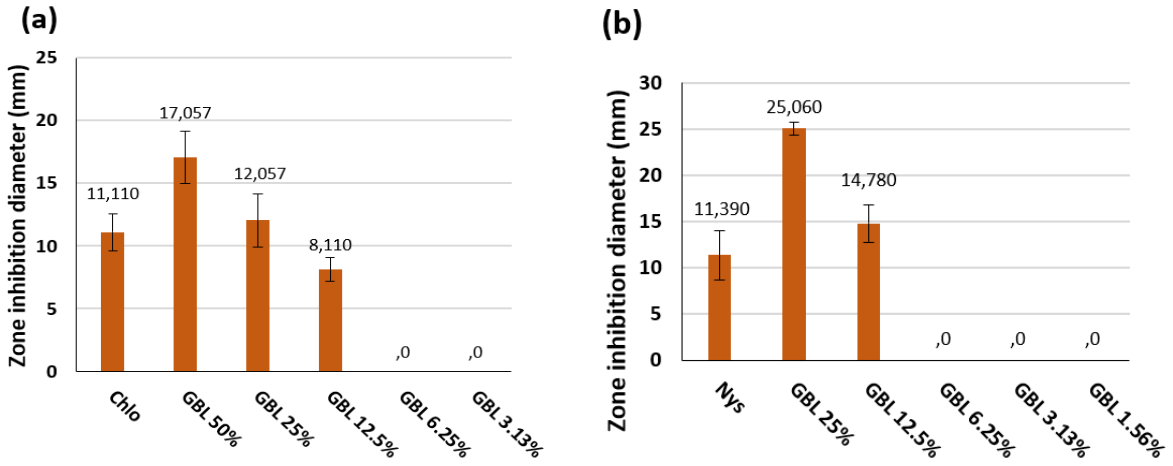

Figure 2 (a) Antibacterial activity test and (b) Antifungal activity test (GBL: green betel leaves extract; Chlo: chloramphenicol; Nys: Nystatin)

The antibacterial and antifungal activity of green betel leaf essential oil is attributed to several bioactive components present in the oil. Antifungal tests against *C. albicans* at concentrations of 25% and 12.5% showed, respectively, *very strong* and *strong* inhibitory activity [30]. In the antibacterial test against *S. epidermidis*, the concentrations of 25% and 12.5% were *strong* and *moderate,* respectively [30]. Thus, the antifungal activity of *C. albicans* is considered better than the antibacterial activity against *S. epidermidis* in a single antimicrobial test of green betel leaf essential oil. The inhibition zone observed in the antimicrobial test of the combination of green betel leaf essential oil and antibiotics is shown in Figure 3. The graphic shows that the inhibition zone formed in the antibacterial combination test against *S. epidermidis* is greater than in the antifungal combination test against *C. albicans*.

The results of the combination test at concentrations of 12.5%, 6.25%, and 3.125% in the antibacterial and antifungal tests showed *strong* inhibitory activity. Figure 3 shows that the inhibition zone diameter was increased in the combination with essential oils compared to the antibiotics alone. Furthermore, the inhibition zone diameters were increased with the increase in the essential oil concentration, indicating that essential oils could strengthen the antimicrobial activity of chloramphenicol. The results of the antibacterial combination test of green betel leaves essential oil are considered synergistic towards inhibiting the growth of *S. epidermidis*. This is because the size of the inhibition zone is larger in the combination test than in the single essential oil test conducted. However, the antifungal combination showed different results. The combination of nystatin and green betel essential

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oil did not show a significant increase in zone inhibition diameter. As shown in Figure 3, the combination of antifungal with green betel essential oil could weaken the antimicrobial activity.

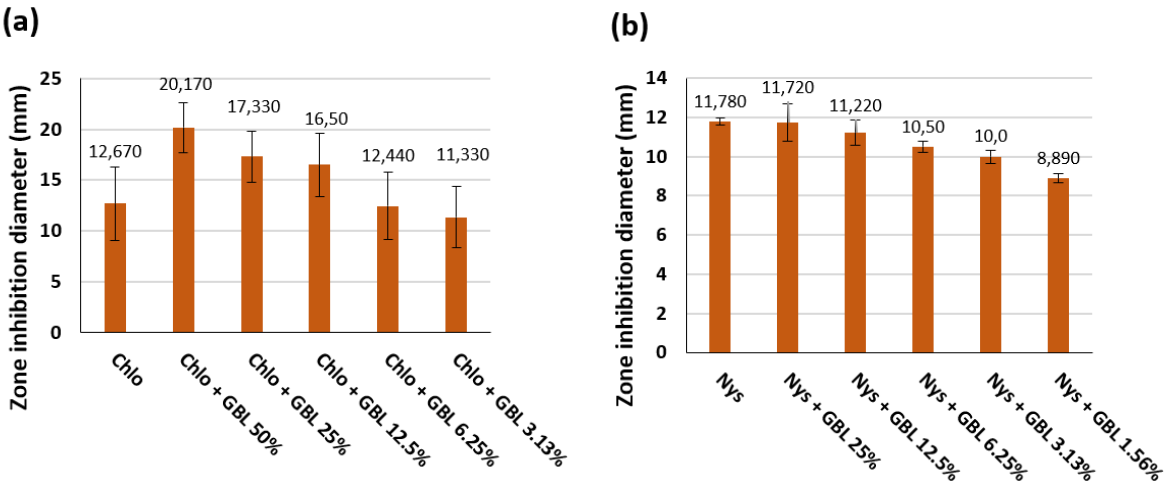

Figure 3 (a) Combination Antibacterial Test and (b) Combination Antifungal Test (GBL: green betel leaves extract; Chlo: chloramphenicol; Nys: Nystatin)

The results show a synergistic effect in the combination of antimicrobial activity test, which might be because there are differences in the mechanism between chloramphenicol and green betel leaf essential oil. Chloramphenicol has a mechanism of action to inhibit bacterial growth through inhibition of cell protein synthesis. This antibiotic readily enters bacterial cells and reversibly binds to the 50S ribosomal subunit. Chloramphenicol antibiotics prevent peptidyl transferase from interacting with its amino acid substrate, thereby inhibiting peptide bond formation [31]. Green betel leaf essential oil acts as an antimicrobial agent by disrupting the cell membrane. The combination of these two activities might strengthen the antimicrobial activity against *S. epidermidis* [20]. Therefore, this difference in mechanism enhances the growth-inhibitory effect of *S. epidermidis* bacteria.

The results of the antifungal combination test of green betel leaves essential oil are considered not synergistic towards inhibiting the growth of *C. albicans*. This is because the inhibition zone is smaller in the combination test than in the single essential oil test. Precisely, the properties shown in this combination test compared to the single test are antagonistic. This might be because the mechanism of green betel leaves essential oil and the antibiotic used, nystatin, have the same mechanism [32]

If the two components in a combined antifungal agent act through different target mechanisms, this will increase their ability to inhibit microbial growth [32]. However, the target and mechanism of action of the antifungal agents used in this study, which are nystatin and green betel leaf essential oil (phenolic group), are the same, that is, by targeting the fungal cell membrane to inhibit its growth. Nystatin binds to ergosterol in fungal cell membranes, forming pores that cause electrolyte leakage, cell lysis, and cell death [27]. Having the same target, the phenolic group inhibits ergosterol biosynthesis and forms pores in fungal cells. That activity leads to disruption of the integrity of the cytoplasmic membrane and will further damage the cytoplasmic membrane of the fungus, ending in fungal cell death [33]. However, the mechanism underlying the antagonistic interaction between essential oils and the antifungal agent nystatin is not yet well understood.

This finding provides an overview of potent alternative antimicrobials and of the combination of conventional antibiotics with natural ingredients. Therefore, it is necessary to expand the study to include natural ingredients, as well as combinations of natural ingredients with conventional drugs, to prevent antimicrobial resistance. Another study should be conducted to explore the potential practical applications of green betel leaf essential oil as a single or complementary natural antimicrobial agent for various pharmaceutical formulations. In practical terms, the green betel leaf essential oil holds significant

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potential for development into topical cosmeceutical formulations, such as anti-acne gels or creams, as well as oral health products such as medicated mouthwashes or mucoadhesive gels to combat oral infections.

4 Conclusion

Based on the results of GC-MS analysis of green betel leaf essential oil, the 5 highest compounds were the compound chavicol acetate (13.28%), chavibetol (13.25%), eugenol acetate (6.68%), α-selinene (4.11%), and β-selinene (3.88%). The compound contributes to the essential oil's antimicrobial and antifungal activity. The antibacterial combination test of green betel leaf essential oil was considered synergistic in inhibiting the growth of *S. epidermidis*, whereas the antifungal combination test was not considered synergistic in inhibiting the growth of *C. albicans*. However, further research was needed to strengthen the evidence for the potential of essential oils as antimicrobial agents, including in vivo and formulation studies. These would strengthen the clinical and pharmaceutical relevance of the findings.

5 Declarations

5.1 Acknowledgements

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5.2 Author contributions

Rima Erviana contributed to the conceptualization, methodology, data collection, data analysis, and manuscript preparation; Ajeng Tri Isna and Shafira Salsabila contributed to experimental work; Rifki Febriansyah contributed to research supervision and data interpretation; Vella Laili Damarwanti contributed to study design; Annisa Krisridwany contributed to sample preparation; Sabtanti Harimurti contributed to critical revision of the manuscript and final approval of the version to be published. All authors have read and approved the final manuscript.

5.3 Ethics

Ethical approval for this study was obtained from the Ethics Committee of the Faculty of Medicine and Health Science Universitas Muhammadiyah Yogyakarta, with approval number No.098/EC/EXEM-KEPK FKIK UMY/XI/2023.

5.4 Conflict of Interest

No conflicts of interest have been declared by the authors.

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