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Isolation, Identification, and Antibacterial Testing of Essential Oil from Green Betel Leaf (*Piper Betle* L.) Using Well Diffusion Method

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Abstract

Green betel leaves have been traditionally employed within communities for various medicinal purposes, owing to their rich composition. These leaves are endowed with secondary metabolites encompassing flavonoids, phenols, saponins, and essential oils. Notably, the essential oils within green betel leaves possess a spectrum of biological properties, including antioxidant, antifungal, antidiabetic, anti-inflammatory, antibacterial, antitumor, anti-Alzheimer's, and anti-carcinogenic activities. In this study, the essential oil from green betel leaf collected from Klaten, Central Java, Indonesia, was isolated using water-steam distillation. The components of this isolate were identified using GC-MS analysis. Antibacterial activity was assessed against *Staphylococcus epidermidis* ATCC 12228 (*S. epidermidis*) and *Escherichia coli* (*E. coli*) using the well-diffusion method at various concentrations (25%, 50%, 75%, 90%, and 100%). Chloramphenicol served as the positive control, while a solution of Tween 20 in distilled water used as the negative control. The essential oil derived from green betel leaves exhibited a brownish-yellow color, possessed a distinctive betel aroma, and had a concentration of 0.21% v/w, a refractive index of 1.5001, and a specific gravity of 0.961 g/mL. The GC-MS analysis revealed the presence of 44 components, with the five most abundant constituents being acetyl chavicol (20.65%), germacrene- D (11.55%), eugenol (8.94%), trans-caryophyllene (7.92%), and chavicol (5.74%). Regarding antibacterial activity, the isolate demonstrated strong activity against *S. epidermidis* ATCC 12228 at a concentration of 75%, yielding an inhibition zone diameter of 12.33 mm. Similarly, it exhibited strong activity against *E. coli* at a concentration of 90%, resulting in an inhibition zone diameter of 12.67 mm.

1. Introduction

Green betel (*Piper betle* L.) is a traditional herbal plant from the Piperaceae genus, originally hailing from Malaysia. This plant species thrives in over 700 varieties distributed across the Northern and Southern hemispheres [1, 2]. Moreover, it is prevalent in numerous Southeast Asian countries and is renowned for its medicinal applications in treating various ailments [3]. The therapeutic potential of green betel is attributed to the diverse array of secondary metabolite compounds present in its leaves, such as essential oils, alkaloids, phenols, steroids, terpenoids, flavonoids, saponins, terpinenes, and tannins [4].

Essential oils are a mixture of many volatile substances (secondary metabolites) with several components with a characteristic odor, are unstable, decompose and evaporate easily [5]. In particular, the essential oil derived from green betel leaves predominantly comprises terpenoid and phenol derivative compounds [6]. This essential oil from green betel leaves possesses a wide array of important biological properties, including antifungal, antibacterial, antiamoebic [7], antidiabetic, anti-inflammatory [8], antioxidant, antitumor, anti-carcinogenic [9], anti- Alzheimer [10] and antihelminthic [11]. Furthermore, essential oils can be applied to various

industries, such as food, perfume, pharmaceutical, and others [12].

Urinary tract infections (UTIs) are primarily triggered by bacterial organisms within the bladder. UTIs can affect individuals of all age groups, encompassing men and women. The bacterial culprits responsible for these infections include *Staphylococcus epidermidis*, *E. coli*, *Staphylococcus saprophyticus*, *Proteus mirabilis*, *Enterococcus faecalis*, and *Klebsiella pneumonia* [13]. Furthermore, beyond their role in causing UTIs, *S. epidermidis* bacteria are also associated with skin acne, nosocomial bloodstream infections [14], and kidney infections in newborns [15]. Meanwhile, *E. coli* bacteria are known to cause pneumonia, diarrhea, wound infections, and meningitis in neonates [16].

Green betel leaves exhibit high efficiency against various types of bacteria, including Gram-negative strains like *E. coli* and *P. aeruginosa*, as well as Gram- positive bacteria like *S. aureus* [17]. Numerous investigations have examined the antibacterial properties of green betel leaf essential oil against *S. epidermidis* ATCC 12228 and *E. coli*, primarily employing the disk diffusion method. However, no research has been conducted using the well diffusion method. It is important to highlight that the well diffusion method offers distinct advantages regarding antibacterial testing. This method allows for a more comprehensive assessment as it involves interactions between the sample and bacteria not only on the surface of the agar medium but also within the lower layers of the medium.

In contrast, the disk diffusion method primarily assesses surface interactions. Additionally, the sample volume can be accurately measured in the well diffusion method, whereas the disk diffusion method relies solely on sample absorption. Hence, the well diffusion method is considered a more effective approach [18, 19].

The purpose of this study was to extract essential oil from green betel leaves, identify the chemical compounds within the essential oil using GC-MS (Gas Chromatography-Mass Spectrometry), and collect data regarding the antibacterial effectiveness against *S. epidermidis* ATCC12228 and *E. coli*, employing the well diffusion method.

2. Experimental

Green betel leaves, prepared in simplicia form, were subsequently isolated using steam distillation to isolate their essential oil. The resulting oil was then subjected to physical tests and identified for compound components using GC-MS. Furthermore, the oil was assessed for its antibacterial properties using the well diffusion method against *S. epidermidis* ATCC12228 and *E. coli*.

2.1. Materials and Tools

The materials were green betel leaves obtained from Klaten, Central Java, distilled water, anhydrous Na_2SO_4, C_2H_5OH 70%, Tween 20 (Merck Germany), nutrient agar (Merck Germany), Muller Hinton agar (Merck Germany), peptone (Merck Germany), yeast (Merck Germany), antibiotics Chloramphenicol ($C_{11}H_{12}C_{l2}N_2O_5$), $BaCl_2$ (Merck Germany), H_2SO_4 (Merck Germany), cotton, gauze, *E. coli* bacterial isolates were obtained from the UNDIP FSM Biochemistry laboratory and *S. epidermidis* ATCC 12228 was obtained from the UNS Microbiology laboratory Surakarta.

The tools used were a set of laboratory glassware, analytical balance, a set of distillation tools, Petri dishes, inoculation loop, spatulas, spreaders, Atago refractometer, GC-MS QP2010 SE Shimadzu, Memmert IN 30 incubator, AMT M-11 shaker incubator, Top micropipette Pette Pipettor and tip, Bunsen, ruler, Biobase V800 laminar airflow (LAF), and All American autoclave sterilizer.

2.2. Isolation and Determination of Physical Properties of Essential Oils

A total of 18 kg of green betel leaf simplicia were cleaned, air-dried, chopped, and then subjected to six hours of steam distillation. The essential oils extracted were assessed for various physical properties, including color, odor, refractive index, and specific gravity. The refractive index was determined using an Atago refractometer, and the specific gravity of the green betel leaf oil was measured using a pycnometer [20].

2.3. Identification of Essential Oil Compounds

The essential oils were identified using GC-MS QP2010 SE with a Restek stabilwax-DA column measuring 30 m in length. Helium served as the carrier gas, and the injection temperature was set at 200°C. The column temperature was programmed to increase from 50°C (maintained for 5 minutes) to 230°C at a rate of 5°C/minute. The flow rate was set at 1.22 mL/min, and the pressure was maintained at 69.4 kPa.

2.4. Preparing Samples with Varied Concentrations

Green betel leaf essential oil was made at different concentrations (25% v/v, 50% v/v, 75% v/v, 90% v/v, and 100% v/v). These concentrations were prepared by diluting pure essential oil with the addition of 0.1 mL of Tween 20 [21]. As a reference, the antibiotic chloramphenicol at 5% w/v was utilized as a positive control, and the Tween 20 solution in distilled water as a negative control.

2.5. Preparing Suspensions

Distilled water was used to dissolve yeast and peptone, forming a liquid medium that was subsequently sterilized in an autoclave for 45 minutes. This medium was then divided into three Erlenmeyer flasks. The test bacteria, obtained from stock agar slants, were inoculated into the liquid medium using a sterile tube needle. The bacterial inoculation procedure was conducted in a laminar airflow cabinet, after which the mixture was incubated in a shaker incubator at 150 rpm until it reached a 0.5 McFarland standard.

2.6. Antibacterial Activity

The antibacterial activity of the samples against *E. coli* and *S. epidermidis* ATCC 12228 bacteria was assessed using the well diffusion method. To prepare the solid

medium, Muller Hinton agar (MHA) was dissolved in distilled water and sterilized by autoclaving at 121°C for 45 minutes. After sterilization, the medium was poured into a petri dish and solidified at room temperature. Subsequently, the test bacteria were adjusted to a 0.5 McFarland standard (equivalent to 1.5 x 108 CFU mL^{-1}) [22] and then inoculated.

The test bacteria were taken using a micropipette and subsequently inoculated onto solid media using the spread plate technique (spread method). After inoculation, a waiting period ensued to allow the bacteria to be absorbed into the solid media. In the solid media, holes were created to facilitate the insertion of the test samples, the positive control (chloramphenicol antibiotic), and the negative control (Tween 20 solution in distilled water). The bacterial inoculation process and the introduction of test and control samples were conducted in a laminar airflow cabinet. The bacterial culture in the test medium was then incubated in an incubator at 37°C for 24 hours. The final step encompassed the observation and measurement of the bacterial inhibition zone, which was indicated by the presence of a clear zone, carried out in triplicate [23, 24].

3. Results and Discussion

3.1. Isolation and Determination of Physical Properties of Essential Oils

The obtained essential oil exhibited a yield of 0.21% v/w. The results indicate that the essential oil content in green betel leaves exceeded that reported by Sujono *et al.* [25], although the value was smaller compared to that study, which ranged from 0.25 - 0.53% [26, 27]. This variation can be attributed to numerous factors, including soil fertility, plant age, climate, storage conditions, drying techniques, chopping size, sample condition, and the method of distillation [28]. Among these factors, soil fertility depends on nutrient content, specifically phosphorus, and nitrogen, as these elements play a pivotal role in influencing the biosynthesis process of essential oils, thereby impacting both the quantity and composition of these oils [29].

The assessment of the physical properties of green betel leaf essential oil revealed that it had a brownish-yellow color, a distinct betel aroma, a specific gravity of 0.961 g/mL, and a refractive index of 1.500. Previous studies by Alighiri *et al.* [30] and Guenther [31] reported these findings, specifying the physical attributes of green betel leaf essential oil as brownish-yellow color, a characteristic betel fragrance, a specific gravity within the range of 0.9400-0.9900, and a refractive index within the range of 1.4800-1.5400. The essential oils obtained align with this specified range.

3.2. Identification of Essential Oil Compounds

The compound components in green betel leaf essential oil were identified using Gas Chromatography-Mass Spectrometry (GC-MS). The GC chromatogram analysis revealed 44 peaks, as illustrated in Figure 1.

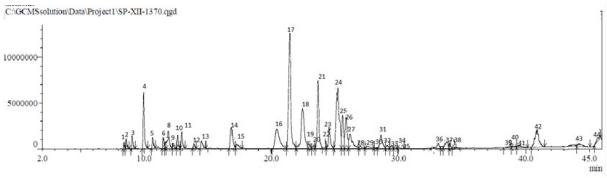

Figure 1. GC chromatogram of essential oils

The five predominant components, specifically acetyl chavicol (20.65%), germacrene-D (11.55%), eugenol (8.94%), trans-caryophyllene (7.92%), and chavicol (5.74%), are shown in Table 1.

Table 1. Identification result of the five predominant components of green betel leaf essential oil

Compound	Retention time (minute)	Width (% area)	SI	B.M	Compound approximation
4	20.434	5.74	93	134	Chavicol
17	21.456	20.65	93	176	Acetyl chavicol
18	22.463	8.94	94	164	Eugenol
21	23.682	7.92	96	204	Trans-caryophyllene
24	25.222	11.55	95	204	Germacrene-D

3.3. Antibacterial Activity

The obtained essential oil was assessed for its antibacterial activity, Gram-positive *S. epidermidis* ATCC 12228, and Gram-negative *E. coli*, utilizing the well diffusion method. The well diffusion method offers several advantages for more effective antibacterial testing. It allows interactions between the sample and bacteria, not limited to the surface of the agar medium, as predominantly seen in the disc diffusion method. Furthermore, the well method provides the measurable volume sample, a feature absent in the disc diffusion method, thus rendering it a more effective approach [18, 19]. Importantly, the well diffusion method is also cost-effective. For the negative control, a solution of Tween 20 in distilled water was chosen because Tween 20 functions purely as a solvent and lacks antibacterial properties, ensuring it does not influence the test outcomes [21]. As for the positive control, chloramphenicol, a bacteriostatic antibiotic, was utilized at a concentration of 5%. Chloramphenicol operates by inhibiting peptidyl transferase during protein synthesis, impairing the functionality of the 50S ribosomal subunit and preventing it from binding to tRNA [32]. The results of the antibacterial activity tests are presented in Figure 2.

Figure 2 shows that an increase in essential oil concentration corresponds to a larger zone of bacterial growth inhibition. The overall results of antibacterial activity assessments against *S. epidermidis* ATCC 12228 reveal larger inhibition zones when compared to *E. coli*. This difference can be attributed to structural characteristics and cell wall composition variations. Gram-positive bacteria only have an outer peptidoglycan layer, whereas Gram-negative bacteria feature an additional outer phospholipid membrane and porins, reinforcing their cell walls. Therefore, Gram-positive bacteria exhibit greater susceptibility to antibacterial agents than Gram-negative counterparts [33]. According

to Davis and Stout [34], antibacterial activity is classified into 4: an inhibition zone diameter <5 mm is categorized as weak, 5-10 mm medium, >10-20 mm strong, and >20 mm very strong.

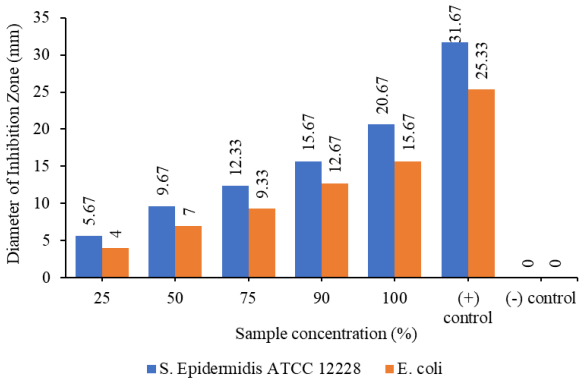

Figure 2. Inhibition zone of green betel leaf essential oil against *S.epidermidis* ATCC 12228, *E. coli*, which was conducted in three repetitions

The antibacterial activity of essential oil exhibited varying degrees of effectiveness at different concentrations. At a 25% concentration, it was categorized as weak against both test bacteria, while at 50%, it was deemed moderate. Interestingly, at a 75% concentration, it was rated moderate against *E. coli* but strong against *S. epidermidis* ATCC 12228. Concentrations of 90% and 100% resulted in a strong classification for both test bacteria. In the positive control (chloramphenicol), the antibacterial effect was very strong [34], whereas the negative control (Tween 20 solution in distilled water) showed no inhibition zone. Comparing these findings to previous research [23], a clear zone of 9 mm was observed at a 75% concentration against *S. epidermidis* ATCC 12228 using the disc diffusion method. Additionally, in another study [35], a clear zone of 12 mm was obtained at a 70% concentration against *E. coli* using the same method. In our study, at a 75% concentration, the inhibition zone measured 12.33 mm for *S. epidermidis* ATCC 12228 and 9.33 mm for *E. coli*, which closely aligns with previous research. However, concentrations above 75% produced larger inhibition zones than the two previous studies.

The compound components of green betel leaf essential oil that act actively as antibacterial agents are thought to be acetyl chavicol, chavicol, eugenol, germacrene-D, and trans-caryophyllene. When bacterial cell walls are exposed to essential oils, a phenomenon occurs wherein the bacterial cells undergo lysis due to an elevation in osmotic pressure induced by the essential oils. The compounds acetyl kavikol, kavikol, and eugenol, which belong to the phenylpropanoid or phenol compound, contribute to the antibacterial mechanism by dissolving phospholipids. This dissolution of phospholipids within essential oils reduces cell permeability, leading to cell lysis and protein denaturation. Consequently, the formation of cytoplasmic proteins and nucleic acids is inhibited [36]. As a consequence of bacterial cell lysis, the bacteria lose moisture and nutrients and eventually die.

The compounds germacrene-D and trans-caryophyllene, classified as sesquiterpene-type terpenoid compounds, exhibit antibacterial mechanisms wherein these terpenoid compounds form strong polymer bonds with porins, which are trans-membrane proteins found on the outer membrane of the bacterial cell wall. This bond formation affects the functionality of porins, which are responsible for facilitating the diffusion of various compounds and ions. As a result of this interaction, smaller molecules essential for cellular metabolism and the elimination of metabolic waste are unable to enter the cell nucleus and consequently become impaired [37]. The impairment of porins leads to a reduction in the permeability of bacterial cells, resulting in insufficient nutrient uptake. This nutritional deprivation hampers bacterial growth and can ultimately lead to bacterial cell death [38].

4. Conclusion

The essential oil extracted from green betel leaves (*Piper betle* L.) yielded 0.21%v/w and exhibited a refractive index of 1.5001 and a specific gravity of 0.961 g/mL. Furthermore, The results of GC-MS analysis revealed the presence of 44 components in the essential oil of green betel leaves (*Piper betle* L.), with five major constituents, namely acetyl chavicol (20.65%), germacrene-D (11.55%), eugenol (8.94%), trans-caryophyllene (7.92%), and chavicol (5.74%). Regarding antibacterial activity, the essential oil from green betel leaves (*Piper betle* L.) demonstrated effectiveness at a concentration of 75% when assessed using the well diffusion method against *S. epidermidis* ATCC 12228 and *E. coli*.

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