Bioprospecting Test of *Piper betle* Leaf Essential Oil Against *Staphylococcus aureus* and *Escherichia coli*-Antibiotic Resistant

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ABSTRAK

Salah satu upaya mengatasi resistensi antibiotik dapat dilakukan melalui pencarian bahan alam tumbuhan yang berpotensi sebagai antibakteri. Salah satu hasil destilasi bahan alam yang berpotensi antibakteri adalah minyak atsiri daun sirih (*Piper betle*). Tujuan penelitian ini untuk mengetahui pengaruh minyak atsiri terhadap *Staphylococcus aureus* dan *Eschericia coli* ressisten antibiotik. Desain penelitian ini adalah eksperimental dengan variabel bebas berupa minyak atsiri konsentrasi 5%,10%,15%,20%,25%, dan 100%, sedangkan variabel terikat adalah diameter zona hambat pertumbuhan bakteri *S. aureus* dan *E.coli.* Ekstraksi minyak atsiri menggunakan destilasi uap, karakterisasi kimia dengan uji GC-MS. Pengujian antibakteri menggunakan metode Kirb-baeur. Hasil Uji GC-MS dominan eugenol (3,50%), Caryophyllene (3,79%), Germacrene D (1,19%). Pemberian minyak atsiri mampu menghambat pertumbuhan *S. aureus* dengan diameter zona hambat 10,5 mm (sedang), 10,6 mm (sedang), 10,8 mm (sedang), 12,3 mm (kuat), 14 mm (kuat), dan 30,2 mm (sangat kuat), sedangkan *E. coli* sebesar 5,7 mm (sedang), 7,5 mm (sedang), 10,5 mm (sedang), 12 mm (kuat), dan 30,03 mm (sangat kuat). Hasil uji one-way ANOVA menunjukkan nilai F_{hitung} sebesar 11762,7 sedangkan F_{tabel} sebesar 3,48 ($F_{hitung} > F_{tabel}$). Kesimpulan dari penelitian ini adalah pemberian minyak atsiri konsentrasi 5%, 10%, 15%, 20%, 25%, dan 100% mampu menghambat pertumbuhan bakteri *S. aureus* dan *E.coli* secara nyata. Pemberian minyak atsiri daun sirih lebih efektif dalam mempengaruh *S.aureus* dibandingkan *E.coli*

Kata Kunci—sirih, piper, minyak atsiri, *S.aureus, E.coli*.

ABSTRACT

One of the efforts to overcome antibiotic resistance can be carried out by proving that natural plant materials have the potential to be antibacterial. One of the results of distilling natural materials with antibacterial potential is betel leaf essential oil (*Piper betle*). This study aimed to determine the effect of essential oils on *Staphylococcus aureus* and *Escherichia coli*-resistant antibiotics. The design of this study is experimental, with independent variables in the form of essential oils with concentrations of 5%, 10%, 15%, 20%, 25%, and 100%. In contrast, the dependent variable is the diameter of the growth inhibition zone *of S. aureus* and *E.coli bacteria.* Essential oil extraction using steam distillation and chemical characterization by GC-MS test. Antibacterial testing uses the Kirb-Bauer method. GC-MS test results were dominant eugenol (3.50%), caryophyllene (3.79%), and Germacrene D (1.19%). The application of essential oils was able to inhibit the growth *of S. aureus* with inhibition zone diameters of 10.5 mm (moderate), 10.6 mm (moderate), 10.8 mm (moderate), 12.3 mm (strong), 14 mm (strong), and 30.2 mm (very strong), while *E. coli* was 5.7 mm (moderate), 7.5 mm (moderate), 10.5 mm (moderate), 12 mm (strong), and 30.03 mm (very strong). The results of the ANOVA one-way test showed a value of 11762.7, while the F_{table} was 3.48 (Fcal > Table). This study concludes that treatment essential oils with concentrations of 5%, 10%, 15%, 20%, 25%, and 100% can significantly inhibit the growth *of S. aureus* and *E.coli* bacteria. The treatments of betel leaf essential oil is more effective in influencing *S.aureus* than *E.coli*

Keywords—betel, betel, essential oil, S.aureus, E.coli.

I. INTRODUCTION

The Indonesian people are increasingly using medicinal plant extracts, which can cause dangerous side effects and high prices for synthetic drugs. However, crude extracts are still dominated by powders or brews. Some plants can produce secondary metabolite compounds that optimize medicinal plant efficacy (Pasaribu, 2020).

According to Ratnasari et al. (2022), essential oils are one of the secondary metabolites that need to be tested intensively for scientific evidence. Scientific evidence regarding the efficacy of essential oils is required as a raw material for developing modern pharmaceutical innovation products such as soap or deodorant. Given the urgency of the importance of scientific evidence of the efficacy of essential oils contained in medicinal plants, research on bioprospection is needed to facilitate the selection of local plants that produce essential oils as active ingredients for the manufacture of pharmaceutical products. This is also reflected in the government's program, Government Regulation No. 8 of 1999; Government of the Republic of Indonesia (2020); Regulation of the Minister of Environment and Life No. P.2 of 2018 states that essential oil bioprospecting activities would facilitate the selection of active ingredient data for developing innovative products as a public health solution.

Indonesian Institute of Sciences (LIPI) (2014) stated that bioprospecting has a basic scheme that includes exploration, research, production, and conservation. The principle of essential oil bioprospecting activities is to test the efficacy of essential oils that have the potential to be raw materials for pharmaceutical products, leading to the commercialization of health innovation products.

The bioprospecting activity to be carried out in this study is to test the efficacy of essential oils as antibacterial, especially for bacteria resistant to antibiotics. The essential oil to be tested in this study comes from betel leaves (*Piper betle*) taken in Setu District, Bekasi City. The choice of betel leaves as a source of essential oil is because betel leaves are easy to grow and are widely cultivated in abundant quantities by the people of Bekasi City. Still, they are only used as a stew simplicia for bathing and eliminating body odor. Therefore, researchers are interested in bioprospecting betel leaves in terms of their essential oil content. Essential oil tests were carried out on *S. aureus* and *E. coli* bacteria taken from clinical samples of health institutions. The selection *of S. aureus* and *E.coli* as test bacteria is based on the results of The Global Burden of Bacterial Antimicrobial Resistance (2019) report which states that *E. Coli* and *S. aureus* are the dominant bacteria causing death in 135 countries in the world, especially *antimicrobial resistance* (AMR) strains that are difficult to treat with antibiotics.

Several previous studies regarding the test of green betel leaf essential oil have been conducted on the bacterium *Streptococcus mutants* (Rizkita *et al*., 2017). *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes* (Sujono *et al*., 2019) and *S. epidermidis* (Nisyak *et al*., 2022) . Betel leaf samples were taken from the cities of Boyolali, Ambarawa, Yogyakarta, Cimanggu (Bandung), and Sidoarjo (East Java). The diameter range of the inhibition zone with an essential oil concentration of less than 30% is 7.1 mm-17 mm (Septiani *et al*.,2024).

Referring to the problem, impact, and various previous studies, the author is interested in testing the effect of betel leaf essential oil on *S. aureus* and *E.coli*. This study aims to determine the impact of betel leaf essential oil on growth against *S. aureus* and *E.coli bacteria.* This study is expected to provide information about the effectiveness of the antibacterial concentration of green betel leaf essential oil against *S. aureus* and *E.coli* bacteria, which can be used to determine raw materials for pharmaceutical products.

II. METHODS

A. Research Design

The design of this study is experimental, with *treatment* in the form of betel leaf essential oil 5%, 10%, 15%, 20%, 25%, 100% and Dimethyl sulfoxide (DMSO) as a negative control. All treatments were given to *S. aureus* and *E.coli* for three replicates. The samples used included green betel leaf (*Piper betle* L.) and *S*. aureus and *E.coli* Multidrug resistance *(*MDR*)*. Bacterial was isolated from wound samples of diabetes mellitus

(DM) patients at a diabetes home in Bekasi City. The Kirby Bauer test confirms antibiotic resistance.

B. Distillation of essential oils

Betel leaf essential oil distillation is carried out using the steam distillation method. One hundred fifty grams of betel leaves are put in a round base flask containing 400 ml of sterile water. The distillation process is gradual. Each stage is carried out for 18 hours. The essential oils obtained are removed from their water content by adding anhydrous Na_2SO_4 powder little by little until the Na_2SO_4 floats, and then decanting is carried out to get pure essential oils that are free from water content (Sujono *et al*., 2019).

C. Essential oil characterization test

Essential oil characterization tests were carried out by organoleptic tests and *Gas Chromatography-Mass Spectrophotometry* (GC-MS)

D. Essential oil antibacterial activity test

The antibacterial bioactivity test of betel leaf essential oil was carried out using *the Kirby-Bauer* method by streaking four quadrants on the surface of MHA media with a sterile cotton swab. Essential oils at 5%, 10%, 15%, 20%, 25%, and 100% concentrations were dripped by 30 µl on a blank disc using a micropipette and waited ± 15 minutes. Negative control is in the form of DMSO. All discs are placed on top of the petri dish containing MHA media using sterile tweezers. Each petri dish is repeated 3 times. All Petri dishes are then incubated for 18 hours at a temperature of 37◦C. The diameter of the inhibition zone was measured after 1x18 hours of incubation by measuring the presence/absence of clear zones formed around the treatment disc using a ruler. The inhibition zone diameter measurement results were then compared with the CLSI (2020) guidelines in Anindita et al. (2022) to see the sensitivity category of the test bacteria in responding to each treatment disc.

III. RESULTS AND DISCUSSION

This study obtained samples of betel leaves in Bekasi City as a source of essential oil. The morphology of betel leaves in this study can be seen in Figure 1.

Figure 1. Morphology of betel leaves

The results of the determination of Figure 1 were carried out at the Depokensis Herbarium, University of Indonesia No. 149 / UN2. F3.11/PDP.02.00/2024 shows that this study's green betel leaf sample has the Latin name *Piper betle* L. (Family: Piperaceae). The morphology of green betel leaves is ovate; the base is notched, the edges are flat, the tips are tapered, the leaf surface is shiny, the leaf bones are pinnate, the Phyllotaxis includes folia sparsa (scattered), and includes incomplete single leaves (Mauludiyah *et al*., 2024). Green betel leaves are then extracted using steam distillation. The results of essential oil vapor distillation can be seen in Figure 2.

Figure 2. Green betel leaf essential oil

Figure 2 shows that the organoleptic characteristics of betel leaf essential oil are brownish-yellow with a distinctive aroma of betel leaf essential oil. The results of this study are by the researchers Azzahra *et al.* (2018), Saraswati

et al. (2019), and Gunawan & Kurniaty (2021) who reported that the organoleptic of the essential oil of green betel leaves is liquid, brownish-yellow in color, with a distinctive aromatic aroma of green betel leaf with a bitter and slightly spicy taste. Essential oils are generally clear, colorless liquids, but during storage, they will thicken, become yellowish or brownish, and smell, according to the plants that produce them.

Green betel leaf essential oil was then tested by GC-MS. The results are in Table 1.

Table 1. GC-MS test results of betel leaf essential oil

Component No.	Separation time	Percentage (%)	Dominant components	Chemical Formula
1	18.78	3.50	Eugenol	$C_{10}H_{12}O_2$
2	19.80	3.79	Caryophyllene	$C_{15}H_{24}$
3	21.30	1.19	Germacrene D	$C_{15}H_{24}$

Based on Table 1, it can be seen that betel leaf essential oil obtained from Bekasi City contains eugenol compounds (3.50%), Caryophyllene (3.79%), and Germacrene D (1.19%). The results of this study are by the review of Nagori *et al.* (2011) that the dominant compounds found in betel leaf essential oils include eugenol, quercetin, caryophyllene, safrole, α-pinene, myrcene, chavicol, Germacrene-D, α-terpineol, ß-pinene, and research includes Germacrene-D (16.07%), Eugenol (7.17%), and germacrene-D (11.55%), eugenol (8.94%-18.9%), caryophyllene (7.92%). The results of other studies on betel essential oil from Vietnam reported the content of domina eugenol (24.56%), germacrene D (5.98%), and caryophyllene (3.32%), Mumbai (India) produced eugenol (11.59%), germacrene D (12.68%), and caryophyllene (11.92%). (Periyanayagam *et al.*,2011; Fachriyah *et al.*, 2023; Ahmad *et al.*, 2024; Jadhav *et al.*, 2020).

The essential oil results were then tested on *S. aureus* and *E.coli bacteria.* Confirmation of *amoxicillin-resistant, cefotaxime, cefixime, and ceftriaxone-resistant* S. aureus and *E. coli*-resistant Ampicilin (AMP), Imipinem (IMI), Cefuroxime (CXM), Ceftriaxone (CRO), Ciprofloxacin (CIP), Tetrasiklin (TE) was performed by the Kirby Beaur test. The results of the test of betel leaf essential oil with concentrations of 5%, 10%, 15, 20%, and 15% against *S. aureus* and *E.coli* can be seen in Table 2.

Table 2. Effect of betel leaf essential oil on the diameter of the inhibition zone *of S. aureus* and *E.coli*

Treatment	Diameter of the inhibition zone (mm)								F-count	F-table
	S. aureus				E.coli					
	I	II	III	Average	I	II	III	Average		
DMSO	0	0	0	0	0	0	0	0		
100%	30	30,1	30,2	30,1	30	30	30,1	30,03		
25%	13,9	14	14,1	14	12	12	12	12		
20%	11,9	12	12,4	12,3	10,5	10,5	10,5	10,5	11762,7*	3,48*
15%	10,8	10,8	10,8	10,8	9	9	9	9		
10%	10,6	10,6	10,6	10, 6	7,5	7,5	7,5	7,5		
5%	10,5	10,5	10,5	10, 5	5,7	5,7	5,7	5,7		

*One-way ANOVA : Fcal >Ftabel (H0 rejected : there is a significant difference)

Based on Table 2, it can be seen that the effect of betel leaf essential oil at concentrations of 5%, 10%, 15%, 20%, 25%, and 100% can inhibit the growth of *S. aureus* with inhibition zone diameters of 10.5 mm (intermediate), 10.6 mm (intermediate), 10.8 mm (intermediate), 12.3 mm (strong), 14 mm (strong), and 30.2 mm (very strong), while *E. coli* respectively 5.7 mm (intermediate), 7.5 mm (intermediate), 10.5 mm (intermediate), 12 mm (strong), and 30.03 mm (very strong). The results of the ANOVA one-way test showed a Fcal value of 11762.7 while the table F = 3.48 (Fcal. > Ftable) or the treatment of essential oils at concentrations of 5%, 10%, 15%, 20%, 25%, and 100% was able to inhibit the growth of *S. aureus* and *E.* significantly. The

results of the study complement the research conducted by Sujono *et al*. (2019), who reported that the administration of essential oil from Cimanggu (West Bandung) 10%, 20%, 30%, and 40% was able to inhibit the growth of non-resistant *S. aureus* with an inhibition zone diameter of 13.85 mm, 17.6 mm, 20.85 mm, 23.9 mm, while Fachriyah *et al.*, 2023 reported the treatment of betel leaf essential oil 25%, 50%, 75%, 90%, 100% can inhibit the growth of *E.coli* by 4 mm, 7 mm, 9.33 mm, 12.67 mm, 15.67 mm, and 25.33 mm. The results of Septiani *et al*. (2024) establish that 10%, 20%, and 30% green betel leaf essential oil can inhibit *S. aureus* by 17.2 mm, 17.4 mm, and 18.8 mm. Nisyak *et al*. (2022) added the antibacterial effect of betel leaf essential oil 0.5%, 1%, 5%, 10%, 15,20%, and 25% able to inhibit *Methicillin Resistant Staphylococcus aureus* (MRSA) Bacteria by 7.3 mm, 7.8 mm, 8.5 mm, 9 mm, 10.4 mm, 11.3 mm, and 11.9 mm.

Another result was reported by (Ahmad et al., 2024), who established that 100% green betel leaf essential oil from Kampung Terusan, Juasseh, Negeri Sembilan, Malaysia was able to inhibit *S. aureus* by 8.67 mm, while *E.coli* was 7 mm. A study by Gupta (2023), tested a 100% concentration of Indian essential oil inhibited *E.coli* by 7 mm. Antibacterial activity is divided into four categories, namely the diameter of the inhibitory zone < 5 (low), 5-10 mm (intermediate), > 10-20 mm (strong), and > 20 mm (very strong) (Salsabila et al., 2024).

The antibacterial effectiveness of betel leaf essential oil in inhibiting pathogenic bacteria is due to the presence of dominant compounds: eugenol, Caryophyllene, and Germacrene D (GC-MS results table 1). This is explained in the study by Musdja *et al.* (2019), which shows that the mechanism of eugenol of betel leaf essential oil inhibits the growth of pathogenic bacteria by damaging the cell membrane so that the release of cell metabolites such as proteins and nucleic acids characterizes cell leakage. Nayaka et al. (2021) reported that the content of betel leaf essential oil consists of monoterpenoids, sesquiterpenoids, phenylpropanoids, and aldehydes. Silva *et al.* (2017) state that betel leaf essential oil compounds comprise hydrocarbon monoterpenes, hydrocarbon sesquiterpenes, oxygenated scuterpenes, and phenylpropanoids. The dominant ingredient that functions as an antibacterial is found in the phenylpropanoid component. Sharifi-rad et al. (2017) added that eugenol is one of the dominant components of phenylpropanoid that inhibits the growth of pathogenic bacteria. The high and low levels of essential oil compounds are affected by the growth location, the plant's age, and the harvest time.

Figure 3 compares the average diameter of the essential oil inhibitory zone between *S. aureus* and *E. coli*.

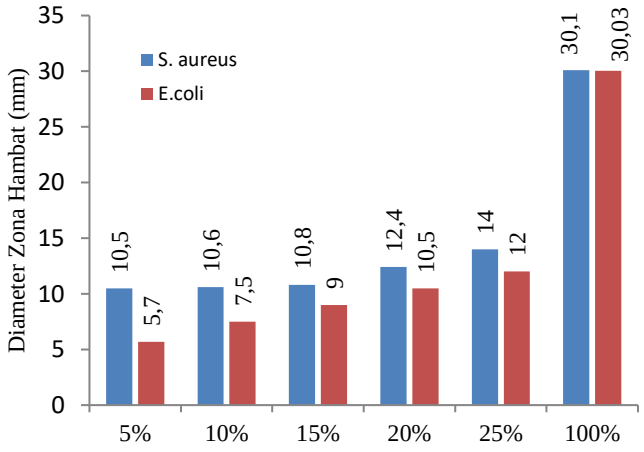

Figure 3. Comparison diagram of the inhibition of green betel leaf essential oil

Figure 3 shows that treatment betel leaf essential oil with concentrations of 5%, 10%, 15%, 20%, 25%, and 100% is more optimal in inhibiting *S. aureus* than *E.coli*. The results of this study complement the review conducted and that Biswas *et al.* (2022) and Singh *et al.* (2023) *P.betle* essential oil is more optimal in inhibiting *S. aureus* and C. *albicans* than *E.coli.* Sateriale *et al.* (2022) stated that essential oils were more effective in gram-positive bacteria than gram-negative. Perwitasari et al. (2023), *S. aureus* is a gram-positive bacterium that does not have a phospholipid barrier in the peptidoglycan layer, so it is easily penetrated by lipophilic essential oils, while *E. coli* is gram-negative and has a phospholipid barrier on the peptidoglycan layer, so essential oils do not easily enter the cytoplasm. Furthermore, Anindita *et al*. (2022) explained that *S. aureus* is more sensitive to essential oil treatment than *E. coli*. The sensitivity of *S. aureus* is because the bacterium does not have an outer membrane as a direct protector of its cell wall (Galgano *et al*., 2022). As a result, essential oils easily damage cell membranes, impacting cytoplasmic leakage (Martínez *et al*., 2021), while *E. coli* has an outer barrier (phospholipids) to prevent the entry of various antibiotic and antibacterial compounds into space (Li *et al*., 2024). In addition, the presence of enzymes in the periplasmic of *E. coli* is thought to be able to break down essential oil molecules (Bai *et al*., 2023; Zhao *et al*., 2023).

IV. CONCLUSION

One Way ANOVA's parametric comparative statistical test produced a value of Fcal=11762.7. At the same time, the F_{table} = 3.48 (Fcal > Ftable) or the treatment of essential oils with concentrations of 5%, 10%, 15%, 20%, 25%, and 100% was able to inhibit the growth *of S. aureus* and *E. coli* significantly. The results of the antibacterial activity test are more effective in *S. aureus* bacteria than *E.coli.*

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