

Crude Aceh patchouli alcohol (*Pogostemon cablin* Benth.) elucidated antibiofilm activity of against *Staphylococcus aureus*

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Abstract. The emergence of biofilm-producing bacteria has prompted the search for novel antibacterial agents, including plant-based compounds, such as patchouli oil (*Pogostemon cablin* Benth.). This study evaluated the phytochemical and antibiofilm activities of crude Aceh patchouli oil (CPO) against *Staphylococcus aureus*. This study employed a laboratory experimental design: two controls (growth and negative) and three varying concentrations (0.5%, 1%, and 2%) of CPO. Phytochemical analysis was conducted using gas chromatography-mass spectrometry, which revealed the presence of terpenes, sesquiterpenes, and fatty acids in CPO, with patchouli alcohol, azulene, and alpha-guaiene as the dominant compounds. CPO exhibited significant antibiofilm activity against *S. aureus*, with a minimum biofilm-inhibitory concentration of 0.5%. Notably, CPO was also effective in eradicating existing *S. aureus* biofilms at a concentration of 1%. The findings of this study suggest that CPO could be a promising candidate for the development of novel anti-staphylococcal agents.

1 Introduction

The increasing resistance of pathogens to antibiotics and the formation of biofilms have led to global efforts to develop anti-virulence agents. Biofilms are a major cause of infections that are difficult to treat and can seriously impact on human health. Researchers have used a variety of approaches to target biofilms to identify new chemical structures of various compounds from natural sources, including patchouli (*Pogostemon cablin* Benth.).

Patchouli (*Pogostemon cablin* Benth.) is a plant species that yields patchouli oil, an essential oil [1]. Several studies have reported that patchouli oil has antimicrobial, antioxidant, analgesic, anti-inflammatory, antimutagenic, antithrombotic, emetic, and cytotoxic activities [2]. Additionally, antibacterial activities of essential oils and their chemical constituents have been documented in studies [3]. Patchouli, a natural resource of Aceh, has been studied for its medicinal properties, including antimicrobial activity. Previous studies have shown that patchouli oil from Aceh can inhibit the growth of *S. aureus* at a

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concentration of 10% [4]. Therefore, it is important to investigate the potential of patchouli oil from Aceh as an antibiofilm agent. This study aimed to evaluate the phytochemicals and potential of crude patchouli oil (CPO) obtained from aceh (*Pogostemon cablin* Benth.) Inhibition of biofilm formation and degradation by *Staphylococcus aureus*.

2 Material and Methods

Patchouli leaves were obtained from Aceh Jaya, Indonesia, and processed at the Atsiri Research Center (ARC), Universitas Syiah Kuala. In brief, patchouli leaves were dried to remove water, chopped into 5 cm pieces, and placed in a distillation tank. The leaves were then compressed and steam was passed through the leaves for 4-6 hours. The steam carried the essential oil from the leaves, which was then condensed using cooling water. The condensed mixture of water and oil was collected using a separatory funnel. Water and oil are separated by gravity owing to their different densities. Crude patchouli oil (CPO) was collected in storage containers.

The phytochemical content of the crude patchouli oil samples was analyzed using gas chromatography-mass spectrometry (GC-MS) to determine the concentrations of patchouli alcohol (PA) and other active components. A 0.1 μ L sample was injected into the GC-MS system. The initial oven temperature was 50°C, which was increased at a rate of 3°C/min to 300°C over a period of 88.33 minutes. The injector temperature was 280°C, the injection mode was split mode at a ratio of 1:200, the ion source temperature was 200°C, and the interface temperature was 250°C. Helium, which was pressurized to 26.7 Kpa and flowed at a linear velocity of 30 cm/s, was used as the carrier gas. The mass spectrometer detected compounds in the *m/z* range of 40–400. The GC-MS results were displayed as peaks in a chromatogram, which were then matched to compounds in the GC-MS library and confirmed by a literature review; the similarity percentage range used was 80–100%.

S. aureus suspensions were prepared by inoculating 2-4 colonies grown on Nutrient Agar (NA) medium into 5 mL 0.9% NaCl solution. The suspensions were then homogenized by vortex for 15-20 seconds. The density of the suspensions was measured using a UV-Vis spectrophotometer at a wavelength of 625 nm and adjusted to the range of 0.08-0.13 absorbance, which is equivalent to $1-5 \times 10^6$ Colony Forming Units (CFU/ml).

A minimum inhibitory concentration (MIC) assay was performed using the macrodilution method. The assay was performed in test tubes. Each test tube was filled with 3 ml of patchouli oil sample at various concentrations (4, 2, 1, and 0.5%), followed by the addition of 3 ml of the test bacterial suspension to each test tube. The assay was performed twice. Muller Hinton Broth (MHB) medium with bacteria (without patchouli oil) and MHB medium alone (without bacterial suspension and patchouli oil) were also used as growth control and negative control (blank), respectively. To ascertain the MIC value, the turbidity of the solution in each test tube was monitored after a 24-hour incubation period at 35°C.

The inhibition of biofilm formation was evaluated by measuring the amount of biofilm formed after exposure to patchouli oil. Patchouli oil was added to microplates containing *S. aureus* suspensions at $1/2 \times$ MIC, $1 \times$ MIC, and $2 \times$ MIC. The microplates were incubated for 24 h at 35°C. After incubation, the microplates were washed with PBS and stained with crystal violet. The crystal violet was then washed away, and the absorbance was measured using a microplate reader at a wavelength of 595 nm [5].

Biofilm degradation was evaluated by measuring the amount of biofilm remaining after exposure to patchouli oil. Biofilms were grown on microplates for 48 h, which was the optimal time for biofilm formation. Patchouli oil was added to the microplates at $1/2 \times$, $1 \times$, and $2 \times$ MIC. Subsequently, the microplates were incubated for an extra 24 hours. The microplates were rinsed with PBS and stained with crystal violet following incubation. The

absorbance was then determined at a wavelength of 595 nm using a microplate reader after the crystal violet was removed by washing.

3 Results and Discussion

Seventeen phytochemical compounds were detected in CPO by GC-MS, with retention times ranging from 7 to 38 min. Phytochemical compounds were detected in CPO from the 7th to the 38th min. Based on the GC-MS chromatogram shown in Figure 1 and Table 1, CPO generally contains terpenoids, alcohols (sesquiterpenoids), and fatty acids with areas ranging from 0.27% to 33.62%.

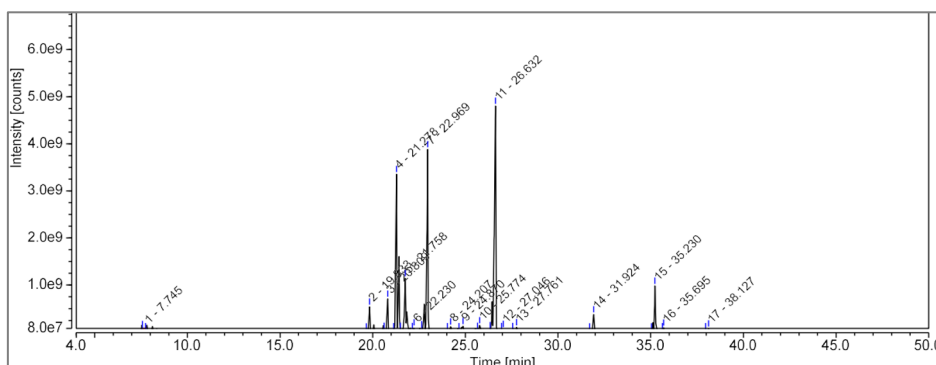


Fig. 1. Chromatogram of crude patchouli oil analyzed by GC-MS

GC-MS analysis of CPO revealed three major phytochemical components: patchouli alcohol (PA) (33.62%), α -guaiene (22.13%), and azulene (20.84%). These three components account for 76.69% of the total CPO composition. The remaining 23.31% were comprised of 14 minor components. PA ($C_{15}H_{26}O$) and guaene ($C_{15}H_{24}$) are sesquiterpenes, whereas azulene ($C_{10}H_8$) is monoterpene. PA is the main component of patchouli oil, and is responsible for its characteristic aroma. PA is insoluble in water, but soluble in alcohol, ether, and other organic solvents. The detailed composition of the CPO is shown in Table 1.

In addition to terpenoids and sesquiterpenoids, CPO contains a number of fatty acids with higher molecular weights. GC-MS analysis revealed the presence of several fatty acids, including hexadecanoic acid methyl ester ($C_{17}H_{34}O_2$), 9-octadecenoic acid (Z), methyl ester ($C_{19}H_{36}O_2$), methyl stearate ($C_{19}H_{38}O_2$), and tributyl acetyl citrate ($C_{20}H_{34}O_8$). The phytochemicals contained in patchouli oil, including terpenoids, sesquiterpenoids, and fatty acids, exhibit antibacterial activity.

The biofilm inhibitory activity of NMN against *S. aureus* is shown in Figure 2. The results showed that CPO was able to inhibit the formation of the test bacteria by 83.69% at a concentration of 0.5% ($1/2 \times \text{MIC}$), 81.28% at a concentration of 1% ($1 \times \text{MIC}$), and 80.99% at a concentration of 2% ($2 \times \text{MIC}$) compared to the growth control (TSB medium without CPO addition). The ANOVA test findings indicated that the inclusion of CPO significantly inhibited the production of *S. aureus* biofilms ($p < 0.05$). This is similar with the results of prior research demonstrating that patchouli oil inhibits the development of *S. aureus* biofilms.[6] and *Streptococcus pyogenes* by 60-80% [7].

Patchouli oil (CPO) has been shown to be effective in inhibiting the formation of *S. aureus* biofilm. CPO is thought to inhibit biofilm formation by interfering with bacterial signaling pathways. This suggests that CPO has potential as an anti-quorum sensing (QS) agent. CPO may inhibit the expression of genes involved in bacterial attachment to surfaces

in the early stages of biofilm formation, synthesis of extracellular polymeric substances (EPS), and expression of genes responsible for the production of virulence factors.

Table 1. Results of GC-MS analysis of phytochemical compounds in crude patchouli oil (CPO)

Peaks	Retention time (min)	Area (%)	Names	Chemical formula	Molecular weight (g/mol)	Compound types
1	7.745	0.92	3,Trifluoroacetoxypentadecane	C ₁₇ H ₃₁ F ₃ O ₂	324.4	Fatty acid
2	19.833	1.73	Naphthalene, 1,2,4a,5,8,8a-hexahydro-4,7- dimethyl-1-(1-methylethyl)-, (1a,4aβ,8aa)- (±)-	C ₁₀ H ₈	12.816	Terpene
3	20.809	2.85	Caryophyllene	C ₁₅ H ₂₄	204.35	Sesquiterpene
4	21.278	22.13	α-Guaiene	C ₁₅ H ₂₄	204.35	Sesquiterpene
5	21.758	7.56	1H-3a,7-Methanoazulene, 2,3,6,7,8,8a- hexahydro-1,4,9,9-tetramethyl-, (1a,3aa,7a,8aβ)-	C ₁₅ H ₂₄	2.5	Diterpene
6	22.230	0.42	α-Guaiene	C ₁₅ H ₂₄	204.35	Sesquiterpene
7	22.969	20.84	Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4- dimethyl-7-(1-methylethenyl)-, [1S- (1a,7a,8aβ)]-	C ₁₀ H ₈	128.16	Terpene
8	24.207	0.40	(1R,4aS,6R,8aS)-8a,9,9-Trimethyl-1,2,4a,5,6,7,8,8a-octahydro-1,6-methanonaphthalen-1-ol	C ₁₅ H ₂₆ O	222.37	Sesquiterpene
9	24.870	0.86	Ledene oxide-(II)	C ₁₅ H ₂₄ O	220.35	Sesquiterpene
10	25.774	0.93	Globulol	C ₁₅ H ₂₆ O	222.37	Sesquiterpene
11	26.632	33.62	Patchouli alcohol	C ₁₅ H ₂₆ O	222.37	Sesquiterpene
12	27.046	0.27	Longipinocarveol, trans-	C ₁₅ H ₂₆ O	220.35	Sesquiterpene
13	27.761	0.33	4-Hydroxy-6-methyl-3-(4-methylpentanoyl)-2H-pyran-2-one	C ₁₂ H ₁₆ O ₄	224.25	Terpene
14	31.924	1.51	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270.5	Fatty acid
15	35.230	4.94	9-Octadecenoic acid (Z)-, methyl ester	C ₁₉ H ₃₆ O ₂	296.5	Fatty acid
16	35.695	0.39	Methyl stearate	C ₁₉ H ₃₈ O ₂	298.5	Fatty acid
17	38.127	0.31	Tributyl acetylcitrate	C ₂₀ H ₃₄ O ₈	402.5	Fatty acid

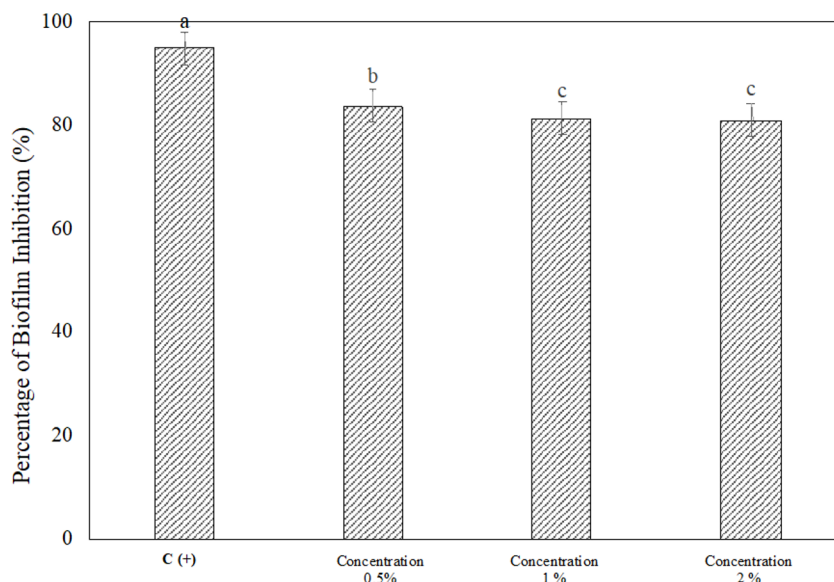


Fig. 2. Percentage biofilm inhibition activity of crude patchouli oil (CPO) with various concentrations: 0.5%, 1%, and 2% and positive control (C+). Different superscripts above each column indicated statistically significant differences ($p < 0.05$).

The ability of CPO to inhibit the formation of *S. aureus* biofilms at sub-MIC concentrations is closely related to its bioactive compounds. CPO is primarily composed of terpenoids, sesquiterpenoids, and fatty acids. Terpenoids, including trans-caryophyllene, limonene, (S)-cis-verbenol, and linalool, have been reported to inhibit the formation of *S. aureus* biofilms by up to 88% at concentrations that do not inhibit bacterial growth, either alone or in combination with other terpenoids. These compounds can inhibit the expression of *sdrD*, *spa*, *agr*, and *hld*, which are involved in quorum sensing (QS) and attachment of *S. aureus* cells to the surface of a substrate, thereby inhibiting biofilm formation [8, 9]. Fatty acids, including hexadecanoic, octadecanoic, and oleic acids, have also been reported to inhibit the formation of *S. aureus* biofilms at sub-MIC concentrations. Hexadecanoic acid, which is also found in CPO, has the best antibiofilm activity among fatty acids against *S. aureus* and other Gram-positive bacteria. This is thought to be due to the ability of fatty acids to inhibit the production of extracellular polymeric substances (EPS) [10] and alter the hydrophobicity of bacterial cells by affecting membrane proteins [11]. In addition, inhibition of *S. aureus* biofilm formation by essential oils at concentrations below the MIC can also occur through inhibition of the expression of the *sarA* gene, which encodes an accessory regulator in *S. aureus* responsible for controlling the production of various virulence factors, including factors involved in biofilm formation. Cinnamaldehyde, which is the main component of the essential oil of *C. burmannii*, has been reported to inhibit the expression of *sarA* in *S. aureus* at sub-MIC concentrations [12].

In addition to the QS pathway, CPO has been shown to affect biofilm formation by inhibiting bacterial growth. This is evidenced by the ability of CPO to inhibit biofilm formation by more than 80% at $1 \times \text{MIC}$ and $2 \times \text{MIC}$. Several studies have shown a linear correlation between bacterial growth inhibition and biofilm formation, whereby inhibition of bacterial growth affects the inhibition of biofilm formation [13, 14]. The Fatty acids and sesquiterpenoids found in CPO are thought to play a major role in the inhibition of bacterial growth, ultimately leading to the inhibition of *S. aureus* biofilm formation.

High concentrations of fatty acids have been reported to dissolve bacterial cell membranes, which can inhibit bacterial growth and ultimately inhibit biofilm formation [11]. The sesterpene compound beta-caryophyllene has also been reported to inhibit the production of *Streptococcus mutans* biofilm by inhibiting the expression of the *gtf* gene (glucosyltransferase) involved in EPS synthesis and to alter the permeability and integrity of the cell membrane in *Bacillus cereus* [15]. In addition, sesterpenes from the plant *Teucrium polium* L. have also been reported to inhibit the formation of *S. aureus* biofilms by destroying fatty acids in the bacterial cell membrane, thereby inhibiting biofilm formation. Sesterpenes can damage fatty acids by damaging the structure and function of the lipid layer of the bacterial cell membrane [16]. Essential oils can affect the fatty acid components of *S. pyogenes* biofilms [7]. This is thought to be due to the hydrophobic nature of essential oils, which allows them to bind to lipid compounds in the bacterial cell membrane, thereby damaging cell structures [17, 18].

The biofilm degradation activity of CPO is shown in Figure 3. The results showed that CPO was able to degrade *S. aureus* biofilm by 80.52% at 0.5% ($1/2 \times \text{MIC}$), 75.49% at 1% ($1 \times \text{MIC}$), and 71.45% at 2% ($2 \times \text{MIC}$). According to the ANOVA results, CPO significantly inhibited the biofilm destruction activity of *S. aureus* ($p < 0.05$), with the concentration of CPO having a significant effect on the degree of inhibition.

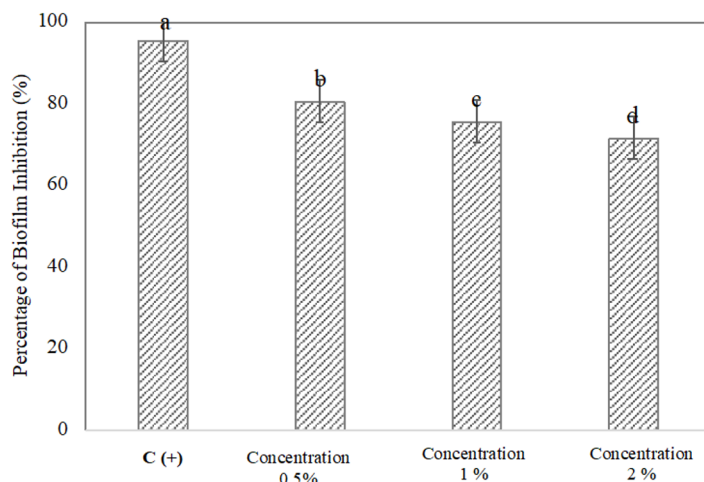


Fig. 3. Percentage biofilm degradation activity of crude patchouli oil (CPO) with various concentrations: 0.5%, 1%, and 2% and positive control (C+). Different superscripts above each column indicated statistically significant differences ($p < 0.05$).

CPO was found to have biofilm degradation activity. This may be due to the presence of bioactive compounds in CPO. CPO had the highest α -guaiene content compared to the other two types of patchouli oil. Guaiene compounds from patchouli oil have been reported to have antibiofilm effects against *S. aureus* [19, 20]. CPO also contains azulene (a terpene). A study revealed that azulene and its derivatives have antibacterial effects against the gram-positive bacterium *Streptococcus beta-haemolyticus* [21]. In addition, CPO also contains fatty acids, especially octadecanoic acid, which inhibit the initial attachment of *S. aureus* to the substrate, thereby inhibiting biofilm formation [22]. In addition, the presence of a variety of phytochemical groups (terpenes, sesquiterpenes, and fatty acids) in CPO makes it more likely that synergistic interactions between these compounds will occur, which can improve the biofilm degradation ability of CPO. The use of essential oils with more than one bioactive component has been reported to have better antibiofilm effects against various Gram-positive and Gram-negative bacteria than the use of single bioactive compounds [23-25].

4 Conclusion

Crude patchouli oil (CPO) is a promising candidate for the development of novel anti-staphylococcal agents because it contains a diverse array of bioactive compounds, including terpenes, sesquiterpenes, diterpenes, and fatty acids. The most prevalent compounds in CPO are patchouli alcohol, azulene, and α -guaiene, which have been demonstrated to possess antibiofilm and antibacterial properties against *S. aureus*. Our study found that CPO inhibited biofilm formation and degraded existing *S. aureus* biofilms. These findings suggest that CPO can be utilized in the development of effective treatments for staphylococcal infections.

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