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Discovery of Active Antibacterial Fractions of Different Plant Part Extracts of clove (*Syzigium aromaticum*) Against *Streptococcus mutans*

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ABSTRACT

Nowadays, dental cavities caused by *Streptococcus mutans* are a major focus of research in Indonesia. While several antibiotics are available to combat this bacterium, concerns about antibiotic resistance have prompted researchers to explore natural remedies. Clove (*Syzigium aromaticum*) is a commonly studied natural remedy against dental cavities and S. mutans. Among the different parts of the clove plant, clove bud is the most widely used against dental cavities or *S. mutans*, and the potential of other clove parts has not been thoroughly explored. Identifying which parts of the clove plant have higher concentrations of active ingredients and exhibit the strongest antibacterial activity is important. Therefore, this study evaluated the antibacterial activity of three different parts, i.e., leaf, stems, and buds of the clove plant ethanolic extracts against *S. mutans*. The ethanolic extracts of clove leaf, stems, and buds were prepared using the maceration method with 70% ethanol, and their activity against *S. mutans* was tested using the disc diffusion method at three different concentrations (10%, 5%, 2.5% b/v). Fractionation was carried out using hexane and water to obtain two fractions: hexane and water fraction. These fractions were then subjected to antibacterial assays. The ethanolic leaf, stems, and bud extracts exhibited varying antibacterial activity levels. The best activity was observed with the 10% clove bud ethanolic extract, which produced an inhibition zone of 20.83 ± 0.77 mm. The leaf and stem extracts showed inhibition zones of 16.38 ± 3.84 mm and 17.95 ± 5.15 mm, respectively. Furthermore, the hexane-soluble fraction of the clove bud displayed the highest activity with an inhibition zone diameter of 23.7 ± 3.21 mm at 10%. This activity was twice as high as ampicillin, used as the positive control. In conclusion, clove bud remains the best source of antibacterial compounds against *S. mutans*.

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Fractionation of the bud extract using hexane can significantly enhance its activity. Further investigation should be conducted to optimize the effectiveness of this active fraction for use as an anti-dental caries treatment.

1 Introduction

Dental caries (DC) is an oral infection characterized by tooth decay, primarily caused by *S. mutans*, and is a highly prevalent disease among children. According to the International Caries Detection and Assessment System (ICDAS), the prevalence of caries exceeds 90% among children aged 6-12 years (Aripin et al. 2024). DC affects both children and adults, targeting primary and permanent teeth. The starch and sugar residue around teeth are fermented by microorganisms, forming acids that cause the decalcification of enamel and dentin, ultimately resulting in caries (Sivapathasundharam and Raghu, 2020).

Pharmacological treatment and prevention of dental caries range from the use of anti-cariogenic compounds such as fluoride, chlorhexidine, or xylitol to antibiotics such as amoxicillin, amoxicillin-clavulanate, clindamycin, cephalexin, and metronidazole (Cui et al. 2019; Qiu et al. 2020; Ahmadi et al. 2021). However, several studies have reported the development of resistance in *S. mutans* to various anti-cariogenic compounds (Lee et al. 2012; Liao et al. 2017; Cieplik et al. 2019), as well as resistance to most commonly used antibiotics (Haque et al. 2019). Furthermore, antibiotics might disrupt the balance of the oral microflora community or cause side effects such as allergies, nephritis, and gastrointestinal disorders (Cheng et al. 2022; Heta and Robo 2018).

When treating dental cavities, natural compounds could be used as an alternative to antibiotics. Several studies have demonstrated that a wide range of natural compounds exhibit antibacterial activity against the growth of *S. mutans* (Dwivedi and Singh 2015; Jacob and Nivedhitha 2018; Folliero et al. 2022). The essential oil from the clove plant *S. aromaticum* is often empirically and clinically used. A previous study by Moon et al. (2011) combined ampicillin and clove oil and found that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were decreased approximately fourfold against *S. mutans*, resulting in a synergistic influence which accounted for a fractional inhibitory concentration index (FICI) of less than 0.5. Another study revealed a synergistic activity of eugenol, clove oil, and clove methanol extract in combination with azithromycin to inhibit biofilm formation resulting from the growth of *S. mutans* (Jafri et al. 2021). Clove oil showed a strong inhibitory effect as fluoride-free toothpaste against *S. mutans* and its biofilm (Dhamodhar et al. 2014; de Oliveira Carvalho et al. 2020).

Nurdjannah et al. (2016) evaluated phytochemical research on the clove plant and found that the highest concentrations of clove essential oil were found in clove buds (10-20%), followed by the stems (5-10%) and leaf (1-4%). In another study, steam distillation of clove resulted in different concentrations of phytochemical compounds from buds, leaf, and stem parts, and it was found that the clove leaf contained a higher percentage of eugenol (82.97%) than the buds (75.30%). However, stems contain the highest amount of eugenol (97.75%). Furthermore, the antibacterial activity test of those three parts showed that the stem possesses the highest inhibitory zone (15.05± 0.200) against *Escherichia coli*, followed by *Salmonella typhimurium* (13.67 ± 0.764) (Sohilait et al. 2018; Mak et al. 2019). The results indicate the potential for other parts of the clove plant to be utilized since clove buds take about four years to grow (Cortés-Rojas et al. 2014). Therefore, this research was carried out to evaluate the potential activity of stems, leaves, and buds extracted from clove plants.

Additionally, the most active part of the plant extract will be subjected to fractionation to separate the polar and nonpolar compounds. This process aims to obtain the most active fraction. In a previous study, Mann (2012) described three different fractions (hexane, chloroform, and methanol fraction) of clove basil (*Ocimum gratissimum*) and revealed varying antimicrobial activities. The hexane soluble fraction exhibited the highest inhibitory activity against *Niesseria gonorrheae*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Klebsiella penumoniae*, and *Vibrio cholerae* compared to the other fractions. This demonstrates the significant benefits of fractionating and separating an active extract to explore the more concentrated active compounds from the plant extract (Silvestre et al. 2009).

This study compared the antibacterial activity of a 70% ethanol extract of clove leaves, stems, and buds against *S. mutans*. Subsequently, the active extract was fractionated, and its antibacterial activity against *S. mutans* was investigated. The results of this study were expected to address the need for active fractions in treating diseases caused by resistant microorganisms, such as dental caries.

2 Materials and Methods

2.1 Sample Preparation

The cloves used in this study were obtained from Sadar Village, Bone Regency, South Sulawesi, Indonesia (Figure 1). The buds, leaf, and stems were dried in an oven at 60°C for three days and then crushed using a blender to obtain the samples.

Figure 1 Figure illustrates Different Parts of the Clove Plant

2.2 Sample Extraction

Two hundred grams of coarse powder from clove buds, leaves, and stems were soaked in 70% ethanol (1:10 ratio). The soaking lasted three days with occasional stirring, and then the mixture was filtered through filter paper. The residue underwent two additional soaking processes. The resulting extracts were evaporated using a Buchi® rotary evaporator until they became thick and viscous. The extracts were then stored in separate amber vials and weighed to determine their yield percentage (Rasul 2018).

2.3 Antibacterial Assay of Clove Extracts

The antibacterial assay was conducted using the agar diffusion method outlined in CLSIM100-S22 (Clinical Laboratory Standard Institute 2012) with some adjustments. Before the assay, *S. mutans* ATCC®25175 was cultured for 24 hours in a Nutrient Agar medium (Merck®). Afterwards, the bacteria were suspended in saline solution and adjusted to the turbidity of the McFarland 0.5 standard (Himedia®) (equivalent to approximately $1.5x10^8$ CFU/mL). Ethanol extracts of clove buds, leaves, and stems were dissolved in 10% DMSO (Merck®) and diluted to obtain various concentrations (10%, 5%, 2.5% v/v). Twenty microliters of the extracts were transferred onto blank disks (Oxoid®) and then dried in a desiccator for 15-30 minutes. A swab of bacterial suspension was spread on the surface of the Mueller Hinton Agar medium (Merck®), followed by the placement of the extract-containing disks. An Ampicillin disk (Oxoid®) served as the positive control, while a disk containing 10% DMSO was used as the negative control. Each assay was carried out in triplicate. The plates were then incubated at 37°C for 24 hours, and the diameter of the inhibition zone was observed and measured using a calliper (Krisbow®). The extract demonstrating the highest activity was subjected to liquid-liquid extraction.

2.4 Liquid-liquid Extraction and Antimicrobial Assay of Fractions

Based on the results of the antibacterial assay, the ethanol extract of clove bud exhibited the most potent activity in inhibiting the growth of *S. mutans*. As a result, this extract underwent further fractionation to separate the polar and nonpolar compounds. Hexane and water (1:1) were solvents to obtain the hexane and water fractions. The obtained fractions were concentrated using a rotary evaporator and air-drying using a fan and water bath (Abubakar and Haque 2020). The dried extract was then subjected to an antibacterial assay using the same method as described in the previous section

2.5 Qualitative Screening of Phytochemical Compounds of Clove Extracts and Fractions

A small quantity of clove extracts, fractions, and eugenol marker was placed into vials, dissolved using acetone, and then spotted on a silica (Merck®) thin-layer chromatography plate. The TLC plate was placed as the eluent in a toluene acetone chamber (3:1). After elution, the plate was dried and observed under a 254 nm UV lamp. The spots were visible after spraying with an anisaldehyde-sulfuric acid reagent (Kumar et al. 2010).

2.6 Statistical analysis

All assays were performed in triplicate, and the data was presented as the mean of three independent experiments ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to

Table 1 Extraction Result of Various Clove Plant Pats

Sample	Sample (g)	Average of Extract (g)	Average of %Yield
Buds	200	19.3±0.04	9.65
Leaf	200	18.9±0.08	9.45
Stems	200	17.8±0.122	8.9

Data are the mean of three replicates, ± Standard Deviation

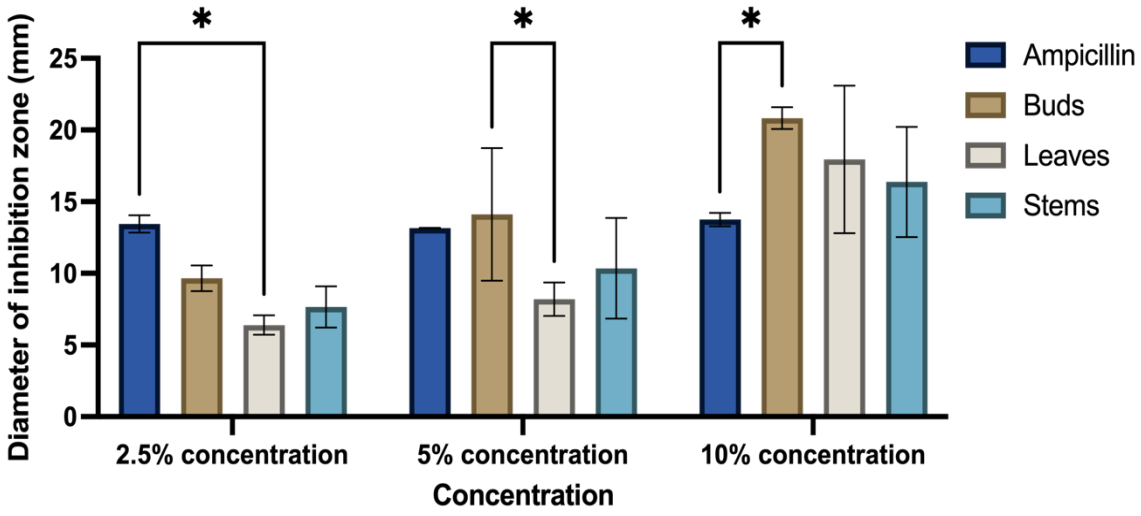

Figure 2 Correlation between plant parts and antibacterial activity of its extracts (mean ± SD, n = 3), significance levels are conceived by stars: * = significant (P < 0.05)

compare the antibacterial activities of individual extracts and fractions, followed by the Tukey HSD test.

3 Results

3.1 Extracted Sample Yield

The quantification results for the extract weight and yield percentage are outlined in Table 1. The findings indicated that the highest extract amount was obtained from clove buds (9.65%), followed by the leaf (18.9%) and stem (17.8%) extract. However, this difference was not significant (Table 1).

3.2 Antibacterial Assay of Clove Extracts

The disk diffusion method is commonly used to test the antibacterial activity of plant extracts. This method measures the diameter of the clear zone that forms around a sample after it has been incubated with bacteria. Different plant extracts may inhibit bacterial growth to varying degrees.

The study found that the bud extract of clove at a concentration of 10% showed the highest activity, with an inhibition zone diameter of 20.83 ± 0.77 mm. This was followed by the clove leaf extract (16.38 ± 3.84 mm) and the stem extract (17.95 ± 5.15 mm). The activity of these extracts varied at different concentrations (Figure 2).

Different plant parts may possess different antibacterial potency. Figure 3 shows the difference in the clear zone size of each plant part of the clove. It can be observed that clove bud extract possesses the widest clear zone compared to positive control (ampicillin), stem extract, and leaf extract.

3.3 Liquid-liquid Extraction and Antimicrobial Assay of Fractions

Out of the three tested extracts, the clove bud extract exhibited the highest inhibitory activities and was thus selected for further fractionation using the liquid-liquid extraction (LLE) method. This process resulted in two fractions: water and hexane. Both fractions underwent antibacterial assays to confirm their potency in inhibiting the growth of *S. mutans*. The results of the antibacterial activity test revealed a significant difference between the two fractions (p-value < 0.001), with the hexane fraction exhibiting the highest inhibitory activity (23.70±3.22 mm). This indicates that the nonpolar compounds in the clove bud have a strong activity in inhibiting the growth of *S. mutans*. When the concentration of the hexane fraction was increased from 2.5% to 5% and 10%, the diameter of the clear zone also increased to 9.66 ± 0.9 mm, 13.4 ± 4.28 mm, and 20.83±0.77 mm, respectively. Figure 4 illustrates the significant difference in antibacterial activity between each experiment. Notably, at a concentration of 10%, the hexane fraction exhibited

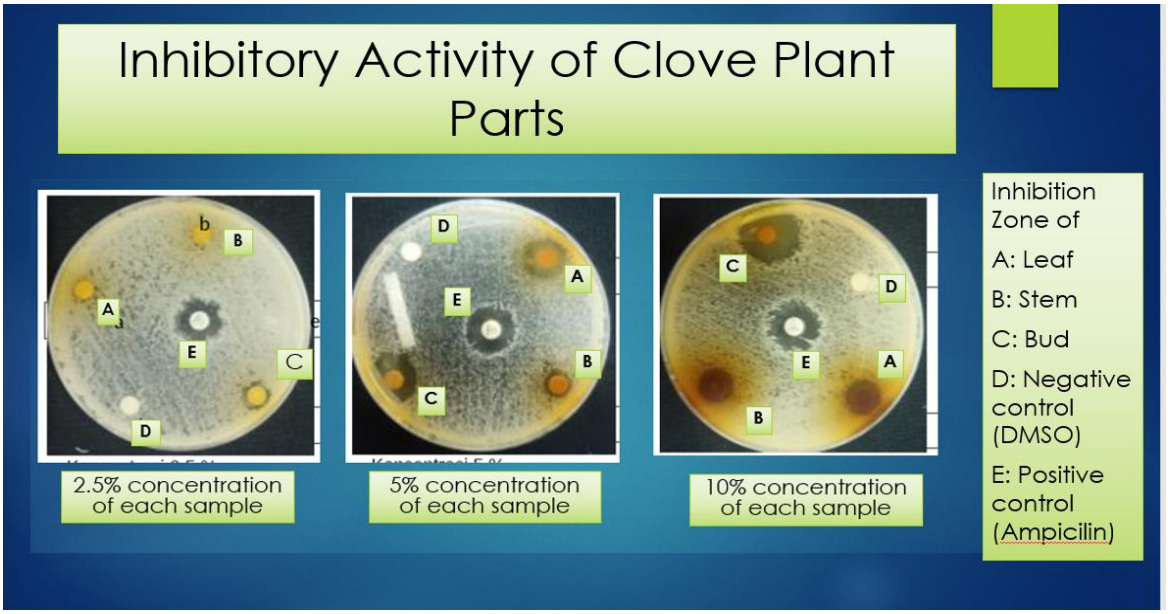

Figure 3 Inhibition activities of different extracts against *S. mutans*

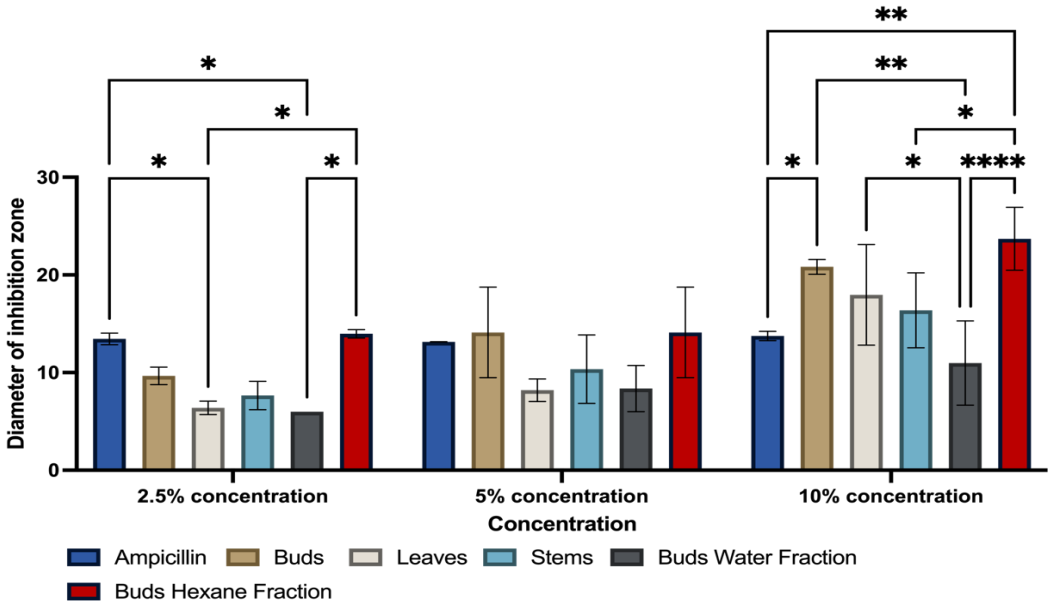

Figure 4 Correlation between plant parts and antibacterial activity of its extracts (mean + SD, n = 3), the Significance grade obtained from two-tailed t-tests are conceived by stars: * = significant ($P < 0.05$), ** = highly significant ($P < 0.01$), *** = very high significant ($P < 0.001$).

higher activity compared to the water fraction and the positive control (ampicillin) with $p< 0.001$ and $p< 0.01$, respectively.

3.4 Qualitative Screening of Phytochemical Compounds of Clove Extracts and Fractions

The results of the phytochemical screening using TLC (thin-layer chromatography) showed that the extracts and fractions contained eugenol. This was indicated by a dark blue spot at a similar Rf value with eugenol when observed at UV 254 (Figure 5). All extracts and fractions contain eugenol with an Rf value of 0.8, which aligns with the eugenol standard. This finding is supported by Pathak et al. (2004), who reported that eugenol was detected with TLC densitometry using toluene: ethyl acetate: formic acid (3:2:0.4) with an Rf value of 0.77. However, in the hexane fraction of clove bud extract, a prominent spot was observed under the eugenol marker, indicating the presence of compounds other than eugenol in the extract with high concentrations.

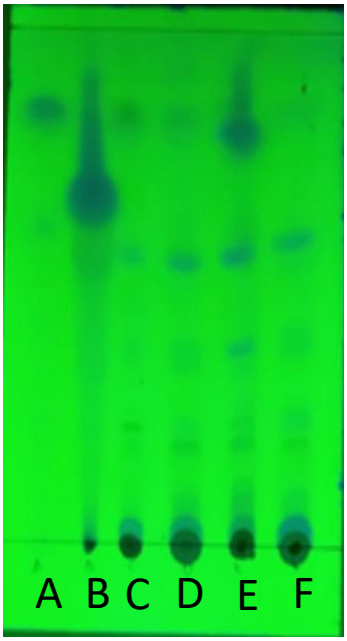

Figure 5 Chromatogram of *S. aromaticum* plant part extracts: (a) eugenol, (b) hexane fraction of clove bud extract, (c) clove leaf extract, (d) clove stem extract, (e) clove bud extract, (f) water fraction of clove bud extract

4 Discussion

4.1 Sample Extraction

This study used the maceration method to extract substances from the cloves plant's buds, leaves and stems. Maceration is an extraction process that dissolves plant substances into solvents. It has several advantages: it is easy to do, requires simple equipment, and can safely extract thermolabile compounds (Rasul 2018). The early extraction process used 70% ethanol to obtain polar and nonpolar compounds from clove plant parts (Sucipto et al. 2022). Ethanol was chosen because it is self-preservative at a concentration above 20%, nontoxic at low concentrations, and requires minimal heat to concentrate the extract (Abubakar and Haque 2020). Additionally, ethanol can prevent the growth of fungi and bacteria in the extract and has an excellent performance of diffusing into plant cell walls and dissolving almost all phytochemical compounds (Utami and Putri 2020). Previous research suggested that the eugenol content in clove extract with ethanol was approximately 87.18%, higher than in clove extracted with n-hexane solvent (76.30%). This study obtained the highest extract yield (9.65%) from the clove bud. The percentage of extract yield can be influenced by the extraction method and the timing of harvesting plant parts (Sulaiman et al. 2015).

4.2 Antibacterial Assay of Clove Extracts

The clove bud extract exhibited the highest inhibition zone against *S. mutans* growth at a concentration of 10%, with a diameter exceeding 20 mm, indicating strong antibacterial activity. There was a significant difference ($p < 0.05$) in the diameter of the inhibition zone between ampicillin (used as the positive control) and the clove bud extract, suggesting that the clove bud extract was highly effective in inhibiting the growth of *S. mutans* bacteria and could be a potential antibacterial agent. The positive control showed a zone of inhibition of less than 14 mm, indicating that the bacteria likely developed resistance to ampicillin (Clinical Laboratory Standard Institute 2012).

While there was no significant difference in the clove plant part at a concentration of 10%, a significant difference was observed between the clove bud and leaf extract at a concentration of 5%. Previous research has shown that 1% essential oil of clove leaf possesses inhibition against *S. mutans* (22.1 ± 1.55 mm). Various studies have reported that the clove bud of *S. aromaticum* has antibacterial and antibiofilm activity against *S. mutans*. Gupta and Prakash (2021) reported intense activity of clove flower extract and clove oil (15-17 mm) against *S. mutans*. In another study, clove bud extract inhibited the growth of multi-drug-resistant *S. mutans* isolated from dental plaque, with an inhibition zone diameter of > 20 mm (Gupta and Prakash 2021). Specifically, the clove extract was reported to be able to damage the cell membrane of *S. mutans* (Suhendar and Sogandi 2019).

4.3 Liquid-liquid Extraction and Antibacterial Assay of Fractions

In this study, clove bud extract was fractionated using the liquid-liquid extraction method and then subjected to an antibacterial activity assay to determine whether the active compound was polar

or nonpolar. The results showed that the compound of clove bud dissolved in the hexane fraction had higher antibacterial activity and was reported to be twice as high as ampicillin. Statistical analysis showed that the hexane fraction significantly differs from ampicillin ($p < 0.05$). Furthermore, the hexane fraction expresses significantly different activity ($p < 0.001$) from the water extract. In previous research, the hexane extract of *Zanthoxylum piperitum* seed also expressed antibacterial activity against *S. mutans* with an inhibitory zone of 15.6 mm. This is higher than the methanol and ethyl acetate extracts, which had 11.6 mm and 13.2 mm, respectively (Park et al. 2008). Based on phytochemical compounds, the major phytochemical constituents of clove were eugenol, eugenyl acetate, caryophyllene, and pyrogallol (Hemalatha et al. 2016). In a review, Hiwandika et al. (2021) reported that the hexane extract contains eugenol, eugenol acetate, β-caryophyllene, and flavonoids. Furthermore, it has been reported that eugenol inhibits the growth of *S. mutans* by preventing bacterial adhesion and biofilm formation. Apart from eugenol, this high activity could also be due to the presence of β-caryophyllene, a nonpolar sesquiterpene compound, which has been reported to have antibacterial activity against *S. mutans* (Pieri et al. 2016).

4.4 Qualitative Screening of Phytochemical Compounds of Clove Extracts and Fractions

The thin layer chromatography method was used to qualitatively analyze phytochemical compounds in clove extract and fractions. It was found that all extracts and fractions contain eugenol, as indicated by a spot with an Rf value of about 0.8, which corresponds to the eugenol spot. This finding is consistent with the results of Pathak et al. (2004), who detected eugenol using TLC densitometry with toluene:ethyl acetate:formic acid (3:2:0.4) and an Rf value of 0.77. Another study identified eugenol and β-caryophyllene in clove acetonic and ethanolic extracts. The different polarity of compounds in the extracts or fractions may result in different spots being observed under UV light. This is due to the flow rate of the spot along the silica plate being dependent on the polarity of the compounds in the extracts or fractions and the polarity of the eluent (Pathak et al. 2004; Hemalatha et al. 2016; Hiwandika et al. 2021; Mostafa et al. 2023).

Conclusion

According to the results of this study, it can be concluded that the clove bud extract showed the highest inhibitory activity against *S. mutans* ($p < 0.05$) compared to the leaf extract, stem extract, and the positive control (ampicillin). Additionally, the activity of the hexane and water fractions against *S. mutans* growth was significantly different ($p < 0.001$). Clove bud is the best source of antibacterial compounds against *S. mutans*. Fractionation of the ethanol extract using hexane can substantially enhance its activity more than ampicillin as the positive control. This suggests that the hexane fraction could be a promising source for treating diseases caused by *S. mutans* infection.

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Ethical Clearance

It is confirmed that no animal or human model was utilized in the study; therefore, no ethical clearance is necessary.

Conflict of interest

The authors declare no conflict of interest in this study.

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