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FULL PAPER

Syzygium aromaticum essential oil prevents halitosis caused by oral bacteria *Streptococcus sanguinis*

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Abstract

Halitosis is caused by oral bacteria including *Streptococcus sanguinis* in mouth producing volatile sulphur compounds (VSCs), such as hydrogen sulfide, ethyl mercaptan, and methyl mercaptan which have pungent odor. Bacteria producing sulphur compounds produce oral biofilms as the accumulation of caries promotion. Caries is caused by the acid produced by oral bacteria that lead to tooth demineralization in low pH condition. Clove bud (*Syzygium aromaticum*), known as endogenous spice in Indonesia, has been traditionally used for centuries for treatment of periondal diseases. In this study, we extracted essential oil from *Syzygium aromaticum* (SAEO), identifed for its major essential oils by pyrolysis gas chromatograpy mass spectrometry (py-GC/MS), determined its antihalitosis efficacy on preventing and eradicating *S. sanguinis* oral biofilms, inhibiting VSCs and acid production *in vitro*. Chromatogram profile showed that SAEO contained major eugenol (22.10%) and aceteugenol (13.31%). For antibiofilm effect toward oral bacteria *S. sanguinis*, SAEO at 40 and 60 µg/mL effectively prevented biofilm formation up to 60% and removed the existed biofilms up to 50%. SAEO at 60 µg/mL also demonstrated a significant inhibition on VSCs production (up to 58%) and acid produced by *S. sanguinis* by increasing the terminal pH from 5.66 to 6.30. These data suggest that SAEO could be applied for a promising candidate for developing oral care functional products for management of halitosis.

1. Introduction

Halitosis is widely known as bad breath and is caused by volatile sulphur compounds (VSCs) which mostly produced by anaerobic Gram-negative bacteria, such as *Phorphyromonas gingivalis*, *Weisselia cibaria*, *Treponema denticola*, *Provetella intermedia*, and *Fusobacterium nucleatum*. Various volatile compounds like hydrogen sulfide, methyl mercaptan, putrescine, indole, cadaverine and propionate are known to have pungent odor. Volatile compounds are byproducts of amino acid or sugar metabolism. The substrate used for the volatile compounds in mouth came from host component such as food particles, mucosal cells, blood and precipitated saliva (Marawar *et al*., 2012). Hydrogen sulfide is a volatile product formed by cysteine desulfhydrase and uses cysteine as the primary substrate. Methyl mercaptan is produced by methionine γ-lyase with methionine as a primary substrate (Thorn and John, 2012). Hydrogen sulfide and methyl mercaptan are known as the major VSCs produced by Gram-negative bacteria and act as an indicator for halitosis (Kang *et al.*, 2006).

Gram-positive bacteria also have a major role in halitosis by promoting caries formation on the tooth, followed by the formation of biofilm and production of acid which accelerates tooth enamel demineralization. The biofilm is recognized as a suitable environment for VSCs producing bacteria to grow (Duarte *et al.*, 2006; Kang *et al.*, 2006). Gram-positive bacteria, such as *Streptococcus mutans* and *S. sanguinis* produce biofilm for attachment on the tooth surface. *S. sanguinis* is one of the initial bacteria to make tooth surface environment suitable for other bacteria to colonize. *S. sanguinis* competes with other bacteria to colonize the tooth surface by producing hydrogen peroxidase to inhibit other bacteria growth (Kreth *et al.,* 2012). Initial attachment for *S. sanguinis* on tooth surface used hydrophobic interaction and attachment on epithelial cells using anchoring adhesin protein produces by the *srt*A gene (Percival *et al*., 2006). The biofilm formed by

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S. sanguinis is also a problem because the acid by-products are contained in the biofilm and make the pH-condition on tooth surface become acid (Paes Leme *et al.,* 2008). The acid condition makes tooth calcium easier to be diluted by water, the process is also known as demineralization. *S. sanguinis* is a natural flora in the oral environment, but it may cause endocarditis if the bacteria get into the bloodstream (Duarte *et al.,* 2006). The most abundant substance in *S. sanguinis* biofilm is glucan which is synthesized by glucosyltransferase (GTF). *S. sanguinis* produces two kinds of GTF including GtfB and GtfP. GtfB produces insoluble glucan with α-1,3 bond. GtfB is a virulence factor for dental caries because glucan becomes the adherence place for other bacteria (Yamaguchi *et al.,* 2006).

Syzygium aromaticum, known as clove bud or cengkeh (in Indonesian), is a native spice grouped in Myrtaceae family from Molucca Islands in Indonesia and has been traditionally used for the treatment of toothache and root canal infection, as well as oral anesthetic purpose for management of periodontal diseases. The parts of *S. aromaticum* plant such as the stems, flower buds, and leaves have been reported to contain high essential oils particularly eugenol and acetyl eugenol (Rahim and Khan, 2006). Eugenol is a monoterpene with a molecular weight of 164 g/mol and has been reported to have antimicrobial, antimutagenic, and anti-inflammatory effects (Pal *et al.*, 2010).

Several recent studies related to the use of *S. aromaticum* extract and its essential oil content for the treatment of periodontal diseases including halitosis have been reported. A cross-sectional survey by Akkaoui and Ennibi (2017) reported that there were 23 medicinal plants including clove commonly used by Moroccan herbalists for the treatment of halitosis. A study by Kaur and Chandrul (2017) showed that the main component of clove oil was eugenol, with β-caryophyllene and eugenyl acetate; but the contents of essential oils in cloves were found to be various depending on their anatomic and geographical origins. Another study by Pulikottil and Nath (2015) showed that clove and its derivatives exerted dual anti-plaque and anti-inflammatory effects for the treatment of periodontal disease. Fahimi and Naseri (2015) also stated that halitosis is a well-known disorder in Iranian traditional medicines (ITM), and the use of 14 herbal plants and their mixtures in ITM has been scientifically proven for their biological activities relating to anti-halitosis effect. Therefore, in this study, we investigated whether essential oils isolated from the flower buds of *S. aromaticum* had anti-halitosis activity toward *S. sanguinis* oral bacteria by preventing the formation of biofilms and eradicating the existed biofilms, inhibiting total VSCs production, and reducing acid production *in vitro*.

2. Materials and methods

2.1 Materials

The dried flower buds of *S. aromaticum* were purchased from traditional market in Jakarta (Indonesia) and stored in a voucher specimen (code: LY05) at Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia, Jakarta (Indonesia).

2.2 Extraction and identification of essential oil from S. aromaticum flower bud

Essential oil from the dried *S. aromaticum* flower bud was extracted in n-hexane using maceration (Guan *et al.,* 2007). The dried flower buds were powdered by grinding and a 20 g of dried powder was extracted in 200 mL of n-hexane, followed by maceration for 48 hrs at room temperature. Essential oil and solvent were separated by evaporation at 350 mbar and 50˚C, followed by drying the essential oil in fume hood overnight. *S. aromaticum* essential oil (SAEO) was obtained and stored in room temperature. Chemical compounds in SAEO was analysed by pyrolysis-gas chromatography-mass Spectrometry (py-GC/MS) according to the method of Kang *et al*. (2006). A 0.5 g of sample was injected to the capillary column (Phase Rtx-5MS) with a film thickness of 60 m x 0.25 mm, ID. Pyrolysis temperature was set to 280°C using helium as a carrier gas.

2.3 Antibiofilm assay

Antibiofilm activity of SAEO was assayed for prevention and eradication effects using *S. sanguinis* (ATCC 5513) biofilm model according to the modified method of Yanti *et al*. (2008). *S. sanguinis* was inoculated in Brain Heart Infusion (BHI) media or BHIA (BHI supplemented with 1.5% of bacteriological agar at 37°C for 24 hrs aerobically. The media were centrifuged in 4500 × *g* for 5 mins. The supernatant was discarded and then the pellet was resuspended using adherence buffer (KH_2PO_4 10 mM, KCl 50 mM, $CaCl_2$ 1 mM, and $MgCl_2$ 0.1 mM).

For prevention assay, the 96-well plate was coated with 150 µl artificial saliva (1% CMC, KH_2PO_4 10 mM, KCl 50 mM, $CaCl_2$ 1 mM, and $MgCl_2$ 0.1 mM) and 50 µl SAEO at various concentrations (20-100 µg/mL). Chlorhexidine at 20 µg/mL was used as a reference standard. The plate was dried at 37°C overnight. For treatment, a 20 µL *S. sanguinis* culture (1 x 10^6 CFU/mL) and 180 µL BHI media supplemented with 3% sucrose (BHIS) were added into each well. The negative control (untreated) was BHIS medium and the cell

suspension without sample, and the blank control contained only BHIS medium. The plate was incubated at 37°C for 24 hrs. The well was gently washed using PBS to discard the planktonic cells. The well was dried in room temperature for 1 hrs. The biofilm formed at the bottom of the well was dyed using 110 µL crystal violet 0.4% for 30 mins. The well was destained using 200 µL ethanol absolute. A 100 µL ethanol absolute was transferred to the new well and the absorbance was measured at 596 nm using microplate reader. The percentage of inhibition was calculated using the equation:

(1 – A_{596} of sample/A_{596} of the untreated control) x 100.

For eradication assay, the 96-well plate was coated with 150 µL artificial saliva**.** The plate was dried at 37°C overnight. After drying, a 20 µL *S. sanguinis* culture (1 x 10^6 CFU/mL) and 180 µL BHIS were added into each well. The plate was incubated at 37°C for 24 hrs. After the biofilm formed, a 50 µL SAEO at various concentrations (20-100 µg/mL) was added to each well for 1 hr. Chlorhexidine at 20 µg/mL was used as a reference standard. For the negative control (untreated), the same steps were taken except that BHIS medium (200 µL) was used without sample or addition of cell culture. The well was gently washed using PBS to discard the planktonic cells in the well. The well was dried in room temperature for 1 hr. The biofilm formed at the bottom of the well was dyed using 110 µL crystal violet 0.4% for 30 mins. The well was destained using 200 µL ethanol absolute. A 100 µL ethanol absolute was transferred to the new well and the absorbance was measured at 596 nm using microplate reader. The remained biofilm cells were quantified as described previously. SAEO activity was defined as the percentage absorbance of the biofilm remaining after sample treatment in comparison with the untreated control.

2.4 Inhibition on S. sanguinis volatile sulphur compounds production

The assay was carried out according to the method of Cord-Ruwisch (1985). *S. sanguinis* culture (1 x 10^6 CFU/mL) was growth in BHI media in the glass tube, followed by the addition of $CuSO_4$ 0.2%, methionine 0.5%, and SAEO at various concentrations (20-100 µg/ml). Tubes were incubated at 37°C for 24 hrs to measure the production of H_2S. The formation of H_2S resulted in a brown precipitate that was measured at 480 nm using a spectrophotometer.

2.5 Inhibition on S. sanguinis acid production

The assay was done to determine the changes in the production of acid by using a pH meter (Percival *et al.,* 2006). *S. sanguinis* bacterial cultures were grown on BHI medium at 37°C for 24 hrs, followed by centrifugation at 4500 × *g* for 5 mins. Pellets were taken and rinsed with a saline solution containing KCl 200 mM and $MgCl_2$ 20 mM. Bacteria that have been rinsed were resuspended in 5 mL of saline solution. One ml of solution was transferred into a beaker containing 100 mM sucrose solution and SAEO at various concentrations (20-100 µg/mL), then the pH change was measured for 30 mins and the pH value was recorded every minute.

2.6 Statistical analysis

Statistical analysis was used to determine the accuracy and the repeatability of data. All experiments were separately repeated triplicate. Data were analysed using descriptive statistics (standard deviation and variance) and one-way ANOVA for assessing the significance of differences. A value of $p<0.05$ was taken as significantly different.

3. Results

3.1 Identification of chemical compounds in SAEO

The py-GC/MS chromatogram revealed that SAEO contained twelve chemical compounds with eugenol (22.1%) and aceteugenol (13.31%) as the major essential oils (Figure 1 and Table 1). SAEO had approximately 45.76% essential oils classified in phenylpropanoid and sesquiterpenoid groups.

Table 1. Identification of chemical compounds in *S. aromaticum* essential oil

Peak	Retention time	Compounds	Concentration (%)	Group
1	2.677	Propanethiol	52.17	Thiol
2	15.708	Trans anethole	2.16	Phenylpropanoid
3	16.466	Eugenol	22.1	Phenylpropanoid
4	16.617	Alpha-copaene	0.36	Sesquiterpenoid
5	17.094	Trans-caryophyllene	6.48	Sesquiterpenoid
6	17.391	Alpha-humulene	0.71	Sesquiterpenoid
7	17.846	Aceteugenol	13.31	Phenylpropanoid
8	18.557	Caryophyllene oxide	0.64	Sesquiterpenoid
9	18.958	Tetracyclo [6.3.2.0E2,5.0E1,8] tridecan-9-ol, 4,4-dimetyl	0.42	Alkane
10	19.291	2',3',4' Trimethoxyacetophenone	0.59	Ketone
11	20.897	Palmitic acid	0.54	Fatty acid
12	22.164	Linoleic acid	0.52	Fatty acid

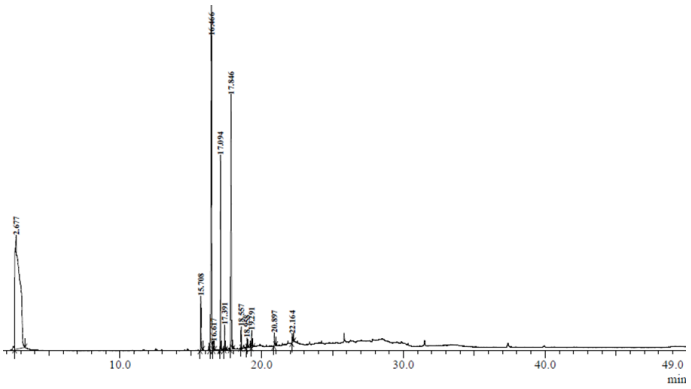

Figure 1. Identification of chemical components in *S. aromaticum* essential oil by py-GC/MS.

3.2 Effect of SAEO against S. sanguinis biofilm formation and eradication

Figure 2a demonstrated the effect of SAEO at various doses on preventing the formation of *S. sanguinis biofilm*. SAEO at 60 µg/mL effectively inhibited *S. sanguinis* formation up to 50% as well as chlorhexidine reference. However, the inhibition pattern was not dose-dependent manner. SAEO was also tested on killing the existed *S. sanguinis* biofilm (Figure 2b). At 40 µg/ml, SAEO removed effectively up to 50% of the existed *S. sanguinis* biofilm, and its efficacy was similar to the chlorhexidine reference.

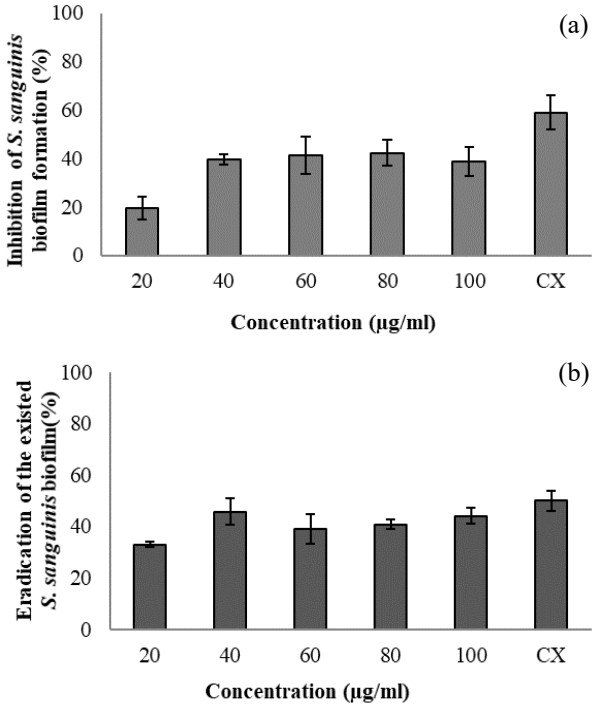

Figure 2. Effect of *S. aromaticum* essential oil on preventing *S. sanguinis* biofilm formation (a) and eradicating the existed *S. sanguinis* biofilms (b). CX, chlorhexidine reference.

3.3 Effect of SAEO on VSCs production inhibition

Oral bacteria *S. sanguinis* increased the VSCs production and SAEO was further tested whether it reduced the VSCs. Our results showed that SAEO at 20-80 µg/mL exerted dose-dependent pattern on inhibiting VSCs produced by *S. sanguinis* (Figure 3). At 60 µg/mL, SAEO significantly decreased the total VSCs >50%.

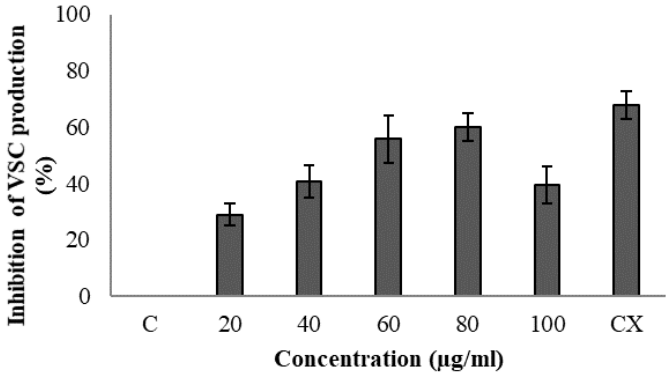

Figure 3. Effect of *S. aromaticum* essential oil on inhibiting volatile sulphur compound production. C, negative control; CX, chlorhexidine reference; $p<0.05$ against negative control.

3.4 Effect of SAEO on acid production inhibition

Figure 4 demonstrated that the production of acid by *S. sanguinis* increased during 0-20 mins, indicating by the decrease of pH value. SAEO effectively inhibited *S. sanguinis* acid production by reducing the terminal pH value compared to that of negative control (Figure 4a). The terminal pH value of negative control pH was reduced until 5.66. After treatment with SAEO at 60 µg/mL, the terminal pH value increased up to 6.3. Its efficacy was similar to chlorhexidine reference which raised the terminal pH value until 6.53. SAEO also decreased acid production rate (Figure 4b). At higher concentration (60 - 100 µg/mL), SAEO showed a similar inhibition pattern with chlorhexidine reference on acid produced by *S. sanguinis*.

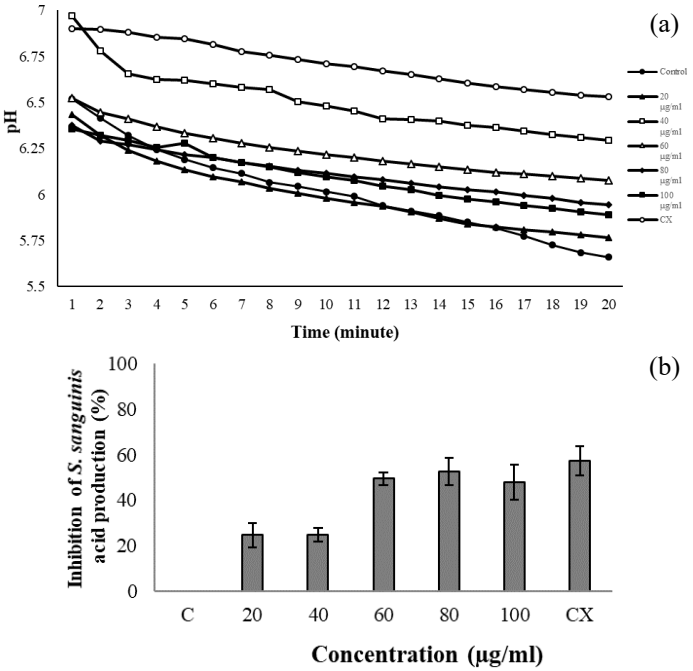

Figure 4. Effect of *S. aromaticum* essential oil on decreasing the pH value (a) and inhibiting acid produced by *S. sanguinis* (b). C, negative control; CX, chlorhexidine reference; $p<0.05$ against negative control.

4. Discussion

S. aromaticum has been widely used as a traditional medicine for the treatment of oral-related diseases and may be applied for a more specific target in oral diseases particularly halitosis. In this study, it was observed that SAEO had a strong inhibitory effect towards *S. sanguinis* ability to produce biofilms, total acid and VSCs (Figures 2-4). The py-GC/MS result showed that SAEO contained major active compounds, including eugenol and aceteugenol which have health benefits (Figure 1 and Table 1). Eugenol was known to have anti-inflammatory, antimutagen, and antimicrobial broad-spectrum activities (Rahim and Khan, 2006). Terpenoids contained in SAEO also has been reported to have antimicrobial activity towards bacteria and fungi (Ndam *et al.,* 2016). Fatty acids (palmitic acid and linoleic acid) contained in SAEO also possess antimicrobial activity and play an important role in the cellular process (Abdullah, 2013). Long chain fatty acids also have been reported to have a strong antimicrobial effect towards *S. sanguinis* (Huang *et al.,* 2011).

In terms of its antibiofilm efficacy against *S. sanguinis* biofilms (Figure 2a), SAEO may inhibit the biofilm formation of *S. sanguinis* by affecting initial attachment and glucan synthesis. Rahim and Khan (2006) had reported *S. aromaticum* methanol extract had an inhibitory effect towards *S. mutans* initial attachment to glass surface, but the effect was not caused by eugenol antimicrobial activity. Eugenol was also known to affect the ATP ion channel and change the fatty acid profile of bacteria by altering the membrane (Nazzaro *et al.,* 2013). More research is needed to uncover the active component responsible for initial attachment inhibition. Glucan synthesis inhibition may be resulted from eugenol capability to alter the membranes which affect the cell membrane permeability towards primary component to produce energy and biofilm (Gill *et al.,* 2004).

Biofilm eradication result showed that SAEO had stronger eradication ability than chlorhexidine reference (Figure 2b). The result may be caused by the difference in lower molecular weight of SAEO active compounds compared to the molecular weight of chlorhexidine reference (505 g/mol). SAEO with lower molecular weight (115 g/mol) had a better penetration ability towards the existed biofilm (Peterson *et al.,* 2015). These existed biofilms are more resistant to the antimicrobial agent that was proved in chlorhexidine reference (20 µg/mL). Based on Figure 2, chlorhexidine showed the inability to eradicate *S. sanguinis* biofilm than to prevent the biofilm formation. Antimicrobial activity may reduce the total viable cell within the biofilm, therefore reduce the biofilm integrity (Steinberg *et al.,* 2004). However, further study could be specifically designed to examine the penetration ability in a more mature biofilm.

VSCs produced by *S. sanguinis* from the degradation of enzyme-containing sulphur. Inhibition of VSCs production by SAEO indicated its potential ability to treat halitosis (Figure 3). The reduction of VSCs production may be caused by eugenol ability to decrease the total viable cells. Inhibition towards VSCs-producing enzyme could not be observed in this research. Specific research is needed to observe the SAEO ability towards VSCs-producing enzyme inhibition and a further characterization and sample purification may be needed to identify the responsible compounds. VSCs production involved several pathogenic bacteria (Kang *et al.,* 2006), therefore, the use of consortium or *in vivo* treatment is a better option than using only single culture bacteria.

The acid produced by *S. sanguinis* also contributed for tooth demineralization (Percival *et al.,* 2006). It was also observed that acid production of *S. sanguinis* was susceptible to SAEO and chlorhexidine. Our data showed that SAEO ability at higher concentration (60-100 µg/mL) was comparable with chlorhexidine in inhibiting acid produced by *S. sanguinis* (Figure 4). It is known that acid production is strongly linked with energy production in *S. sanguinis* because compounds that could inhibit nutrient uptake and act as an inhibitor on nutrient metabolism enzyme may be used as potential caries reducing agent (Percival *et al.,* 2006). Reduction of acid may be resulted in the antimicrobial activity of SAEO active compounds. Another study also stated that eugenol ability to damage the membrane play an important role in nutrient uptake and maintain cell integrity and lower the production of ATP in cells (Nazzaro *et al.* 2013).

5. Conclusion

SAEO contained major eugenol exerted potential anti-halitosis effect by preventing *S. sanguinis* biofilm formation, eradicating the existed *S. sanguinis* biofilms, inhibiting *S. sanguinis* VSCs production, and reducing *S. sanguinis* acid production. SAEO may be applied as a promising natural material in developing oral care functional products for management of halitosis.

Conflict of Interest

The authors declare no conflict interest. The authors alone are responsible for the content of the paper.

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