

ANTIHALITOSIS EFFECT OF ESSENTIAL OIL EXTRACTED FROM *ZANTHOXYLUM ACANTHOPODIUM* FRUITS

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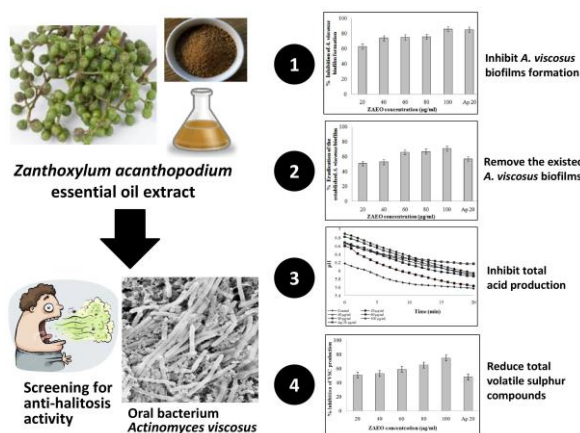
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Graphical abstract



Abstract

Halitosis is associated with the excessive production of acid and volatile sulphur compounds (VSCs), as well as accumulation of biofilm plaque by oral bacteria in mouth cavity. *Zanthoxylum acanthopodium*, locally known as andaliman or lemon pepper, is an Indonesian endemic spice with high content of essential oils, including carveol that has been reported for its potential antimicrobial and anti-inflammatory activities. We determined whether extract of *Z. acanthopodium* essential oil (ZAEO) exerted anti-halitosis effect on reducing biofilm plaque, acid production, and VSCs by using *Actinomyces viscosus* model *in vitro*. ZAEO was extracted in n-hexane followed by evaporation. ZAEO at various doses (20-100 µg/ml) was tested and quantified for its antibiofilm, acid production, and total VSCs production toward *A. viscosus* oral bacteria *in vitro* by conducting biofilm assays for preventing and eradicating effects, pH-stat analysis, and VSCs assay. At lowest dose (20 µg/ml), ZAEO inhibited and removed >50% of *A. viscosus* biofilms. *A. viscosus* was able to produce acid rapidly in 20 minutes, resulting in the pH terminal of 5.57, and ZAEO treatment at 40 µg/ml exerted significant inhibition on acid production with the terminal pH of 5.93, respectively. ZAEO at lowest dose also reduced >50% of total VSCs produced by *A. viscosus*. Our results suggest that ZAEO extract could be applied as a natural ingredient for halitosis treatment.

Keywords: *Actinomyces viscosus*, anti-halitosis activity, biofilms, acid production, volatile sulphur compounds, *Zanthoxylum acanthopodium* essential oil.

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1.0 INTRODUCTION

Halitosis (oral malodour) is indicated by bad smell from mouth due to the production of volatile sulphur compounds (VSCs) by oral bacteria. Halitosis is mostly caused by less oral hygiene, periodontal infection, food residue, oral carcinoma, and throat infection [1]. Physiologically, halitosis is caused by food component that is consumed and morning breath.

During sleep, the flow of saliva decreased, consequently the oral hygiene can not be kept due to the growth of bacteria. Meanwhile, oral pathology of halitosis is caused by accumulation of dental plaque, periodontal infection, the growth, and biofilm of oral bacteria. Dental plaque is caused by less of oral hygiene consequently the bacteria decompose food residue. Periodontal disease is characterized by the swollen gum. Several early and

late oral bacterial colonizers, including *Streptococcus mutans*, *S. sanguinis*, *Actinomyces viscosus*, *Fusobacterium nucleatum*, *Prevotella intermedia*, and *Tannerella forsythensis* have reported to be highly significant to produce VSCs [2]. If combine with *Porphyromonas gingivalis* and *P. endodontalis*, they may cause periodontal disease.

Halitosis occurs in all ages and appears due to the decomposition protein by bacterial and host proteases. Halitosis can be treated by the use of natural substances, such as plant extracts and chemical substances. However, the use of chemical substances often causes side effects. The chemical substances, like triclosan, cetilpiridinium chloride, chlorhexidine contained alcohol as an antiseptic agent. The use of these agents in may cause tooth staining and decrease saliva flow [1, 3]. Alternative strategics for halitosis treatment are focused on the use of natural products derived from plant extracts. *Zanthoxylum acanthopodium* fruit, locally known as andaliman or lemon pepper, belongs to an endogenous spice grouped in Rutaceae family in Indonesia and has a unique lemon aromatic flavor [4]. Previous studies showed that *Z. acanthopodium* fruit contained up to 69% of essential oils, including monoterpenes and diterpenes, and possessed pharmacological effect, such as antibacterial, antioxidant, antidiabetic, anti-inflammatory, and xanthine oxidase inhibitory activities [5, 6, 7, 8]. However, the essential oil derived from *Z. acanthopodium* fruit has not been reported for its potency and mechanism to combat halitosis. This study was focused on determining the antihalitosis effect of *Z. acanthopodium* essential oil (ZAEO) on inhibiting and removing biofilm plaque, reducing acid production and inhibiting production of VSCs using oral bacteria *Actinomyces viscosus*.

2.0 METHODOLOGY

2.1 Plant Material

The fruits of *Z. acanthopodium* were purchased from traditional market in Central Tapanuli region, North Sumatera province (Indonesia). The fruits were identified by Herbarium Bogoriense, Bogor Botanical Garden, Bogor (Indonesia) and stored in a voucher specimen (code: LY25) at Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia. *Z. acanthopodium* fruits (pericarp and seed) were dried using freeze dryer and grinded to obtain the powder.

2.2 Extraction of Essential Oil from *Z. acanthopodium*

Essential oil from *Z. acanthopodium* (ZAEO) fruit was extracted using n-hexane. A 40 g of dried fruit of *Zanthoxylum* was weighted and soaked in 200 mL n hexane and agitated 120 xg using shaker incubator at room temperature. After 48 h, the solid particle

was sieved by filter paper. Essential oil and the n-hexane were separated using rotary evaporator at 50°C and 350 mbar. After extraction, n-hexane residue was evaporated and ZAEO was obtained. Our previous results showed the ZAEO contained 69.03% of essential oils with major compounds of carveol (47.7%), myrtanyl acetate (12.55%), phytol (2.81%), and citronellyl acetate (2.77%) [9].

2.3 Antibiofilm Assay

Antibiofilm activity of ZAEO was assayed for prevention and eradication effects using *A. viscosus* biofilm model according to the modified method of Yanti *et al.* [10]. *A. viscosus* (KCCM 12074) was cultured in brain heart infusion (BHI) broth or BHIA (BHI supplemented with 1.5% of bacteriological agar at 37°C for 24 h aerobically. For preventing effect, a 1% of artificial saliva was diluted in adherence buffer (10 mM KPO₄, 50 mM KCl, 1mM CaCl₂, 0.1 mM MgCl₂, pH 7.0) and autoclaved at 121°C for 15 min. ZAEO was first dissolved in dimethyl sulfoxide (DMSO) 100%, followed by a serial dilution of ZAEO using sterilized artificial saliva until the final concentrations of 20-100 µg/mL. ZAEO was added into each well of the 96-well plate, incubated and shaken gently at room temperature for 3 h, followed by air-drying overnight. After coating, a single-species inoculum (1×10⁶ CFU/ml) was grown using BHIS media (BHI broth supplemented with 3% sucrose). For treatment, a 20 µl of cell inoculum was added to the wells (final concentration cells 1×10⁵ CFU/ml). The negative control was BHIS and cell suspension without addition of ZAEO. Ampicillin at 20 µg/ml was used as the reference. After incubation at 37°C for 10 h, absorbance at 596 nm was recorded to assess culture growth. The culture supernatants from each well were decanted, then the planktonic cells were removed by washing with 200 µl of 50 mM PBS (pH 7.2) and air-dried for 1 h. Biofilm was stained using 110 µl of 0.4% (w/v) crystal violet solution for 30 min, then rinsed with 300 µl of sterile distilled water until the control wells appeared colorless. The quantification of biofilm formation was done through the addition of 200 µl of 95% ethanol as destaining solution to each crystal violet stained well, and 100 µl of destaining solution was immediately transferred to the new well. This assay was repeated in triplicate.

For the eradication of biofilm, each plate of the 96-well plates was conditioned with 200 µl of artificial saliva, incubated, and shaken gently at room temperature 3 h. Then, excess artificial saliva was removed and the plate was air-dried overnight. For negative control, 200 µl BHIS broth was used without inoculum. For treatment, a 20 µl of single species inoculum was added with 180 µl BHIS medium. The plate was incubated at 37°C for 10 h. Biofilm cells were treated with 50 µl of ZAEO at various final concentrations (20-100 µg/ml), followed by incubation for 60 min. Biofilm cells were stained and quantified. Ampicillin at 20 µg/ml was used as the reference. The percentage of ZAEO activity on

eradicating *A. viscosus* biofilm was defined as the percentage absorbance of the remained established biofilm after treatment in comparison with the untreated control. This assay was repeated in triplicate.

2.4 Inhibitory Assay of Acid Production

Inhibitory effect of ZAEO on acid produced by *A. viscosus* was determined by using pH-stat analysis [11]. A single-species of inoculum was inoculated in 50 ml of BHI broth and incubated at 37°C for 24 h. Bacterial inoculum was centrifuged at 4500 \times g for 5 min. Pellet were taken and washed with 200 mM KCl and 20 mM MgCl₂. Bacteria was resuspended with 5 mL salt solution. Sucrose and bacteria without ZAEO were used as control. For treatment, ZAEO at various concentrations (20-100 μ g/ml) was added to 5 ml sucrose and 1 ml inoculum bacteria. Ampicillin at 20 μ g/ml was used as the reference. The pH-stat was measured for 20 min within one minute interval. This assay was done in triplicate.

2.5 Inhibitory Assay of Total VSCs Production

ZAEO was further tested for its anti-halitoses efficacy through inhibiting the production of VSCs by conducting the modified method of Cord-Ruwisch [12]. The test medium buffer composed of minimal medium (pH 7.0) containing 0.5% methionine and 1% sucrose with CuSO₄ reagent were added in sterile tube in order to detect H₂S production. The test tubes was tightly sealed with parafilm and incubated at 37°C for 72 h under aerobic conditions. H₂S production was determined by quantitative analysis of colloidal white copper sulphide (CuS) precipitates in the test tubes. For the standard curve, β -mercaptoethanol at various concentrations (20-100 μ g/ml) was added to the test medium buffer. For the negative control, minimal medium and CuSO₄ reagent without inoculum was added to the sterile tube. The test tubes with ZAEO was compared with the test tubes without ZAEO. The test tubes were treated with 100 μ l of ZAEO at various concentrations (20-100 μ g/ml). Ampicillin at 20 μ g/ml was used as the reference. This assay was run in triplicate.

2.6 Statistical Analysis

Data were expressed by computational analysis (SPSS 12.0), and the significance of the differences was assessed via a student's *t*-test. A value of $p < 0.05$ was taken as statistically significant.

3.0 RESULTS AND DISCUSSION

3.1 Antibiofilm Activity of ZAEO

Figure 1 demonstrated the antibiofilm activity of ZAEO against *A. viscosus* oral bacteria by preventing

A. viscosus biofilm formation and eradicating the established *A. viscosus* biofilm. For preventing effect, at lowest dose (20 μ g/ml), ZAEO strongly inhibited up to 63% of *A. viscosus* biofilm formation *in vitro* (Figure 1a). ZAEO dose dependently prevented the growth of *A. viscosus* biofilm. ZAEO at lowest dose also exerted similar eradicating effect on removing ~50% of the established *A. viscosus* biofilm (Figure 1b). In general, ZAEO exerted potential antibiofilm activity toward preventing effect of biofilm growth than that of eradicating effect of the existed biofilm. Its antibiofilm efficacy was comparable to that of ampicillin reference used in this study.

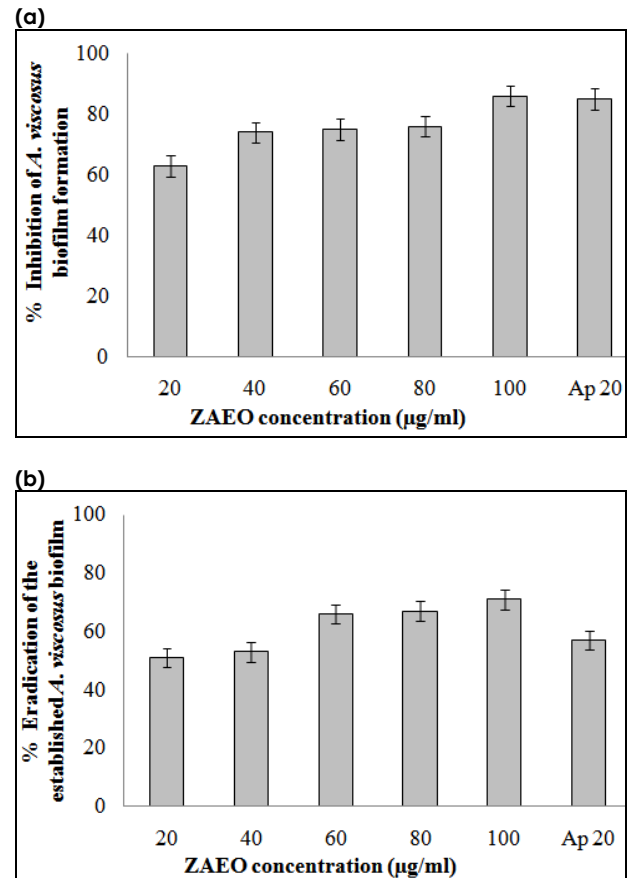


Figure 1 Antibiofilm activity of ZAEO at various concentrations (20-100 μ g/ml) with preventing effect on *A.*

Halitosis is mainly caused by the accumulation of pathogenic oral bacteria in biofilm plaque that may lead to periodontal infection and inflammation. Halitosis is characterized by the increase of accumulated biofilm plaques, acid production, and VSC production by the bacteria. In this study, we tested whether ZAEO may possess anti-halitoses activity toward *A. viscosus* oral bacteria model by inhibiting biofilm formation, removing the established biofilm, reducing acid production, and decreasing total VSCs production. Our previous study showed that the ethanolic extract of *Z. acanthopodium* fruits possessed anti-inflammatory activity by suppressing

several inflammatory mediators at protein and gene levels in macrophages induced by lipopolysaccharide [6].

Halitosis is known to be associated with inflammation and infection, thus, active fraction including essential oil extracted from *Z. acanthopodium* fruits using n-hexane might be the potential candidate for combating halitosis through several mechanism. Chemical profiling of ZAEO indicated that 69.03% of essential oil contents in ZAEO and major components were identified as monoterpenes, such as carveol, phytol, myrtenyl acetate, and citronellyl acetate (data not shown). Meanwhile, ZAEO extracted with diethyl eter mostly consisted of geranyl acetate (32.04%) and limonene (15.80%) [5]. The difference of solvent polarity may affect the yield and targeted compounds as the results of extraction. Carveol (47.70%) is a monocyclic monoterpene alcohol that has been reported for its antimicrobial activity towards *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Bacillus cereus*, and *B. subtilis* [8, 13].

In this study, ZAEO exerted a significant preventive effect toward the growth of *A. viscosus* biofilms (Figure 1a). Major components in ZAEO contain hydroxyl active sites of alcohol that may kill cells by increasing the permeability of cytoplasmic membrane and increasing endogenous oxidative stress [14]. ZAEO was found to be easier to remove planktonic cells mode of growth than the existed biofilms. It is noted that planktonic cells cannot maintain to produce the strong toxin and they are easy to detach from the surface. ZAEO was also able to kill the established *A. viscosus* biofilms, although its eradicating effect was not higher than its preventing effect (Figure 1b). Biofilms are built from the matrix cells and lipopolysaccharides, and the matrix is massive and difficult to destroy [15]. It seems that biofilm matrix adsorbs the active components in ZAEO and the bacteria increase their local concentration. ZAEO is extremely difficult to penetrate into the matrix. Planktonic cells are a reversible fashion which is easily killed by ZAEO. If they find the available nutrition or other advantages, they will produce auto-inducer protein (AIP) and start quorum sensing. AIP is a signal in Gram positive to communicate to each other, thus, they increase their density. ZAEO is difficult to interact with bacteria in matrix-enclosed biofilm formations [16]. Biofilms grow and colonize in tooth surface and release occasionally planktonic cells that react with ZAEO. Bacteria living as biofilm are often more difficult to eradicate compared to the planktonic cells.

In terms of ZAEO effect on eradicating the established *A. viscosus* biofilm, it is assumed that penetration of ZAEO into matrix biofilms is also influenced by their molecular weights (MWs) [17]. Molecules with small size may easily penetrate into the matrix. Carveol as the main essential oil component in ZAEO has lower MW (152.23 g/mol) than that of ampicillin reference (MW 349.41 g/mol). Terpenes as the major component of essential oil

have a specific mechanism to inhibit and eradicate biofilms. Other factor might be correlated with characteristic of terpenes in ZAEO. Terpenes influence the fatty acid composition of cell membrane, disrupt the membrane by lipophilic compounds, and thereby inhibit ion transport. Terpenes are also potential to prevent aggregation and biofilm formation [18]. Carveol as a monoterpene has been reported to exert antibacterial activity toward *Escherichia coli* and *Staphylococcus aureus* through changing the hydrophobicity, surface charge, and membrane integrity with the subsequent K^+ leakage [19].

3.2 Effect of ZAEO on Acid Production

The ZAEO was further tested on its potency on inhibiting the acid produced by *A. viscosus* *in vitro*. In Figure 2, *A. viscosus* was able to produce acid rapidly in 20 min, resulting in terminal pH of 5.57 and treatment with ZAEO effectively reduced the terminal pH and the acid production rate. At 40 $\mu\text{g/ml}$, ZAEO effectively increased the terminal pH to 5.93 and its ability was quite similar to ampicillin reference on inhibiting acid produced *A. viscosus*.

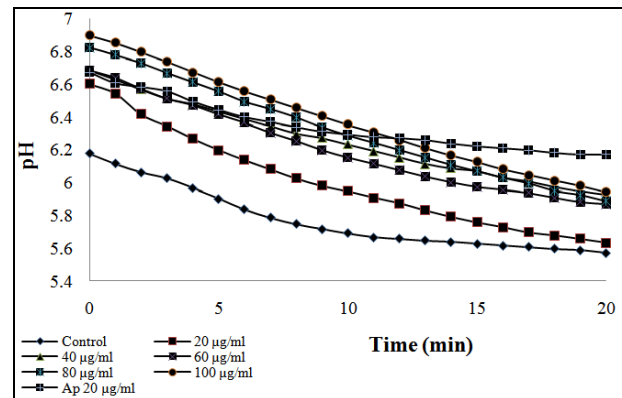


Figure 2 Effect of ZAEO at various concentrations (20-100 $\mu\text{g/ml}$) on inhibiting *A. viscosus* acid production by pH stat assay. Ap 20 was ampicillin reference at 20 $\mu\text{g/ml}$. $P < 0.05$ against control (untreated *A. viscosus*)

These data indicate that ZAEO may inhibit glucosyltransferase (GTF) activity that leads to the decrease of glucan formation and acidogenicity [20]. Oral bacteria *S. mutans* encode *gtfB*, *gtfC*, *gtfD* genes to produce GTF, and GTF converts sucrose to the modified glucans. Next, glucanase converts glucans to glucose, and glucose is converted to acid via heterofermentative metabolism [21]. It is known that sucrose metabolism is associated with caries and halitosis in management of periodontal diseases. Oral bacteria breakdown the sucrose and other carbon sources to synthesize energy via glycolysis pathway. Acid is a by-product of metabolism that can dissolve calcium phosphate in teeth and cause caries [22]. Acid caused halitosis is produced by Gram positive via tricarboxylic acid (TCA) cycle.

Sucrose is converted to glucose and fructose by invertase. In glycolysis pathway, glucose and fructose are converted to phosphoenolpyruvate (PEP). Pyruvate kinase converts PEP to pyruvate, then pyruvate enters the TCA generating methionine, cysteine, and serine [23]. The anaerob metabolism of sucrose by Gram positive produces lactic acid and other organic acids [24]. In addition, VSCs are also produced by other bacteria that live together with *A. viscosus* that supply lactic acid to *Veillonella* species. Hence, *Veillonella* produces hydrogen sulfide from lactic acid [25]. Inhibition of acid production may cause the decrease of dextran production as the adhesion factor of bacteria to teeth surface. The decrease of *A. viscosus* density may lead to the reducing of protein metabolism.

3.3 Effect of ZAEO on Total VSCs Production

ZAEO was also investigated for its ability to decrease total VSCs produced by *A. viscosus in vitro*. ZAEO inhibited total VSC production in the dose dependent manner (Figure 3). Interestingly, at lowest dose (20 µg/ml), both ZAEO and ampicillin demonstrated the similar effect on inhibiting up to 50% of VSCs production.

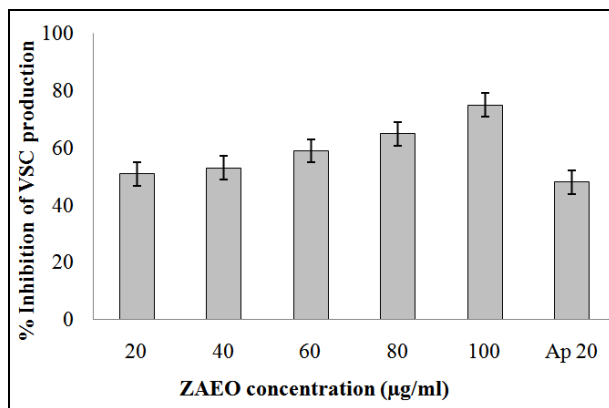


Figure 3 Effect of ZAEO at various concentrations (20-100 µg/ml) on inhibiting total VSCs produced by *A. viscosus*. Ap 20 was ampicillin reference at 20 µg/ml. $P < 0.05$ against control (untreated *A. viscosus*)

It is known that *A. viscosus* produce VSCs from degradation of protein containing sulphur. Cysteine is degraded by *A. viscosus* via desulfhydration mechanism. Degradation of cysteine generates hydrogen sulfide as a VSC product [26]. Washio *et al.* also reported that *A. viscosus* is a predominant bacteria which produces hydrogen sulfide [25]. We assumed that ZAEO efficacy on inhibiting VSCs production is associated with its potential antibiofilm activity either to prevent *A. viscosus* biofilm formation or to eradicate the existed *A. viscosus* biofilm (Figure 1). Antibiofilm effect of ZAEO toward *A. viscosus* biofilm may suppress total VSCs produced by *A. viscosus* itself.

4.0 CONCLUSION

In summary, essential oils rich in carveol isolated from the fruits of *Z. acanthopodium* may exert antihalitosis potential through several mechanisms, including preventing the growth of *A. viscosus* biofilms, eradicating the established *A. viscosus* biofilms, inhibiting acid production, and reducing total VSCs production.

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