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Research Report

Effect of *Citrus aurantifolia swingle* essential oils on methyl mercaptan production of *Porphyromonas gingivalis*

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ABSTRACT

Background: *Halitosis is a term used to describe an unpleasant odors emanating timely from oral cavity. The unpleasant smell of breath most common caused from volatile sulphure compound (VSC). Methyl mercaptan is the major component of VSC. P. gingivalis produced large amount of methyl mercaptan. The essential oils of Citrus aurantifolia swingle contain antibacterial component.* ***Purpose:*** *The purpose of this study was to determine the effect of essential oil of Citrus aurantifolia swingle on the production of methyl mercaptan compounds in P. gingivalis.* ***Methods:*** *Bacterial suspension of P. gingivalis in TSB medium with 10^8 CFU/ml concentration cultured in a microplate and added by the essential oils of Citrus aurantifolia swingle with 1%, 2%, 3% and 4% concentration. Distilled water was used as negative control and 0.2% Chlorhexidine mouthwash was used as a positive control. Microplate was incubated anaerobically for 48 hours. After the periode of incubation, 0.6% methionine as the exogenous substrate and 0.06% DTNB as a reagen for determining methyl mercaptan concentration were added to each wells. The microplate was futher incubated for 12 hours. Concentration of methyl mercaptan produced by the P. gingivalis was measured spectrophotometrically using microplate reader at 415 nm.* ***Results:*** *One-way ANOVA showed that the essential oil of Citrus aurantifolia swingle take effect on the concentration of methyl mercaptan produced by P. gingivalis. LSD test results indicated that there was a significant difference of methyl mercaptan concentration between treatment groups of the essential oils of Citrus aurantifolia swingle and distilled water that used as negative control.* ***Conclusion:*** *The essential oil of Citrus aurantifolia swingle has decreased the production of methyl mercaptan produced by P. gingivalis.*

Key words: *Citrus aurantifolia swingle, Porphyromonas gingivalis, methyl mercaptan, halitosis*

ABSTRAK

Latar belakang: *Halitosis adalah istilah yang digunakan untuk menggambarkan bau tidak sedap yang berasal dari rongga mulut. Penyebab utama halitosis adalah senyawa volatile sulphur compound (VSC) dan metil merkaptan merupakan komponen VSC yang paling dominan menyebabkan halitosis. P. gingivalis dapat memproduksi metil merkaptan dalam jumlah banyak. Minyak atsiri kulit jeruk nipis (Citrus aurantifolia swingle) memiliki kandungan antibakteri di dalamnya.* ***Tujuan:*** *Untuk mengetahui pengaruh minyak atsiri kulit jeruk nipis terhadap produksi senyawa metil merkaptan pada bakteri P. gingivalis.* ***Metode:*** *Suspensi bakteri P. gingivalis dalam media TSB dengan konsentrasi 10^8 CFU/ml dibiakkan dalam microplate. Selanjutnya dilakukan penambahan minyak atsiri kulit jeruk nipis konsentrasi 1%, 2%, 3%, dan 4%. Obat kumur chlorhexidine 0,2% digunakan sebagai kontrol positif dan akuades sebagai kontrol negatif. Microplate diinkubasi selama 48 jam untuk selanjutnya dilakukan penambahan metionin 0,6% dan DTNB 0,06% dan diinkubasi kembali selama 12 jam. Konsentrasi senyawa metil merkaptan yang diproduksi oleh bakteri P. gingivalis dihitung dengan menggunakan microplate reader pada panjang gelombang 415 nm.* ***Hasil:*** *ANOVA satu jalur menunjukkan bahwa minyak atsiri kulit jeruk nipis berpengaruh terhadap konsentrasi metil merkaptan yang diproduksi oleh bakteri P. gingivalis. Hasil uji LSD menunjukkan adanya perbedaan yang signifikan antara kelompok perlakuan minyak atsiri kulit jeruk nipis dengan akuades sebagai*

kontrol negatif. ***Kesimpulan:*** *Minyak atsiri kulit jeruk nipis dapat menurunkan produksi senyawa metil merkaptan yang dihasilkan oleh bakteri penyebab halitosis P. gingivalis.*

Kata kunci: *Kulit jeruk nipis (Citrus aurantifolia swingle), Porphyromonas gingivalis, metil merkaptan, halitosis*

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INTRODUCTION

Halitosis is one of oral health problems that many people complain after caries and periodontal diseases.[1] Halitosis is a general term used to describe the bad smell of breath coming from the oral cavity.[2] Detection upon the presence of halitosis can be a manifestation of a systemic illness and is a good indicator of oral diseases or certain systemic diseases.[3] Halitosis is mainly (85-90%) initiated by the bacteria that live in the oral cavity.[4] The oral cavity is home to hundreds of bacteria species that produce some bad-smelling substances as a result of protein degradation.[5]

Microbial degradation products which primarily cause halitosis are volatile sulphur compound (VSC).[6] VSC compounds are mainly produced by negative Gram anaerobic oral bacteria through a process of sulfur-containing amino acid degradation derived from peptides and proteins.[7] These proteins are obtained from organic substrates derived from saliva, crevicular fluid, epithelial cells that exfoliate from the oral mucous membranes, and food debris.[8] Bacteria that produce VSC is negative Gram anaerobic.[7] Volatile sulphur compound components, among of them, include hydrogen sulfide (H_2S), methyl mercaptan (CH_3SH), and dimethyl sulfide [$(CH_3)_2S$][3]. CH_3SH is believed to be the major component of the VSC causing halitosis.[2] CH_3SH compound could cause halitosis three times more halitosis than VSC H_2S.[7]

Porphyromonas gingivalis (*P. gingivalis*) is a negative Gram anaerobic bacterium, has no spores, and is non-motile. These bacteria has fimbriae which is important as adhesion molecule when these bacteria interact with oral epithelial cell, fibroblast ligament periodontal, endothelial cell, extracellular matrix protein, saliva protein, and others bacteria.[9] *P. gingivalis* has strong proteolysis' activities that can degrade proteins,[10] so that this bacterium can produce large amounts of CH_3SH compound derived from the enzymatic METase reaction upon amino acid L-methionine.[11]

Citrus aurantifolia swingle is known for long as a plant with many benefits. As a natural herb, limes are efficacious to relieve sore throat and cough, mucus laxative (mucolytics), and urine laxative (diuretics).[12] *Citrus aurantifolia* swingle belong to the Citrus genus, which is one of essential oil producers.[13] The essential oil content in citrus is concentrated in the peel and leaves of the fruits.[14] The fresh *Citrus aurantifolia swingle* contains about 1.25% of essential oil with limonene as its main components.[15] Limonene acts as an anti-bacterial agent by expanding the cell membranes, increasing the membrane voltage and penetrating cell membranes of bacteria in order to inhibit bacterial respiration enzyme which is important as the energy system of the cell.[16]

This study aims to examine the effect of *Citrus aurantifolia swingle* (lime peel essential oil) on the production of CH_3SH (methyl mercaptan) compound on halitosis causing bacteria *P. gingivalis*.

MATERIALS AND METHODS

The material used in this study was lime peel from Purworejo, breeding suspension of *Porphyromonas gingivalis* in the liquid medium of Trypticase Soy Broth (TSB), 5% of PEG organic solvent to dilute the essential oil into the required concentration, Reagent 5, 5 '-dithiobis (2-nitrobenzoic acid) (DTNB) 0.06% (w/v), methionine 0.6% (w/v) as the source of sulfur-containing amino acids, 1 µM EDTA and 0.1 M NaOH as DTNB reagent solvent, distilled water as a negative control, and Chlorhexidine 0.2% (Minosep®) were used as a positive control.

Citrus aurantifolia swingle used first were identified to ensure that the materials used in this study were the right limes in question. This study used 10 kg of *Citrus aurantifolia* swingle and yielded as much as 2.5 kg of *Citrus aurantifolia swingle*. Furthermore, a small piece of *Citrus aurantifolia swingle* was put in distillation container that has been assembled with a cooler (condenser), and then they were heated. The distillation process carried out in this study was a distillation with steam and water (water and steam distillation). The distillation process resulted the essential oil with 100% concentration in which dilution were then performed using an organic solvent of 5% PEG to obtain the required concentrations (1%, 2%, 3% and 4%).

The next step was to prepare the bacterial suspension of *P. gingivalis*. The bacteria used were pure-cultured bacteria derived from Balai Laboratorium Kesehatan Ngadinegaran, Yogyakarta. Before used, the bacteria were inoculated and optimized first in blood gelatin plate media for 1 week. After the optimization process for one week, a few bacterial colonies were taken using a sterile loop and suspended into a liquid medium of trypticase soy broth (TSB) and then the bacterial concentration was calculated

using Densichek ® to obtain bacterial concentration 10^8 CFU/ml which is equivalent to McFarland standard 0.5 and Brown III standard solutions.

This study uses DTNB (Ellman reagent). DTNB is a reagent which is used to measure the concentration of thiol compounds in a sample. Thiol compound is a compound that contains the functional group consisting of sulfur and hydrogen atom (-SH). Methyl mercaptan is a thiol compound. The chemical reaction occurring between the thiol group and 5.5'-dithiobis (2-nitrobenzoic acid) (DTNB) will form yellow 2-nitro-5-thiobenzoate compound (NTB-).

Suspension of *P. gingivalis* was cultured in 96-well microtiter plates, each was 30 µl. The concentration of *P. gingivalis* bacteria of each well was 10^8 CFU/ml. Then, *Citrus aurantifolia* swingle essential oil with concentration 1%, 2%, 3%, and 4%, was added, as the positive control 0.2% Chlorhexidine mouthwash was used and as the negative control was distilled water, 30 µl for each well and was incubated in a CO_2 incubator of Thermo scientific Barnstead lab-line® for 48 hours. After the 48 hours-incubation, 30 µl of methionine (0.6% w/v) and 30 µl of DTNB (0.06% w/v) were added at each pitting. Then, they were incubated once more for 12 hours. The concentration of the CH_3SH compound produced by bacteria P. gingivalis was calculated using the Bio-rad microplate reader Benchmark model 680Xr® at a wavelength of 415 nm carried out at the Laboratory of Integrated Research and Testing (LPPT) Unit III, Universitas Gadjah Mada, Yogyakarta.

The data obtained in the form of absorbance values were equal to the concentration of methyl mercaptan produced by the *P. gingivalis* bacteria. The overall data obtained were ratio-scaled data which were then grouped according to the respective treatment to be calculated for the mean.

RESULTS

Research on the effect of essential oil of *Citrus aurantifolia swingle* upon the production of CH_3SH (methyl mercaptan) compound in vitro toward halitosis-causing bacteria, *Porphyromonas gingivalis*, has been carried out in the microbiology laboratory of Balai Laboratorium Kesehatan Ngadinegaran and LPPT III Universitas Gadjah Mada. The data obtained were quantitative in the form of absorbance values which were equivalent to the concentration of methyl mercaptan produced. The research findings suggest that the concentration of methyl mercaptan compound produced varies in each treatment group. Highest production of methyl mercaptan compounds were found in the negative control.

Based on the test results using one way ANOVA, their significance was 0.001 ($p<0.05$), there was a significant difference in the concentration of methyl mercaptan production between group of treatments. It means that the essential oil of *Citrus aurantifolia swingle* take effect on the concentration of methyl mercaptan produced by *P. gingivalis*. Afterward, advanced analysis test of Post-Hoc Least Significant Difference is conducted to know which group of treatment that had a significant mean difference. In this test, a significant differences in average ($p<0.05$) was found between the negative control group treatment and essential oils of *Citrus aurantifolia swingle* skin concentration of 1%, 2%, 3% and 4%. LSD test results indicated that there was a significant difference of methyl mercaptan concentration between treatment groups of the essential oils of *Citrus aurantifolia swingle* and distilled water that used as negative control.

DISCUSSION

This study occupied Ellman reagent or DTNB (5.5'-dithiobis 2-nitrobenzoic acid) to detect the concentration of methyl mercaptan compounds produced by the bacteria *P. gingivalis* in microplate wells. DTNB is a reagent which is used to measure the concentration of thiol compounds within a sample. Thiol compounds are a compound that contains the functional group consisting of sulfur and hydrogen atom (-SH). Methyl mercaptan is a thiol compound which, when bonded with DTNB, will produce yellow TNB compounds (2-nitro-5-thiobenzoic acid). The yellow color results from the formation of TNB (2-nitro-5-thiobenzoic acid) then its absorbance values were calculated using the Bio-rad microplate reader Benchmark model 680Xr ® at a wavelength of 415 nm.[17] The chemical reaction between the reagent DTNB and thiol compound (Figure 1).

The research findings in Table 1 showed that the average concentration of methyl mercaptan compounds produced by *P. gingivalis* in the negative control treatment had the highest values compared with the other treatment groups which can be seen qualitatively from the most concentrated yellow color resulted than other treatment groups. This was because in the negative control treatment, the bacterial suspensions within the wells were added only by distilled water which is neutral and has no antibacterial power so that *P. gingivalis* living in the wells remains alive and able to produce methyl mercaptan in large quantities derived from the amino acid methionine. Based on previous theory regarding the action mechanism of DTNB reagent that is the more thiol compounds are produced, the more thiol compounds bounding with DTNB and subsequently form a yellow TNB compound so that reading using a microplate absorbance reader produces a high value.[18]

The average value of methyl mercaptan production in the treatment group of the positive control and the *Citrus aurantifolia swingle* essential oil of concentration 1%, 2%, 3% and 4% had a lower value than the negative control. This was because 0.2% chlorhexidine and *Citrus aurantifolia swingle* essential oil have antibacterial activities so that the generated methyl mercaptan compound will decrease. These results are in accordance with the previous statement

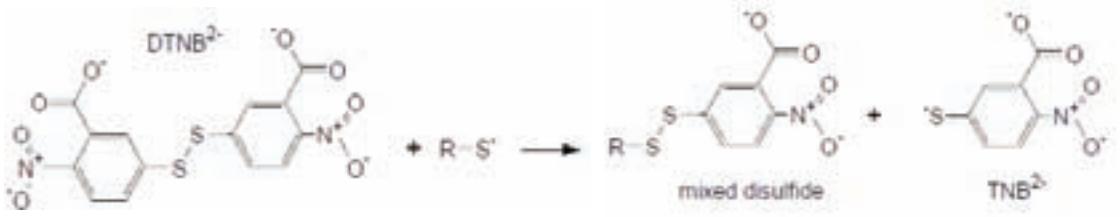

Figure 1. Chemical reaction between DTNB and thiol compound.[17]

Table 1. Means and standard deviations of methyl mercaptan concentrations after treatment with the essential oil of various concentrations, positive controls, and negative controls

No.	Treatment	n	$\bar{X} \pm SD$
1.	Negative control	5	2.244 ± 0.092
2.	Positive control	5	0.322 ± 0.065
3.	*Citrus aurantifolia swingle* essential oil 1%	5	0.327 ± 0.128
4.	*Citrus aurantifolia swingle* essential oil 2%	5	0.246 ± 0.067
5.	*Citrus aurantifolia swingle* essential oil 3%	5	0.241 ± 0.039
6.	*Citrus aurantifolia swingle* essential oil 4%	5	0.262 ± 0.050

that the use of mouthwash with antibacterial ingredients contained within them can reduce halitosis by reducing the amount of bacteria and inhibit bacterial activities so that the productions of compounds that cause halitosis including methyl mercaptan will decline.[3]

Citrus aurantifolia swingle essential oil was used to be one of the treatment groups in this study because *Citrus aurantifolia swingle* essential oil has antibacterial power. The ability as an antibacterial agent is suspected due to of the existence of limonene content as the main compounds. Limonene is antibacterial in nature by expanding the cell membranes, increasing the voltage across the membranes and inhibiting various enzymes in the membranes. These compounds can penetrate the cell membranes of bacteria so that it can inhibit the respiratory enzyme of the bacterial cell and cause death of the bacteria.[16]

Essential oil is not a single compound, but rather a combination of various compounds and therefore limonene is not the only component contained in the essential oil of *Citrus aurantifolia swingle*. In Citrus genus, the essential oil content is concentrated in the peel and leaves of fruits.[14] Fresh *Citrus aurantifolia swingle* contains about 1.25% of essential oils with the main component is liomonene.[15] The other components of the *Citrus aurantifolia swingle* essential oil, geranial (α-citral) and neral (β-citral) can inhibit the growth of bacteria, both negative Gram and positive Gram.[19]

Based on the results of LSD test, there was a significant difference in term of the average between the negative control and all other treatment groups. This was supported by the significant value which was less than 0.05 ($p < 0.05$) which was 0.001. It means that the essential oil of lemon peel and 0.2% chlorhexidine may affect the concentration of methyl mercaptan produced by *P. gingivalis*. LSD analysis also showed there is no significant difference on the average concentrations of methyl mercaptan among *Citrus aurantifolia swingle* essential oil used in the study, hence it can be interpreted that *Citrus aurantifolia swingle* essential oil concentration of 1%, 2%, 3% and 4% influence relatively similar to the decline in the production of methyl mercaptan in *P. gingivalis*. The insignificant difference in term of their average may be resulted from the essential oil used in this study which concentration interval was too short that the effect upon the decreased production of methyl mercaptan was insignificant.

But, when viewed descriptively according to the table on the average concentration of methyl mercaptan compound in Table 1, it can be concluded that the concentration of methyl mercaptan produced tended to decrease with the increase of *Citrus aurantifolia swingle* essential oil concentration of 1%, 2%, and 3%. This is consistent with the statement pelczar and Chan[20] said that the higher the concentration of a certain antibacterial agent, the higher the capability to inhibit or to kill so that in this study the production of methyl mercaptan compound would decrease along with the increasing concentration of essential oil used.

According to the data presented in Table 1, the production of methyl mercaptan in the treatment of essential oil concentration 4% increased. However, based on the LSD test analysis, such increase in the concentration of methyl mercaptan taking place was not significant to the treatment of essential oil concentration of 3%.

Based on the findings showed in the data in Table 1, it is known that the average value of methyl mercaptan concentration in the positive control was 0.322; it is lower than that in the negative control treatment. Chlorhexidine is an antibacterial active ingredient belonging to the gold standard contained in mouthwash.[21] Chlorhexidine has a broad-spectrum antibacterial activity both against the Gram positive and Gram negative.[22]

Antimicrobial activities of chlorhexidine are performed by damaging the cytoplasmic membrane. Bacterial cells characteristically contain negative loads, while the

chlorhexidine molecule is a cation that will be quickly attracted to the surface of the negative load bacterial cell. This process will change the integrity of the bacterial cell membrane. Chlorhexidine will engage to the phospholipids in the inner membrane/cytoplasmic membrane resulting in increased cytoplasmic membrane permeability and leakage in components with low molecular weight such as potassium ions. Then, cytoplasm coagulation resulting in irreversible cell damage happens.[23] However, besides being antibacterial in nature, chlorhexidine also has local side effects when used as a mouthwash that is it can cause extrinsic staining on the teeth so that the teeth become dark yellow brown.[21]

Based on these research, it can be concluded that the essential oil of lemon peel (*Citrus aurantifolia swingle*) of concentration 1%, 2%, 3% and 4% can reduce the production of CH_3SH (methyl mercaptan) compound on halitosis-causing bacteria, *Porphyromonas gingivalis*.

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