Effect of Indonesian medicinal plants essential oils on *Streptococcus mutans* biofilm

Pengaruh oil minyak atsiri dari beberapa tanaman obat Indonesia terhadap biofilm *Streptococcus mutans*

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Abstract

Essential oil's component such as menthol and eucalyptol were already used as dental plaque inhibitors. In searching of potential dental plaque inhibitor from natural products, a study to explore the potency of essential oils extracted from several Indonesian medicinal plants against planktonic growth and biofilm adherence of *S. mutans* was performed. A total of 14 essential oils from some selected Indonesian medicinal plants were extracted by steam-hydro distillation. Antibacterial assay was performed against *S. mutans* by micro dilution technique on nutrient broth media. Biofilm formation inhibition assay was conducted on a flexible U-bottom 96-wells PVC micro plate by using BHI enriched with sucrose 2% at 36.6 °C for 18-24 h. After staining with 1% crystal violet, the optical density was read at 595 nm. A mouthwash commercial product containing essential oil component was used as a positive control. Result showed that the essential oils of *C. sintoc* exhibited the highest biofilm formation inhibition (IC_{50} = 0.005%), and *Z. officinale* showed the highest biofilm degradation with EC_{50} value of 0.013%. Both were active against bacterial planktonic growth but *C. sintoc* showed lower MIC_{90} value (0.6%) in comparison to *Z. officinale* (0.06%). Meantime, *C. citratus* was showed promising antibacterial and antibiofilm activities with MIC_{90} value of 0.06%, MBC 0.6%, IC_{50} 0.008% and EC_{50} 0.026%. It is concluded that the essential oils of *C. citratus*, *Z. officinale* and *C. sintoc* are potential to be developed as dental plaque inhibitors.
Key words: essential oils, antibacterial, biofilm, *Streptococcus mutans*

Abstrak

Komponen minyak atsiri seperti mentol dan eukaliptol telah dilaporkan mampu menghambat pembentukan plak gigi. Penelitian untuk mengetahui potensi minyak atsiri dari beberapa tanaman obat Indonesia dalam penghambatan pertumbuhan planktonik dan biofilm *S.mutans* telah dilakukan. Sebanyak 14 minyak atsiri dipilih dari beberapa tanaman obat Indonesia yang diekstraksi menggunakan metode destilasi air - uap air. Uji antibakteri dilakukan terhadap bakteri *S.mutans* menggunakan metode mikro dilusi dengan media *nutrient broth*. Uji penghambatan biofilm dilakukan pada *flexible U-bottom 96-wells PVC micro plate* menggunakan BHI yang diperkaya dengan sukrosa 2% pada suhu 36,6 °C selama 18-24 jam. Setelah diberi pewarnaan kristal violet 1%, *optical density* dibaca pada panjang gelombang 595 nm. Obat kumur yang mengandung komponen minyak atsiri dan telah beredar di pasaran digunakan sebagai kontrol positif. Pengujian dilakukan sebanyak tiga kali. Hasil penelitian menunjukkan bahwa minyak atsiri dari *C. sintoc* memiliki penghambatan biofilm paling tinggi (IC_{50} = 0,005%), dan *Z. officinale* memiliki kemampuan degradasi yang paling tinggi dengan nilai EC_{50} sebesar 0.013%. Keduanya mampu menghambat pertumbuhan bakteri planktonik tetapi *C. sintoc* memperlihatkan nilai MIC_{90} yang rendah (0,6%) dibanding *Z. officinale* (0,06%). Sementara itu,

C. citratus menunjukkan aktivitas antibakteri dan antibiofilm yang potensial dengan nilai MIC_{90} 0,06%, MBC 0,6%, IC_{50} 0,008% dan EC_{50} 0,026%. Minyak atsiri dari *C. citratus, Z. officinale* dan *C. sintoc* berpotensi untuk dikembangkan sebagai inhibitor plak gigi.
Kata Kunci: minyak atsiri, antibakteri, biofilm, *Streptococcus mutans*

Introduction

One major problem in oral hygiene is dental plaque. Accumulation of this slimy layer (biofilm) on teeth is not only give unpleasant look, but also can lead to gingivitis and carries and even further damage such as dental loss (Marsh, 2006). Developed dental biofilm plaque can consist of many different microbes including those pathogenic in systemic. These microbes can harbor in mouth cavity and hide from immunological reaction (Nasrolahei, *et al*., 2008; Li, *et al*., 2000; Nakano, *et al*., 2007).

Streptococcus mutans is the most frequent bacteria found in biofilm dental plaque (Loesche, 1996). It is also known as initiator of dental biofilm formation and has cariogenic property by demineralizing enamels (Islam, *et al*., 2007). Therefore to overcome dental carries it is important to control mouth flora especially *S. mutans* and at the same time control their capabilities to build biofilm.

Mechanical treatments with dental floss and brush may reduce the plaque but adding chemicals can significantly reduce bacterial counts (Addy, *et al*., 1987; Alexander, 1971). Moreover, considering that electrostatic and hydrophobic interactions govern the attachment of pathogenic bacteria onto the saliva-coated tooth surface suggests chemical agents application in dental plaque control routine (Ouhayoun, 2003). At recent time, available chemicals for dental paste and mouthwash such as chlorhexidine have been correlated with side effects on restorations (Rasooli, *et al*., 2008), imbalance mouth flora and dental staining (Kidd, *et al*., 1997; Ciancio, 2000). On the other hand, those unwanted effects are not commonly seen with essential oils mouthwashes (Rasooli, *et al*., 2008).

This study aimed at finding natural products to prevent the formation of dental biofilm which can be used in an oral hygiene product. The main focus was on the effect of essential oils extracted from selected Indonesian traditional plants against planktonic growth, biofilm formation and adherence of *S. mutans*. Those selected plants and parts were: rhizomes of *Curcuma xanthorrhiza rhizome (Temulawak)*, *Zingiber aromaticum (Lempuyang wangi)*, and *Z. officinale* var *rubrum* L. *(Jahe merah)*; fruits of *Amomum cardamomum (Kapulaga)* and *P. cubeba*; leaves of *Piper betle (Sirih)*, *P. crocatum, P. retrofractum, Syzygium aromaticum*, *Citrus aurantifolia* and *Cymbopogon citratus*; barks of *Cinnamommum zeylanicum*, *C. sintoc*, and *Litsea cubeba*.

Methodology

Plant materials

Fresh rhizomes of *Curcuma xanthorrhiza* Roxb., *Zingiber aromaticum* Vahl, *Zingiber officinale* var *rubrum* L., and *Amomum cardamomum* Wall fruits (Zingiberaceae); fresh leaves of *Piper betle* L., *Piper crocatum* (Ruiz & Pav.), fruits of *Piper retrofractum* Vahl and *Piper cubeba* L. (Piperaceae); barks of *Cinnamommum zeylanicum* Blume, *C. sintoc* Bl., and *Litsea cubeba* (Lauraceae); fresh leaves of *Syzygium aromaticum* (L.) Merrill & Perry, *Citrus aurantifolia* (Lamiaceae); and *Cymbopogon citratus* (Poaceae) were collected on February, 2009 in Yogyakarta, Indonesia. The species taxonomy identification was done by Joko Santoso, M.Sc. (Pharmacognosy Laboratory, Faculty of Pharmacy, Gadjah Mada University, Indonesia).

Isolation of the essential oils

About 5 kg of each fresh sample was separated, triturated and steam-hydro distilled for 6 hours. The appearances of essential oils yielded were observed. Those oils were sealed and kept in dark glass vials for further analyses.

Bacteria and Culture condition

S. mutans ATCC 21752 (Laboratory of Food and Nutrition, Inter University Centre, Gadjah Mada University, Indonesia) were grown in Nutrient Broth (Oxoid) for 24 h at 36.6°C. A McFarland standard 2 was used to adjust inoculum density in NaCl solution for MIC assays. Bacteria for biofilm assays were grown in BHI enriched with sucrose 2% and inoculums density was adjusted to McFarland standard 5.

Determination of the minimum inhibitory concentration (MIC)

MICs were determined by the micro titer broth method (Amsterdam, 1996) in sterile flat bottom 96-well polystyrene plates. Serial dilution of essential oils in methanol were used to determine the MICs of samples after 18-24 h growth at 36.6°C. Negative controls (cells + media), positive control (cells + media + mouthwash product), vehicle controls (cells + media + methanol), and media controls were included. Blank samples were prepared with same treatment as for samples, only without cells added. All tests were performed at least in triplicate. Optical density readings were taken using micro plate reader (Biorad Benchmark, Japan) at 595 nm for 18-24 h post-inoculation. To account for the effect of the essential oils color, a formula for calculating percent inhibition was used. The mean % inhibition of replicate tests was used to determine the final MIC values with formula modified from Quave and collaborators (2008) as follows:

$$\%Inhibition = \left(1-\left(\frac{ODsample-ODsample-blank}{ODsample-ODvehicle-blank}\right)\right) X\ 100\% \quad \text{.....(1)}$$

where OD_{sample}: Optical Density of samples + bacterial suspension; $OD_{sample\text{-}blank}$: Optical Density of samples + saline; $OD_{vehicle}$: Optical Density of control + bacterial suspension; $OD_{vehicle\text{-}blank}$: Optical Density of vehicle control + saline. MIC value was determined as 90% of inhibition or more.

Samples mixtures were re-inoculated on Nutrient Agar plates. MBC value was determined as the lowest concentration with no visible growth.

Determination of the biofilm formation inhibition

IC_{50} was determined by the adherence assay method modified from O'Toole and Kolter, (1998). Serial dilutions of sample solutions were put on flexible U-bottom 96-well polystyrene plates (Becton Dickinson, USA) and were incubated for 18-24 h growth at 36.6°C. Controls were prepared as in the MIC assay. Blank samples were obtained by performing the same treatment as for samples, only without cells added. All tests were performed at least in triplicate. After 18-24 h incubation, the contents of the wells were aspired, rinsed 3 times with distilled water, and were fixed for 10 min. Then, 125 µL of 1% crystal violet stain was added to the wells and left for 15 min. The excess stain was rinsed off with tap water. A 200 µL methanol was added to each wells, left for 15 min and then 150µL was transferred to a flat bottom 96-well plates. Optical density readings were taken using micro plate reader at 595 nm. To account for the effect of the essential oils color, a formula for calculating percent inhibition was used. The mean % inhibition of replicate tests used was the same as for MIC calculation. IC_{50} was determined as a concentration of sample which inhibited 50% of biofilm formation in comparison to vehicle controls and was calculated by using probit analysis with SPSS (Statistical Package for the Social Sciences) version 15.0.

Determination of the biofilm degradation activity

EC_{50} was determined by the adherence assay method modified from O'Toole and Kolter, (1998). Serial dilutions of sample solutions were put on flexible U-bottom 96-well polystyrene plates (Becton Dickinson, USA). Controls were prepared as in the MIC assay. Blank samples were obtained by performing the same treatment as for samples, only without cells added. All tests were performed at least in triplicate. After 24 h incubation at 36.6° C, the contents of the wells were aspired. Serial dilution of essential oils in media were added to the wells and incubated for another 24 h in 36.6° C incubator. Further treatment was as done for biofilm formation inhibition determination. EC_{50} was determined as a concentration of sample which degrade 50% of 24 h-old-biofilm formed in comparison to vehicle controls and was calculated by using probit analysis with SPSS (Statistical Package for the Social Sciences) version 15.0.

Bioautography

Bioautography assay was performed according to Gibbons and Gray (1998) with modification. Several TLC systems were evaluated to find out the best separation of the sample components (stationary phase used was precoated silica gel 60 F 254, Merck, Germany; toluene : ethyl acetate (93:7)$^{v}/_{v}$ as mobile phase). After drying the solvents, one replication of the eluted samples were put on to the Nutrient Agar containing bacteria in Petri dish for 1 h, while others were sprayed with anisaldehyde H_2SO_4 and $FeCl_3$ separately. After incubation in 36.6°C for 18-24 h, the diameters of inhibition zones of each spot were measured in mm.

Results and Discussion

This research has revealed that all essential oils tested were active against the planktonic growth and biofilm of *S. mutans*. Nevertheless, the essential oils of *P. crocatum*, *P. retrofractum*, and *A. cardamomum* had MIC_{90} value higher than 0.6 % (maximum concentration tested). All of the essential oils showed lower planktonic growth inhib-ition in comparison to the biofilm inhibition (Table I).

Table I. Inhibition data of the essential oils against the planktonic growth and biofilm of *Streptococcus mutans*

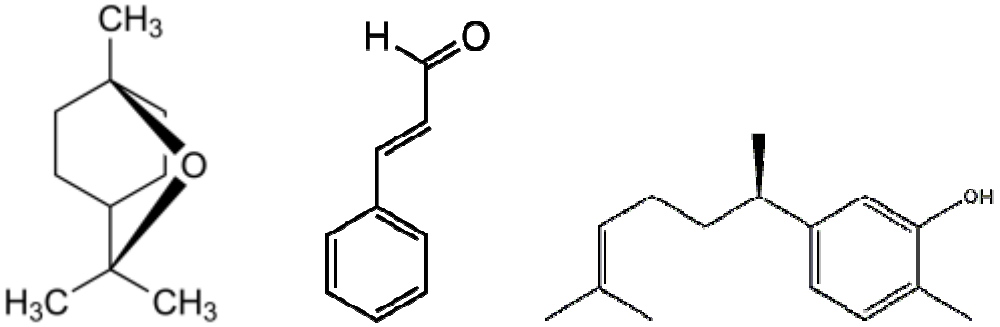
Samples	MIC_{90}	MBC	IC_{50}	EC_{50}
Z. officinale	0.06%	0.6%	0.011%	0.013%
C. xanthorriza	0.06%	> 0.6%	0.010%	0.083%
A. cardamomum	> 0.6%	> 0.6%	0.015%	0.031%
Z. aromaticum	0.06%	> 0.6%	0.014%	0.034%
Piper betle	0.06%	0.6%	0.061%	0.023%
P. crocatum	> 0.6%	> 0.6%	0.012%	0.017%
P. retrofractum	> 0.6%	> 0.6%	0.055%	0.015%
P. cubeba	0.06%	> 0.6%	0.026%	0.038%
Cinnamomum zeylanicum	0.06%	0.6%	0.011%	0.046%
Litsea cubeba	0.06%	0.6%	0.010%	0.03%
C. sintoc	0.6%	0.6%	0.005%	0.083%
Cymbopogon citratus	0.06%	0.6%	0.008%	0.026%
Syzygium aromaticum	0.06%	0.6%	0.030%	0.032%
Citru aurantifolia	0.06%	> 0.6%	0.027%	0.026%

Table II. Result of Bioautografi Assays (stationary phase: silica gel F 254, mobile phase: toluene-ethyl acetate=93:7 v/v, loading zone: 2 μL of essential oils 1% in toluene)

Sample	hRf	Color change			Inhibition zone (ϕ mm)	Active compound prediction
		UV_{254nm}	Anisaldehyde H_2SO_4	$FeCl_3$		
Z. officinale	44	√	Brown	-	6.0	Terpenoid
C. xanthorriza	59	√	Yellow	Blue	4.0	Phenolic
A. cardamomum	46	√	-	-	3.0	unidentified
Z. aromaticum	41	√	Yellow	-	6	Terpenoid
Piper betle	48	√	-	Brown	4.4	Phenolic
P. crocatum	-	-	-	-	-	-
P. cubeba	27	-	Purple	-	4.0	Terpenoid
P. retrofractum	31	-	-	Ungu	4.5	Phenolic
Cinnamomum zeylanicum	30	-	-	Purple	18	Phenolic
Litsea cubeba	44	-	Brown	Purple	6.5	Phenolic
C. sintoc	45	√	Black	-	8.5	Terpenoid
Cymbopogon citratus	44	√	Black	Purple	6.25	Phenolic
Syzygium aromaticum	46	√	-	Brown	8.8	Eugenol
Citrus aurantium	62	-	Brown	Purple	5.35	Phenolic

Some of the essential oils exhibited higher degradation activity than biofilm formation inhibition, while others showed the reverse.

Bioautography assays results were as described in table II. The spot of *C. zeylanicum* essential oil exhibited the highest activity, followed by *S. aromaticum*. *C. sintoc* exhibited the lowest IC_{50} value of biofilm formation inhibition (0.005%). It showed moderate biofilm degradation activity, while the essential oil from *Z. officinale* exhibited the highest activity (EC_{50} 0.013%).



(a) (b) (c)

Figure 1. Chemical structure of eucalyptol (a), cynamaldehyde (b), and xanthorrhizol (c).

Biofilm plaque in mouth cavity is actually consisted of diverse microbes, but mostly initiated by *S. mutans*. Moreover, ability of this Gram positive bacterium to convert sugars can generate acidic environment and cause cariogenic effect (Marsh, 2006). Therefore, finding of agents active against the planktonik as well as biofilm growth of this bacterium is expected to be valuable agents in oral hygiene products.

Essential oils were reported to kill microorganisms by disrupting cell walls and inhibiting enzymatic activities, therefore can prevent bacterial aggregation, slow multiplication and extract endotoxins (Ouhayoun, 2003). Several essential oils component such as menthol, eucalyptol (Ouhayoun, 2003), cynamaldehyde (Niu and Gilbert, 2004) and xanthorrhizol (Hwang and Rukayadi, 2006) have been reported to be potential antibacterial and antibiofilm.

Essential oils assayed in this research were selected based on the antibacterial activity reported in the literatures (Niu and Gilbert, 20004; Martins, *et al*., 2001; Nalina and Rahim, 2006, 2007; Juliantina, *et al*., 2009: Wang and Liu, 2010; Khan and Siddiqui, 2007; Gupta, *et al*., 2009; Chandarana, *et al*., 2005; Inouye, *et al*., 2001; Aneja and Joshi, 2009; Hwang and Rukayadi, 2006; Suwondo, 2007; Akin-Osanaiye, *et al*., 2007). Their abundant availability as well as their wide usage as traditional medicine in Indonesia were also the consideration of selection.

Regarding the prominent antibacterial activity of the *C. zeylanicum* essential oil, can be explained by the presence of cynamaldehyde compound which was supported by the bioautography result. Niu and Gilbert (2004) reported prominent planktonic and biofilm inhibition activity of cynamaldehyde, a major constituent of *C. zeylanicum* (cinnamon) against *Escherichia coli* and selected *Pseudomonas* species. Clove oil showed also prominent activity in bioautography result. Eugenol as the major constituent is a responsible component which contributes to the antibacterial activity. Moreover, Khan and collaborators (2007) reported anti quorum sensing activity of cinnamon and clove oils. Anti quorum sensing activity can inhibit communication between bacteria which further can inhibit bacterial biofilm formation (Williams, *et al*., 2007).

It is interesting to note that not all of active spots were phenolic compounds, even though all of those essential oils contain phenolics. Some factors such as diffusion capacity of the essential oil components through media may influence the bioautography results (Ramírez-Mares, *et al*., 2010). Synergistic effects of several compounds in the essential oils to expose the antibacterial activity may also occur. TLC method prior to the assay separated the active components which were then too dilute to exhibit the activity.

Curcuma xanthorrhiza ethyl acetate extract was reported by Kim and collaborators (2008) to be a potential anti plaque agent based on its antibacterial activity against planktonic and biofilm of mouth cavity microbes. The constituent responsible for the activity was

reported to be the xanthorrhizol. This compound is phenolic and should be extracted as essential oils components.

Conclusions

The essential oils of *C. citratus*, *Z. officinale,* and *C. sintoc* were proven to be potential dental plaque inhibitors. Result showed that the essential oils of *C. sintoc* exhibited the highest biofilm formation inhibition (IC_{50} = 0.005%), and *Z. officinale* showed the highest biofilm degradation with EC_{50} value of 0.013%. Both were active against bacterial planktonic growth but *C. sintoc* showed MIC_{90} value at 0.6%, while *Z. officinale* (0.06%). *C. citratus* was showed promising antibacterial and antibiofilm activities with MIC_{90} value of 0.06%, MBC 0.6%, IC_{50} 0.008% and EC_{50} 0.026%.

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