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ANTIBACTERIAL ACTIVITY OF ZINGIBERACEAE LEAVES ESSENTIAL OILS AGAINST *STREPTOCOCCUS MUTANS* AND TEETH-BIOFILM DEGRADATION

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ABSTRACT

This experiment was conducted to determine the potency of essential oils derived from Zingiberaceae leaves as antibacterial against *Streptococcus mutans* and biofilm degradation on teeth. The essential oils were derived from *Curcuma domestica*, *Curcuma zedoaria*, *Curcuma xanthorrhiza*, *Elettaria cardamomum*, *Kaempferia galanga*, *Zingiber officinale* varietas rubrum, and *Zingiber purpureum*. The assay was performed through dilution method. Chlorhexidine and a commercial mouthwash were used as positive control. The highest yield of essential oil was obtained from *Elettaria cardamomum* (2.43%). Essential oil of *Kaempferia galanga*, *Curcuma domestica*, *Elettaria cardamomum*, and *Zingiber purpureum* were successfully inhibited *Streptococcus mutans* with a minimum inhibitory concentration of 2000 µg/mL. Furthermore, *Elettaria cardamomum* showed the best antibacterial activity and degradation of biofilms. This essential oil was further analyzed using gas chromatography-mass spectrometer. The results showed eucalyptol as the main compound responsible for antibacterial activity and was predicted to effect biofilm degradation activity.

KEYWORDS: **Antibacterial, Biofilm Degradation, Essential Oils, Leaves; Zingiberaceae.**

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INTRODUCTION

Indonesia is a mega diversity country with full of various plants species. Many of plant species in Indonesia are used as medicinal plants. Zingiberaceae is the plant family that most widely used as medicinal plants, not only in Indonesia but also in other country. [1] Rhizome of Zingiberaceae species commonly used traditionally as ingredient of traditional medicine. The essential oil of Zingiberaceae rhizome was reported as an antibacterial. [2,3] Sniffing essential oils also had been reported had slimming or weight gain effects.[4-8]The report for utilization of Zingiberaceae family leaves as traditional medicine is still limited. In Indonesia, *Curcuma domestica* (turmeric) leaves used as herbs in fish cuisine. On the other hand, it reported that *Curcuma domestica* leaves essential oil namely ar-turmerone displayed mosquitocidal activity with an LD100 of 50 µg mL^{-1} on *Aedes aegypti* larvae and LC50 of 0.017mg mL^{-1} on *Anopheles gambiae*. [9,10] The hexane extract of turmeric leaves exhibited an antifungal activity against *Candida albicans* at 1 µg mL^{-1} and inhibited the growth of *Candida kruseii* and *Candida parapsilosis* at 25 µg mL^{-1}.[9] Meanwhile, the essential oil of *Curcuma phaeocaulis* leaves showed an antibacterial activity.[11] A chance to investigate antibacterial activity potency from other leaves of Zingiberaceae species is a challenge for future research. Antibacterial as well as the degradation of dental biofilm are the main problem in oral health.[12,13] Microorganisms form biofilms on teeth by forming a pellicle on the tooth surface, the colonization of bacteria and plaque or biofilm maturation.[14] Bacteria found in the mouth, namely *Streptococcus mutans* is a bacterium that causes the formation of dental plaque or biofilm.[15,16] Inhibition of *Streptococcus mutans* growth will inhibit the dental caries. Antibacterial is a substance that interfere the growth or kill bacteria by interfering with the metabolism of microorganism.[17] In addition, dental caries can also be inhibited by degradation of biofilm on the teeth. The objective of this study is to determine the potency of essential oils derived from Indonesian Zingiberaceae leaves as antibacterial against *Streptococcus mutans* and biofilm degradation on teeth.

MATERIALS AND METHODS

The Zingiberaceae leaves were collected from Tropical Biopharmaca Research Center Conservation and Cultivation unit, Darmaga Campus (Figure 1) on December 2014 and determined by Biology Research Center of Indonesian Institute of Research, Cibinong, Jakarta. The Zingiberaceae leaves used are *Curcuma domestica*, *Curcuma zedoaria*, *Curcuma xanthorrhiza*, *Elettaria cardamomum*, *Kaempferia galanga*, *Zingiber officinale* var *rubrum*, and *Zingiber purpureum*. The *Streptococcus mutans* was obtained from Faculty of Medicine, University of Indonesia No. 63301 and used as tested bacteria.

Figure 1
Location of Bogor Agricultural University (Institut Pertanian Bogor), the sampling sites of Zingiberaceae leaves: 6º33'33"S, 106º43'33"E.

The research steps including distillation of essential oil by steam distillation method, antibacterial activity test, biofilm degradation activity test and chemical constituent determination. Further, the chemical compounds in the most active essential oils were determined by Gas Chromatography-Mass Spectrometry (GC-MS).

METHODS

Isolation of essential oils from Zingiberaceae leaves
About 1.2 Kg of leaves was distilled for 3 hours using water (ratio 1:2) at 95-105 ºC with steam distillation method. The distillate was stood for 24 hours and the oil was separated using a separation funnel. The yield of Zingiberaceae leaves was determined based on the dry weight.

Antibacterial activity test
The antibacterial test against *S. mutans* was performed using micro-dilution method.[18] Essential oils diluted in DMSO to obtain a concentration stock of 10000 µg mL^{-1}. The stock samples were made into several concentrations (16-2000 µg mL^{-1}). In each well of sterile 96 well plates, samples, TSB medium and bacterial inoculant were added. The mixture was incubated at 37

ºC for 24 hours and the Minimum Inhibitory Concentration (MIC) determined. The Minimum Bactericidal Concentration (MBC) was determined after 24 hour incubation of MIC clear zone on new media.

Biofilm degradation activity test

Method used for biofilm degradation activity test is micro-dilution method.[19] Biofilms are formed by synthetic saliva (Mc Dougall solution) with TSB medium, 3 % glucose and bacterial inoculant in 96 well plates. The mixture was incubated for 24 h at 37 ºC. Once a biofilm is formed, the remaining medium is discarded. Essential oils are added at a concentration of 16-2000 µg mL^{-1} and then incubated 24 hours at temperature of 37 ºC. Biofilms attached to the wall of the wells is washed using phosphate buffer. Crystal violet 1 % was added to the wells and left for 15 minutes. Well rinsed with sterile water three times and 95 % ethanol was added. The suspension was incubated for 45 minutes and the solution was transferred to a new micro-plate. Suspension absorbance of each well was measured using a micro-plate reader at a wavelength of 595 nm to determine the % degradation. Chlorhexidine was used as positive control and 20 % DMSO as a negative control.

GC-MS Analysis

The most active essential oil was further analyzed by GC-MS (Shimadzu-QP-5050A, column: HP-5MS, 60m x 250 µm ID x 0.25 µm film thickness). The temperature was programmed from 70 ºC to 290 ºC (for 40 minutes) at the rate of 15 ºC.$minute^{-1}$. The injection and injection port temperature was 290 ºC, meanwhile the detector temperature was 250 ºC. Injection mode was split (50:1) and the inlet pressure was 18.03 psi. The carrier gas was helium with a flow rate of 1 mL $minute^{-1}$. The mass spectrometer conditions were as follows: ionization voltage 70 eV, MS source temperature at 250 ºC, MS quadrupole temperature at 150 ºC, interface temperature at 290 ºC, electron ionization mass spectra were acquired over the mass range of 40-800 m/z. Identification of the compounds was done by comparing with the mass spectra with spectra from NIST library data in literature.

Data Analysis

The data of activity test were analyzed with analysis of variance (ANOVA) at a confidence level of 95 % (α level of 0.05) using SPPS 20 software. The Duncan's multiple range test was also used.

RESULTS AND DISCUSSION

Essential Oils from Zingiberaceae Leaves

Seven fresh leaves species from Zingiberaceae family in Indonesia are used on this study. The local name and scientific name of all the samples is shown in Table 1. Three species are come from genus of *Curcuma*, two species from genus of *Zingiber*, and one species each from genus of *Electtaria* and *Kaempferia.*

Table 1
The essential oils yield and color of some Zingiberaceae leaves oil

No	Leaves name		Essential oils	
	Local	Scientific	Yield (%)	Color
1	Kunyit	*Curcuma domestica*	2.09	Pale yellow
2	Temu putih	*Curcuma zedoaria* Rosc.	0.33	Colorless
3	Temulawak	*Curcuma xanthorrhiza*	0.80	Tawny
4	Kapulaga	*Electtaria cardamomum*	2.43	Colorless
5	Kencur	*Kaempferia galanga*	0.19	Pale yellow
6	Jahe merah	*Zingiber officinale* var *rubrum*	0.13	Pale yellow
7	Bangle	*Zingiber purpureum* Roxb.	0.07	Pale yellow

The yield of essential oils from Indonesian Zingiberaceae leaves are varied (Table 1). The high yield of essential oils can be found on *Electtaria cardamomum* leaves followed by *Curcuma domestica* leaves. The lowest essential oil yield found in the *Zingiber purpureum* leaves. The essential oil yield of *Curcuma domestica* leaves from Indonesian was higher than from India clone reported by Akbar (2015).[20] The yield of essential oil of *Curcuma domestica* clone leaves from India was about 1.1 – 1.9 %[20], meanwhile the yield from Indonesian leaves was >2 %. The yield of *Zingiber officinale* var *rubrum* leaves oil on this study fourfold higher than from Malaysia reported by Sivasothy et al. (2011).[21] Oil yield from Malaysia was only about 0.03 %.[21] Otherwise, *Kaempferia. galanga* leaves essential oil yield from Indonesia is lower compared with the oil yield of Bangladesh *Kaempferia galangal* leaves (0.55 %) as reported by Bhuiyan et al. (2008).[22] The oil yield of *Curcuma zedoaria* leaves is also lower compared with *Curcuma zedoaria* dried leaf from Bangladesh (0.8 %).[23] There is no information related to the oil yield of other samples. The color of the essential oil is varied from colorless to tawny (Table 1). The essential oil from *Curcuma zedoaria* leaves was colorless. This result was different with *Curcuma zedoaria* essential oil from Bangladesh which color was yellowish.[23] It because on this study, the leaf used is the fresh leaves while in the research from Rahman et al. (2014) is from the dried leaves.

Antibacterial and Biofilm Degradation Activities of Essential Oils from Zingiberaceae Leaves

The antibacterial activity of all essential oil is reported by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). MIC is the minimum concentration that can inhibit the bacterial growth, while MBC is the minimum concentration to kill the bacteria. The lowest MIC and MBC value the most active the samples.[18] The antibacterial activity of all essential oils used on this study is shown in Table 2. Four essential

oils can inhibit the *Streptococcus mutans* growth at concentration of 2000 µg mL^{-1} and the other three essential oils could not inhibit the *Streptococcus mutans* growth until the highest concentration of 2000 µg mL^{-1}. The activity of the Zingiberaceae leaves essential oils is lower compared to chlorhexidine as positive control. All of essential oils could not kill the *Streptococcus mutans* until the highest concentration. It indicated that the essential oils from Zingiberaceae leaves possessed mild antibacterial activity. To select the prospective essential oil for oral care, the activity on biofilm degradation was performed. The biofilm degradation activity of essential oils was reported by inhibition concentration 50 % (IC_{50}), the concentration that can degrade 50 % of the biofilm. The results of this activity are shown in Table 2. All of the essential oils from Zingiberaceae diminished the biofilm with various value of IC_{50}. The most prospective oil for degradation of biofilm is *Zingiber officinale* var *rubrum* leaves oil. However, IC_{50} value of *Zingiber officinale* var *rubrum* leaves oil was not significantly different with *Electtaria cardamomum* leaves oil and other zingiberaceae leaves oils, except with *Curcuma zedoaria* leaves oil. The biofilm degradation activity of zingiberaceae leaf oil on this study are lower compare to the activity of chlorhexidine as positive control but higher compare to the activity of commercial mouth wash.

Table 2
Antibacterial and biofilm degradation activity of essential oil from Zingiberaceae leaves

No	Leaves name	Antibacterial activity (µg mL^{-1})		Biofilm degradation activity
		MIC	MBC	IC_{50} (µg mL^{-1})
1	*Curcuma domestica*	2000 [b]	-	354.8[b]
2	*Curcuma zedoaria* Rosc.	-	-	689.6[c]
3	*Curcuma xanthorrhiza*	-	-	289.1[b]
4	*Electtaria cardamomum*	2000 [b]	-	243.3[b]
5	*Kaempferia galanga*	2000 [b]	-	254.9[b]
6	*Zingiber officinale* var *rubrum*	-	-	218.0[b]
7	*Zingiber purpureum* Roxb.	2000 [b]	-	314.8[b]
8	Chlorhexidine	16[a]	16	29.8[a]
9	Commercial mouth wash	2000[b]	-	2001.4[d]

Number that is followed by the same superscripts were not significantly different (P > 0.05) (Duncan's multiple range test)- means the value is higher than 2000

The activity of Zingiberaceae leaves oil on this study gives additional information about its potency. Limited information about antimicrobial activity of the zingiberaceae essential oils is reported. *Zingiber officinale* var *rubrum* leaves essential oil had antibacterial activity against *Bacillus licheniformis*, *B. spizizenii*, *S. aureus*, *E. coli, Klebsiella pneumonia*, and *Pseudomonas stutzeri*.[21] Meanwhile, *Curcuma domestica* leaves essential oils inhibited the fungal (*Aspergillus flavus*) growth.[24] Other paper reported that the essential oil of zingiberaceae had activity not only as antibacterial but also as antioxidant. *Curcuma zedoaria* leaf essential oil has antioxidant activity with IC_{50} about 14.8 mg mL^{-1} against radical of 1,1-diphenyl-2-picrylhydrazyl, DPPH radical.[23] The other activity reported is from *Kaempferia galangal* leaves extract which has antinociceptive property.[25]

Component on Active Essential Oil

Several compounds on the zingiberaceae leaves essential oils had been reported. *Curcuma domestica* leaves essential oil had α-phellandrene as the major compound[20], *Kaempferia galangal* leaves oil possess linoleoyl chloride as the major component[22], *Curcuma zedoaria* leaves oil contains eucalyptol as the major compound[23], and *Zingiber officinale* var *rubrum* leaf oils had β-caryophyllene (31.7 %) as the major compound.[21] The structure of the major compound reported is shown in Figure 2.

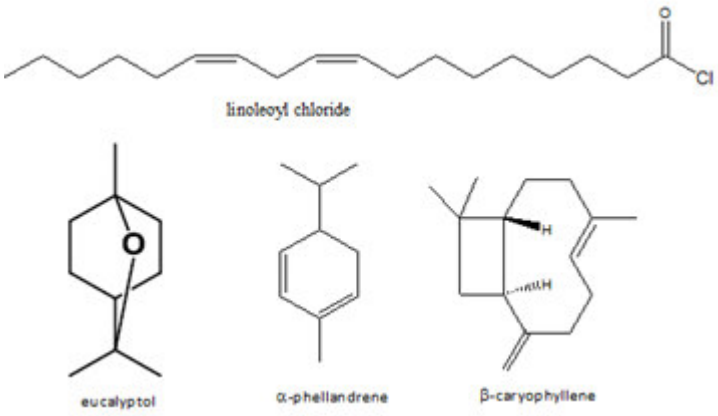

Figure 2
Structure of major compound found in some Zingiberaceae leaves essential oils.

To search the active compound for antibacterial against *Streptococcus mutans* and biofilm degradation, the *electtaria cardamomum* leaf essential oil is chosen because it has antibacterial activity, good to degrade biofilm, and has high yield. The total ion chromatogram of *electtaria cardamomum* leaf essential oil is shown in Figure 3. There is one major compound found in *electtaria cardamomum* essential oil at retention time of 8.781 minutes. This major component was about 83% in abundance. It is suggested that this compound would be the active compound as antibacterial against *Streptococcus mutans* and as biofilm degradation.

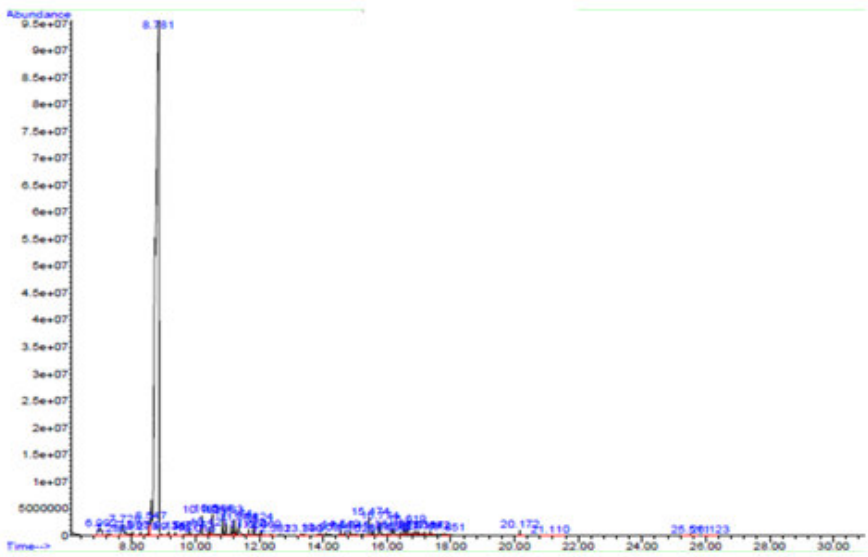

Figure 3
Total ion chromatogram of Electtaria cardamomum leaves essential oil.

Based on the mass spectrum of the major peak and compared with the reference mass spectrum, the major peak is eucalyptol. Eucalyptol is also major compound found in *Curcuma zedoaria* leaves oil, but the content of eucalyptol in *Curcuma zedoaria* leaves oil was only about 22.4 %.[23] It indicated that the concentration of eucalyptol in *Electtaria cardamomum* leaves oil is four times higher than in *Curcuma zedoaria* leaves oils. Therefore, the activity of *Curcuma zedoaria* leaves oil is not as good as the *Electtaria cardamomum* leaves oils. In conclusion, most of zingiberaceae leaves essential oils have antibacterial activity against *S. mutans*, and all of the essential oils are good to degrade the biofilm. The most prospective essential oil is from *Electtaria cardamomum* leaves with eucalyptol as the active compound.

CONFLICT OF INTEREST

Conflict of interest declared none.

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