Journal of the Indonesian Chemical Society
J. Idn. Chem. Soc. 2019, 02(1), 42-47 FULL PAPER

# Antibacterial and Biofilm Degradation Activity of Extract From Steam Distillation Residue of Zingiberaceae Leaves Against *Streptococcus mutans*

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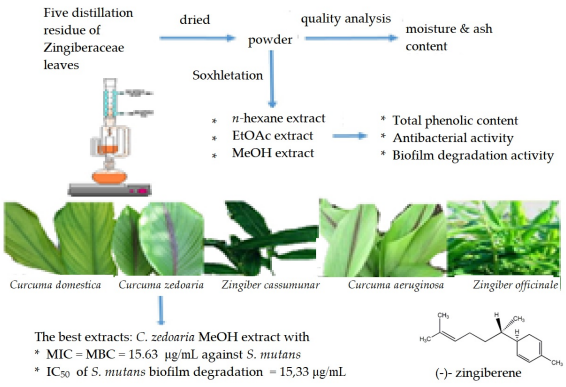
## Abstract

Zingiberaceae is a family of plant that has been widely used to treat various diseases and as an element of spice in cooking. In this paper, the potential of the extract from the steam distillation residue of Zingiberaceae leaves as antibacterial and biofilm degradation agent was studied and determined against *Streptococcus mutans*. Five different species of Zingiberaceae, which consisted of *Curcuma longa, Curcuma zeodoaria, Curcuma aeruginosa, Zingiber officinale, Zingiber cassumunar* were taken for samples and their distillation residues were extracted by soxhlation using 3 different solvents namely *n*-hexane, ethyl acetate, and methanol. The antibacterial and biofilm degradation activity of the assay from each of the samples was determined by the microdilution technique. Among the 15 Zingiberaceae leaves distillation residue extracts, five are categorically active against *Streptococcus mutans* with their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values being the same with that of chloramphenicol, 15.63 µg/mL. All extracts were found to degrade the biofilm. The methanol extract of *C. zeodoaria* leaves was found to have the highest antibacterial activity with MIC and MIB vaues of 15.63 ppm and the best to degrade the biofilm with inhibitory concentration 50% ($IC_{50}$) of 15.33 ppm. The antibacterial and biofilm degradation activities of extracts are not related to the phenolic content and it was suggested that terpenoid such as (-)-zingiberene may have been the active component.

Extract from the steam distillation residue of Zingiberaceae leaves was used as antibacterial and biofilm degradation agent against *Streptococcus mutans* using microdilution technique. The methanol extract of *C. zeodoaria* leaves shows the highest antibacterial activity with MIC and MIB vaues of 15.63 ppm and the best to degrade the biofilm with inhibitory concentration 50% ($IC_{50}$) of 15.33 ppm.




**Article History:**
Received: 22 April 2019, Revised 28 May 2019, Accepted 31 May 2019, Available Online 31 August 2019
http://dx.doi.org/10.34311/jics.2019.02.1.42



***Keywords***: Antibacterial, Biofilm degradation, *Streptococcus mutans*, Zingiberaceae leaves, Distillation residue

## Introduction

Indonesian people often use medicinal plants to maintain their health. Among all plant families, Zingiberaceae is the most used plant family wherein two genera commonly in use are Curcuma and Zingiber. People have used this family of plant to cure various diseases such as fever, skin diseases, digestive disorders, and respiratory disorders. The rhizome is the most used part of this plant, while the leaves had been reported to have high content of essential oils and shown tyrosinase inhibition, antioxidant, antiglycation, and antimicrobial activities [1, 2]. Antioxidant and antiglycation are important for health and often associated with antiaging. The essential oils from cardamom leaves (*Electtaria cardamomum*) are active against *Streptococcus mutans* and able to destroy the biofilm formed by this bacteria [1]. On the other hand, the essential oil of turmeric leaves is reported to inhibit the growth of *Aspergillus flavus* and aflatoxin [3].

During the production of essential oils from this plant, which are usually by distillation, a residue is produced as the by product of the process that is usually discarded or burned. This has become an enviromental concern. On the other hand, the residue may still have active components that can be utilized for further applications. This study was aimed to determine the antibacterial and biofilm degradation activities of the extract from the steam distillation residue of Zingiberaceae leaves against *Streptococcus mutans*.

## Experimental Section

### Materials and apparatus

Zingiberaceae leaves residue from distillation process was obtained from tropical Biopharmaca Research Center (TropBRC) – Conservation and Cultivation Unit and the voucher specimen was deposited in TropBRC IPB University. The test organism *Streptococcus mutans* for the determination of antimicrobial and biofilm degradation activities was procured from Microbiology Laboratory, Faculty of Medicine, University of Indonesia (ATCC® 35668TM). Analytical grade solvents for the extraction of the residue: *n*-hexane, ethyl acetate, and methanol were purchased from Merck. Gallic acid, Folin-Ciocalteu phenol reagent, sodium carbonate, dimethyl sulfoxide (DMSO), Tryptic Soy Broth (TSB) medium, synthetic saliva (McDougall solution), glucose, phosphate buffer, tris(4-(dimethylamino)phenyl)methylium chloride (crystal violet) and chlorhexidine were purchased from Sigma Aldrich.

### Preparation of samples

Five different types of Zingiberaceae leaves (Table 1) residue from distillation process were dried by oven at temperature below 40 °C. The dried leaves were then analyzed for their moisture and ash content.

### Extraction of samples

Some of dried leaves were extracted by using Soxhlet apparatus and three different organic solvents: *n*-hexane, ethyl acetate, and methanol. The yields of extraction were determined

### Determination of phytochemical and total phenolic content

Qualitative analysis of tannin, saponin and terpenoids were done according to the methods described by Edeoga [4] and that by Ayoola *et al*. [5] for flavonoid and alkaloid. Total phenolic content was determined by spectrometry according to the previous method [6]. Briefly, deionized water and Folin-Ciocalteu phenol reagent were added to the extract and the control sample of gallic acid which was followed by the addition of sodium carbonate 7% after 5 minutes of incubation. The mixture was kept in total darkness for 1 hour and then was subjected to spectrophotometric analysis by measuring the absorbance at 750 nm using a AquaMate 8000 UV-Vis spectrophotometer. The total phenolic content in the mixture was determined by plotting the absorbance data of sample into the calibration curve of gallic acid. Gallic acid equivalents (GAE) (% (w/w)) of dried plant material were used as the expression of total phenolic content.

### Determination of the antimicrobial and the antibiofilm activities

The tests for antibacterial activity of the extracts against *S. mutans* were performed using micro-dilution method [1]. The extracts were diluted in DMSO to obtain several concentrations with a range of 16-2000 $\mu$g/mL. In each well of sterile, 96 well plates, bacterial inoculant, TSB medium, and samples were added and incubated at 37 °C for 24 hours, after which the Minimum Inhibitory Concentration (MIC) was subsequently determined. Clear zone after 24-hours incubation of MIC clear zone on new media was determined as Minimum Bactericidal Concentration (MBC).

The same method of microdilution was performed for the activity test of biofilm degradation. In 96 well plates, synthetic saliva (McDougall solution), TSB medium, glucose, and bacterial inoculant was put and incubated for 24 hours at 37 °C to form the biofilms. The remaining medium was discarded and the extract (16-2000 μg/mL) was added and then incubated for 24 hours at 37 °C. Phosphate buffer was used to wash the biofilms attached to the wall of the wells, and crystal violet 1 % was added to the wells. The absorbance of suspension was measured using a micro-plate reader at a wavelength of 595 nm to determine the percentage (%) of degradation. Chlorhexidine was used as positive control and 20 % DMSO as a negative control.

**Statistical analysis**

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

## Results and Discussion

**Name of plants, moisture content, and ash content of samples**

Five different Zingiberaceae plants as shown in Table 1 were selected. The essential oils from the leaves of these plants have been reported to show antioxidant, antiglycation, and antibacterial activity against *S. mutans* as well as demonstrate the ability to degrade the biofilm produced by *S. mutans* [1, 2]. As previously mentioned, the distillation process not only produces essential oils but also residue.

**Table 1.** Common and local name of Zingiberaceae leaves distillation residue as sample

| Sample name | Common name | Local name |
|---|---|---|
| *C. domestica* | Turmeric | Kunyit |
| *Z. officinale* | Ginger | Jahe |
| *Z. cassumunar* | - | Bangle |
| *C. zedoaria* | White turmeric | Temu putih |
| *C. aeruginosa* | Blue ginger | Temu hitam |

The samples used on this research are residues from the distillation process of the five plants in Table 1. The dried residue in this research has different moisture and ash content as shown in Table 2. The moisture content of all dried materials is less then 10% and is used as the correction for ash content and yields. The ash content is reported based on the dried materials and ranging from from 7 to 12 %.

**The phytochemical content, extraction yield, and total phenolic content of extracts**

The phytochemical content of all extracts is shown in Table 3. All *n*-hexane and EtOAc extracts contained steroid, triterpenoid, flavonoid and alkaloid, while methanol extract contained tannin, steroid, triterpenoid, flavonoid and alkaloid. The chemical components from the leaves residue have the potential for different biological activities including antimicrobial agent.

**Table 2.** Moisture and ash content of Zingiberaceae leaves distillation residue as sample

| Sample name | Moisture content (%) | Ash content (%) |
|---|---|---|
| *C. domestica* | 8.98 | 9.67 |
| *Z. officinale* | 7.58 | 11.03 |
| *Z. cassumunar* | 9.32 | 11.76 |
| *C. zedoaria* | 8.38 | 7.64 |
| *C. aeruginosa* | 7.98 | 8.26 |

**Table 3.** Phytochemical content of extracts

| Sample name | Solvent | Group compound | | | | |
|---|---|---|---|---|---|---|
| | | a | b | c | d | e |
| *C. domestica* | *n*-hexane | - | - | + | + | + |
| | EtOAc | - | - | + | + | + |
| | MeOH | + | - | + | + | + |
| *Z. officinale* | *n*-hexane | - | - | + | + | + |
| | EtOAc | - | - | + | + | + |
| | MeOH | + | - | + | + | + |
| *Z. cassumunar* | *n*-hexane | - | - | + | + | + |
| | EtOAc | - | - | + | + | + |
| | MeOH | + | - | + | + | + |
| *C. zedoaria* | *n*-hexane | - | - | + | + | + |
| | EtOAc | - | - | + | + | + |
| | MeOH | + | - | + | + | + |
| *C. aeruginosa* | *n*-hexane | - | - | + | + | + |
| | EtOAc | - | - | + | + | + |
| | MeOH | + | - | + | + | + |

Note: + is present and – is absent
Group compound (a) tannin, (b) saponin, (c) steroid/triterpenoid, (d) flavonoid, (e) alkaloid

The yields from the extraction varied according to the source as well as the solvent as shown in Table 4. The methanol extract has the highest yield in all the five samples with *C. domestica* leaves being the highest while the lowest was found in the residue of *Z. cassumunar* leaves from EtOAc extract.

Total phenolic content is reported as gallic acid equivalent which is commonly used for the determination of phenolic content [7]. The phenolic

content of the extracts also varied. The highest total phenolic content was found in the residue of *Z. officinale* leaves of methanol extract while the lowest was found in the residue of *C. zedoaria* leaves of *n*-hexane extract. It must be noted that the total phenolic content of extract of distillation residue of Zingiberaceae leaves is lower than the fresh extract [6]. It means that the distillation process reduces the total phenolic content in the sample.

**Table 4**. Extraction yield and total phenolic content of extracts

| Sample name | Solvent | Yield (%) | Total phenolic content (mgGAE/g sample) |
|---|---|---|---|
| *C. domestica* | *n*-hexane | 4.14$^{g}$ | 0.60$^{a}$ |
| | EtOAc | 2.20$^{d}$ | 0.78$^{ab}$ |
| | MeOH | 7.82$^{i}$ | 2.81$^{g}$ |
| *Z. officinale* | *n*-hexane | 3.69$^{f}$ | 1.46$^{c}$ |
| | EtOAc | 2.12$^{d}$ | 2.53$^{f}$ |
| | MeOH | 4.99$^{h}$ | 3.78$^{h}$ |
| *Z. cassumunar* | *n*-hexane | 1.62$^{b}$ | 1.09$^{bc}$ |
| | EtOAc | 1.37$^{a}$ | 2.98$^{g}$ |
| | MeOH | 3.43$^{ef}$ | 1.81$^{d}$ |
| *C. zedoaria* | *n*-hexane | 1.89$^{c}$ | 0.49$^{a}$ |
| | EtOAc | 1.63$^{b}$ | 0.94$^{b}$ |
| | MeOH | 1.86$^{c}$ | 1.27$^{c}$ |
| *C. aeruginosa* | *n*-hexane | 3.02$^{e}$ | 0.60$^{a}$ |
| | EtOAc | 2.23$^{d}$ | 2.21$^{e}$ |
| | MeOH | 3.71$^{f}$ | 1.26$^{c}$ |

Note: The same letter (a-i) after the number in the same colom is shown not significantly different at 95 %

### The antibacterial and the antibiofilm activities

As shown in Table 5, the antibacterial activity against *S. mutans* varied accordingly. The residue of *C. domestica* leaves did not show any antibacterial activity as it did not inhibit the growth of bacteria even at concentration higher than 2000 µg/mL. In contrast, the residue of *C. zedoaria* and *C. aeruginosa* leaves showed a strong antibacterial activity against *S. mutans* as it inhibited the growth of the bacteria at a concentration as low as 15.6 µg/mL. The methanol extracts of the residue from *Z. officinale*, *C. cassumunar*, and *C. zedoaria* leaves together with the *n*-hexane extracts of the residue from *C. aeruginosa* and *C. zedoaria* leaves are the best antibacterial agent. Because they have the same MIC and MBC values of 15.6 µg/mL they are categorized as bactericidal agents. This level of antibacterial activity is the same as that shown by the positive control chloramphenicol and is interestingly better than the activity of commercial mouth-wash. There is no correlation between the antibacterial activity and total phenolic content. It means that the active ingredient might not be due to the presence of phenolic group. These results are contradictory to the work reported by Mondal and Kaur [8] which stated that phenolic, flavonoid, and tannin content is responsible to the antimicrobial activity.

**Table 5**. Antibacterial and biofilm degradation activities of samples

| Sample name | Solvent | Antibacterial against *S. mutans* | | % degradation of biofilm (µg/mL) |
|---|---|---|---|---|
| | | MIC (µg/mL) | MBC (µg/mL) | |
| *C. domestica* | *n*-hexane | >2000 | >2000 | 408.93$^{i}$ |
| | EtOAc | >2000 | >2000 | 198.32$^{h}$ |
| | MeOH | >2000 | >2000 | 438.79$^{i}$ |
| *Z. officinale* | *n*-hexane | 1000.0$^{b}$ | >2000 | 61.11$^{d}$ |
| | EtOAc | 15.6$^{a}$ | 250.0$^{d}$ | 81.40$^{e}$ |
| | MeOH | 15.6$^{a}$ | 15.6$^{a}$ | 20.96$^{ab}$ |
| *Z. cassumunar* | *n*-hexane | 1000.0$^{b}$ | >2000 | 112.01$^{g}$ |
| | EtOAc | 15.6$^{a}$ | 250.0$^{d}$ | 92.11$^{f}$ |
| | MeOH | 15.6$^{a}$ | 15.6$^{a}$ | 193.05$^{h}$ |
| *C. zedoaria* | *n*-hexane | 15.6$^{a}$ | 15.6$^{a}$ | 26.72$^{c}$ |
| | EtOAc | 15.6$^{a}$ | 125.0$^{c}$ | 15.33$^{b}$ |
| | MeOH | 15.6$^{a}$ | 15.6$^{a}$ | 17.05$^{b}$ |
| *C. aeruginosa* | *n*-hexane | 15.6$^{a}$ | 15.6$^{a}$ | 26.06$^{c}$ |
| | EtOAc | 15.6$^{a}$ | 62.5$^{b}$ | 39.97$^{d}$ |
| | MeOH | 15.6$^{a}$ | 62.5$^{b}$ | - |
| Chloramphenicol | | 15.6$^{a}$ | 15.6$^{a}$ | 1864.42$^{j}$ |
| Commercial mouth-wash | | 1000.0$^{b}$ | >2000 | - |
| DMSO | | >2000 | >2000 | - |
| chlorhexidine | | - | - | 2.02$^{a}$ |

Note: The same letter after the number is shown not significantly different at 95%

The antibacterial activity of the essential oils of *C. domestica* and *Z. cassumunar* leaves are acting as bacteriostatic at concentration of 2000 ppm, but the essential oils of *Z. officinale* and *C. zedoaria* leaves do not inhibit the *S. mutans* growth [1]. Unlike the essential oils, the residue of *C. domestica* leaves has no antibacterial activity either for *n*-hexane, EtOAc and methanol extract. Extracts of *Z. officinale* leaves residue of EtOAc and methanol extracts is more active as antibacterial against *S. mutans* compared to

the *n*-hexane extract. The same tendency was found in *Z. cassumunar* and *C. zedoaria* leaves.

The activity of all extracts to degrade the biofilm varied with an $IC_{50}$ value ranging from 15 to 438 μg/mL. The lowest $IC_{50}$ value was found in the extract of *C. zedoaria* leaves residue. The activity of this extract was not as good as the positive control chlorhexidine. *C. zedoaria* leaves methanol extract as has been reported by Zahra et al (2016) has the activity as an antioxidant with $IC_{50}$ of DDPH 544.05 mg/L and 7.0 mg Trolox Equivalents Antioxidant Capacity/g extract [6]. It means the *C. zedoaria* leaves before distillation process has antioxidant activity and its distillation residue has activity to degrade the biofilm.

*C. zedoaria* had been reported to consist of (-)-Zingiberene (Figure 1) [8], which was also cited as having antibacterial activity against *Bacillus cereus*, *Enterococcus faecalis*, Methilin-resistant *Staphylococcus aureus* (MRSA) and *Salmonella entiriditis* [10]. It means that one of the compounds that is responsible for the antibacteria activity against *S. mutans* and its biofilm degradation from *C. zedoaria* is (-)-Zingiberene.

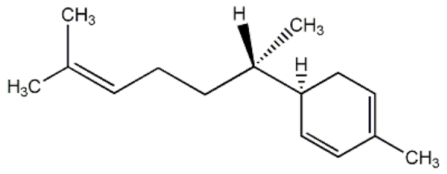



**Figure 1.** Structure of (-)-zingiberene

## Conclusions

The moisture content of distillation residue of Zingiberace leaves used in this research was less than 10% while the ash content varied between 7.6 – 11.8%. The extraction yield varied between 1.3 – 7.8%. The terpenoid, flavonoid, and alkaloid content are found on all extract and the phenolic content on the extracts is from 0.4 to 3.0 mg gallic acid equivalen/g extract. Residue of *Z. officinale* leaves of MeOH extract, *C. cassumunar* leaves of MeOH extract, *C. aeruginosa* leaves of *n*-hexane extract, *C. zedoaria* leaves of *n*-hexane and MeOH extracts were found to be the best antibacterial agent with MIC and MBC being the same as chloramphenicol activity, which is 15.63 μg/mL. The activity of all extracts to degrade the biofilm varied ($IC_{50}$ value from 15 to 438 μg/mL) and the best biofilm degradation was found on extract of *C. zedoaria* leaves residue. The antibacterial and biofilm degradation activities is not related to the phenolic content, perhaps terpenoid such as (-)-zingiberene is the active component.

### Authors Contribution

Conceptualization and methodology: Irmanida Batubara and Irma Herawati Suparto; Data curation, Dian Yunita; Formal analysis, Irmanida Batubara, Dian Yunita, Irma Herawati Suparto; Validation, visualization, writing, review and editing: Irmanida Batubara.

### Conflict of Interest

The authors declare that there is no conflict of interest.

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