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ZINGIBER OFFICINALE ETHANOLIC EXTRACT INHIBITS FORMATION OF PSEUDOMONAS AERUGINOSA BIOFILM

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ABSTRACT

This study was carried out to determine antibiofilm activity of Zingiber officinale ethanolic extract against Pseudomonas aeruginosa. Retention time of phytochemical compounds separated by gas chromatography mass spectrometry ranged from 17.00 to 39.00 and a major compound identified was 1, 3- Cyclohexadiene, 5–(1,5-dimethyl-4-hexenyl)-2-methyl. Microbroth dilution showed minimum inhibitory concentration of Z. officinale ethanolic extract against P. aerugionsa was 12.5 mg/ml. In disc diffusion assay, Z. officinale ethanolic extract was found to exhibit inhibitory effect against P. aerugionsa under both aerobic (diameter of zone of inhibition ranging from 6.2±0.2mm to 22.0±1.6mm) and anaerobic (diameter of zone of inhibition ranging from 13.6±2.9mm to 22.4±2.8mm) conditions. In the context of antibiofilm activity which was determined using static microplate biofilm formation assay, Z. officinale ethanolic extract demonstrated lower optical biofilm density than that of control under both aerobic (optical biofilm density ranging from 0.059±0.002 to 0.080±0.008) and anaerobic (optical biofilm density ranging from 0.060±0.003 to 0.066±0.005) conditions at all test concentrations. Furthermore, the treatment of Z. officinale ethanolic extract caused a reduction in the amount of extracellular biofilm DNA compared to the negative control under aerobic (200 mg/ml- 0.6424±0.019; 50 mg/ml-0.6688±0.112) and anaerobic (50 mg/ml-0.6248±0.003 conditions. Taken together, Z. officinale ethanolic extract possesses a promising potential in combating P. aeruginosa biofilm.

KEY WORDS

antibiofilm activity; biofilms; Pseudomonas aeruginosa; Zingiber officinale

INTRODUCTION

For decades, traditional folks frequently use spices as remedies for various human diseases because these plant species contain components of therapeutic values. The household spice such as ginger (*Zingiber officinale*) has been accepted as an alternative form of health care which has raised a great interest in exploring its potential in combating bacterial infection. Originating from South East Asia, the ginger which belongs to family Zingiberaceae is also used in Ayurvedic medicine [1]. It is widely known to have thick tuberous rhizomes which possess important medicinal values such as anti-inflammatory, cholesterol-lowering, and antithrombotic properties [1,2]. According to [3], the rhizome is rich in the secondary metabolites such as curcumene, non-volatile hydroxyaryl compounds e.g. zingerone, gin- geroles and shogaoles (phenylalkanones), volatile ses- quiterpenes (e.g. zingiberene and bisabolene) and mono-terpenoids (e.g. citral). Although the antibacterial activity of *Z. officinale* has been investigated [3-6], the potential of *Z. officinale* in

controlling biofilm growth remains to be elucidated.

Nowadays there is a growing interest in studying the microbial biofilms in order to overcome various human diseases including cystic fibrosis which is commonly associated with *Pseudomonas aeruginosa* pathogen. This facultative anaerobic bacterium colonizes and subsequently forms a biofilm layer lining the lungs prior to adaption with environment of partial or total oxygen depletion [7] and development of resistance towards many antibiotics. Considering the previously reported antibacterial activity of *Z. officinale* and the severity of chronic lung infection by *P. aeruginosa* biofilm, the present study was carried out to determine inhibitory effect of *Z. officinale* against *P. aeruginosa* biofilm formation.

In this study, the rhizome of *Z. officinale* was extracted using 100% ethanol solvent and subjected to gas chromatography mass spectrometry (GCMS) analysis and determination of minimum inhibitory concentration (MIC). The antibacterial activity was assessed using disc diffusion assay, while the antibiofilm activity was evaluated using a static microplate biofilm formation assay. Both disc diffusion and microplate biofilm formation assays were performed under aerobic and anaerobic conditions.

Materials and methods

a. *Plant extract and test microorganism*

Fresh rhizomes were washed with tap water, ground and extracted using 100% ethanol solvent for three days at room temperature. The solvent was then filtered and removed using rotary evaporator. *Pseudomonas aeruginosa* strain ATCC 10145 *was obtained* from culture collection of the School of Biology, Faculty of Applied Sciences, UiTM Shah Alam and maintained on nutrient agar media. A batch culture of *P. aeruginosa* strain ATCC 10145 was grown at 37 °C in nutrient broth and its purity was assessed regularly by Gram-staining and colony morphology.

b. *Gas chromatography mass spectrometry (GCMS) analysis*

The phytochemical profile of the plant extract was studied using gas chromatography mass spectrometry (GCMS) which was performed by Agilent 7809A GC/5975MSD system at the flow rate of 1ml/min. Initial temperature was set to 50°C and was held for 2 minutes. The first rate was set to 4°C/min until it reached 200°C. Then the temperature was increased by 10°C/min until it reached 300°C. The start and end m/z ratios were 40 and 350. Helium was used as the carrier gas. Identification of phytochemical compounds was achieved by comparing the mass spectra and GC retention times with data system library of the GC-MS equipment, NIST05.

c. *Determination of minimum inhibitory concentration (MIC)*

Minimum inhibitory concentration (MIC) value of plant extract was determined using microbroth dilution method in Mueller-Hinton broth (MHB). Inoculum was prepared at a density adjusted to 0.5 McFarland turbidity standard [(10^8colony forming units (CFU/ml)] and diluted 1:10. The plant extract concentration ranged from 3.125 to 100 mg/ml whilst the final volume in each well of microplate was 200 µl. A positive control (containing 20 µl of inoculum and 180 µl of MHB) and negative control (containing 20 µl of plant extract and 180 µl of MHB without inoculum) were included on the microplate which was then incubated at 37°C. The MIC value was defined as the lowest concentration of *Z. officinale*

ethanolic extract at which the test microorganism did not show visible growth or turbidity and it was recorded after 24 h of incubation.

d. *Disc diffusion assay*

Antibacterial activity of the plant extract was determined using disc diffusion assay with sterilized paper discs containing plant extract at various concentrations (12.5 mg/ml to 200 mg/ml). The discs were applied on the surface of nutrient agar plates, which were previously swabbed uniformly with the bacterial suspension of 10^8 cfu· ml^{-1} (turbidity = McFarland standard 0.5). Erythromycin and DMSO were used as a positive and negative control respectively. The inoculated plates were then incubated in an upright position at 37°C for 24 hours. The diameter of inhibition zones (diameter of paper disc, 6 mm was included) was measured in mm and the results were recorded. The disc diffusion assay procedure was performed in five replicates under both aerobic and anaerobic conditions. The anaerobic condition was developed using candle jar and a strip of paper soaked in methylene blue dye.

e. *Determination of optical biofilm density*

Antibiofilm activity of plant extract was assessed using a static microplate biofilm formation assay. Inoculum was prepared at a density adjusted to 0.5 McFarland turbidity standard [(10^8colony forming units (CFU/ml)] and diluted 1:10. The plant extract concentration ranged from 12.5 to 200 mg/ml. The final volume in each well of microplate was 210 µl (containing 150 µl of fresh nutrient broth, 30 µl of inoculums and 30 µl of plant extract). Erythromycin and DMSO were used as a positive and negative control respectively. Following 24 hours incubation at 37°C, the media containing planktonic cells and *Z. officinale* extracts was discarded while surface-attached biofilm cells were stained with crystal violet for five minutes. The excess stain was rinsed off with tap water and optical biofillm density was determined using microplate reader at a wavelength of 570 nm. The static microplate biofilm formation assay procedure was performed in five replicates under both aerobic and anaerobic conditions. The anaerobic condition was developed using candle jar and a strip of paper soaked in methylene blue dye. The same assay procedure was repeated for another microplate to determine extracellular biofilm DNA.

e. *Determination of extracellular biofilm DNA*

Extracellular biofilm DNA from the static microplate biofilm formation assay was measured using spectrophotometry. Following 24 hours biofilm incubation and removal of media containing planktonic cells and *Z. officinale* extract as described earlier, a volume of 200 µl of phosphate buffer saline (PBS) was added to each well of the microplate to solubilize the biofilm cells. The biofilm suspension was then incubated at room temperature for one hour and its extracellular DNA was measured using microplate reader at a wavelength of 260 nm.

f. *Statistical analysis*

All data from disc diffusion assay and biofilm formation assay were expressed as means±S.E.M. and ANOVA was performed to determine the degree of significance between the groups whereby $p<0.05$ was considered significant.

RESULTS AND DISCUSSIONS

a.Phytochemical profile of Z. officinale ethanolic extract

Phytochemical compounds are of key importance in explaining the biological activites

of medicinal plants. In this study, the phytochemical profiling of *Z. officinale* ethanolic extract was carried out using GCMS. **Figure 1** shows chromatogram of phytochemical compounds present in *Z. officinale* ethanolic extract. The phytochemical compounds were identified by mass spectral interpretation and library searches. The retention time of the chromatographically separated phytochemical compounds ranged from 17.00 to 39.00. However, there were only four compounds identified with acceptable quality value (>85) (**Table 1**). The major compound recorded from *Z. officinale* ethanolic extract was 1, 3 - Cyclohexadiene, 5 – (1, 5 – dimethyl – 4 - hexenyl) – 2 - methyl (RT: 18.01; peak area: 45.24%) which is commonly known as Zingiberene. Our GCMS data has revealed the presence of phytochemical compounds in *Z. officinale* which are known to possess important medicinal value.

Figure 1 Chromatogram of *Z. officinale* ethanolic extract

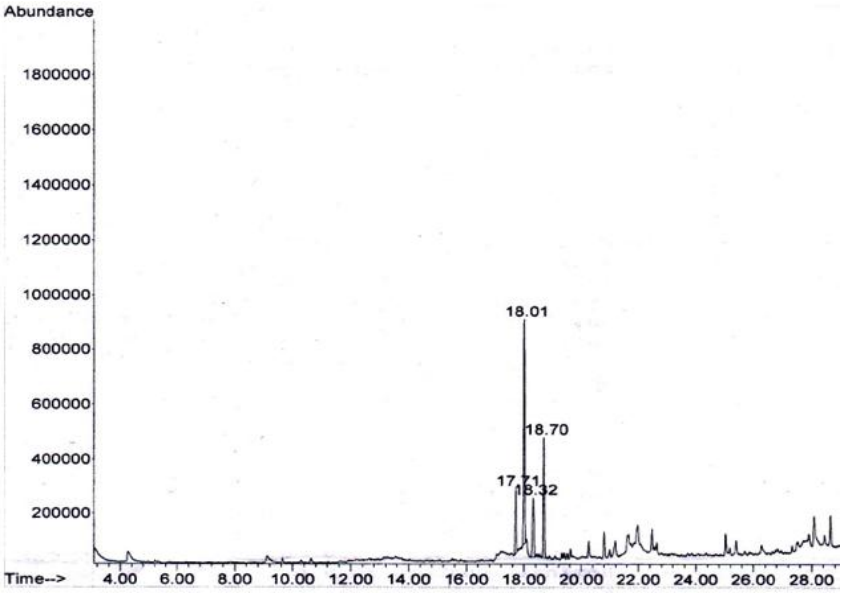

Table 1: Identified phytochemical compounds with their retention time, composition and quality values

Compounds	Common name	Retention time	Area %	Quality
Benzene, 1-(1,5-dimethyl-4-hexenyl) -4-methyl	Curcumene	17.70	12.9	99
1, 3- Cyclohexadiene, 5 – (1,5-dimethyl-4-hexenyl)-2-methyl	Zingiberene	18.01	45.24	94
Cyclohexene, 1-methyl-4-(5-methyl-1-methylene-4-hexenyl)	Bisabolene	18.32	17.2	95
Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene	β-esquiphellandrene	18.7	24.65	93

Recently, Bhargava et al. [6] performed a study on *Z. officinale* with respect to its phytochemical profile and antibacterial activity. In that study, the rhizome of *Z. officinale* was extracted using 100% ethanol and methanol. Following the GCMS analysis, the Zingiberene compound was

found to be present in both plant extracts (20.57% and 15.32% respectively). Zingiberene was found to be the major compound in both plant extracts, which is agreement with the findings herein.

b. *Minimum inhibitory concentration (MIC) value and zone of inhibition*

Minimum inhibitory concentration (MIC) value is important to determine efficacy of antibacterial agent. Low MIC value may be an indication of high efficacy or that microorganism has no potential to develop resistance towards the bioactive compound. The MIC value against *P. aeruginosa* measured for *Z. officinale* ethanolic extract was 12.5 mg/ml. This MIC value was then being used for disc diffusion assay to determine antibacterial activity of *Z. oficinale* ethanolic extract.

Disc diffusion assay has been the most common method for antibacterial screening. Considering the pathogenicity of *P. aeruginosa* in both aerobic and anaerobic environments, the antibacterial potential of *Z. officinale* ethanolic extract was evaluated under both aerobic and anaerobic conditions. Based on **Table 2**, *Z. officinale* ethanolic extract exhibited inhibitory effect against *P. aerugionsa* under both aerobic (diameter of zone of inhibition ranging from 6.2±0.2mm to 22.0±1.6mm) and anaerobic (diameter of zone of inhibition ranging from 13.6±2.9mm to 22.4±2.8mm) conditions. At all test concentrations except 200 mg/ml, its antibacterial activity under anaerobic condition was significantly ($p < 0.05$) higher than that of aerobic condition. It was likely that *Z. oficinale* ethanolic extract has a greater antibacterial activity under anaerobic condition.

Table 2: Antibacterial activity of *Z. officinale* ethanolic extract measured as diameter (mm) of zone of inhibition with =5

Concentration	Aerobic	Anaerobic	Significant difference ($p < 0.05$)
200mg/ml	22.0±1.6mm	22.4±2.8mm	No
100mg/ml	15.8±1.9mm	21.4±2.3mm	Yes
50mg/ml	14.4±2.2mm	19.6±2.8mm	Yes
25mg/ml	10.4±1.7mm	17.8±3.1mm	Yes
12.5mg/ml	6.2±0.2mm	13.6±2.9mm	Yes
+ve control	18.0±9.0mm	15.2±0.6mm	No
-ve control	0.0±0.0mm	0.0±0.0mm	No

Gao and Yang [5] reported the antibacterial activity of polysaccharide, flavonoid, aqueous and ethanolic extract from *Z. officinale* against various microorganisms including *P. aeruginosa.* However, that study did not include the antibacterial activity of *Z. officinale* ethanolic extract under anaerobic condition. Meanwhile, the antibacterial activity of vancomycin antibiotics has also been studied under aerobic and anerobic environments [8]. As shown by flow cytometric data and CFU number, inhibitory effect of vancomycin antibiotics against *Staphylococcus aereus* under aerobic condition was greater than that of anaerobic condition despite its MIC value was similar in both environments. In the context of our study, the *Z. officinale* ethanolic extract has also the antibacterial activity against *P. aeruginosa* in the anaerobic environment thereby updating the

current information of medicinal value of *Z. officinale* ethanolic extract.

c. *Optical biofilm density*

Nowadays, microbial biofilm cells have been a great interest in development of antimicrobial agent due to its higher antibiotics resistance as compared to their planktonic counterparts. In the present study, inhibitory effect of *Z. officinale* ethanolic extract against formation of *P. aeruginosa* biofilm was determined using static microplate assay system. **Table 3** illustrates optical biofilm density at wavelength of 570 nm. Inhibition of biofilm formation was indicated by optical biofilm density of the test concentrations lower than that of negative control. It was observed that the *Z. officinale* ethanolic extract inhibited *P. aeruginosa* biofilm formation under both aerobic (optical biofilm density ranging from 0.059±0.002 to 0.080±0.008) and anaerobic (optical biofilm density ranging from 0.060±0.003 to 0.066±0.005) environments at all test concentrations. At test concentration of 50 mg/ml, 100 mg/ml and 200 mg/ml, the antibiofilm activity of *Z. officinale* ethanolic extract under aerobic condition was not significantly ($p > 0.05$) different from that of anaerobic environment. This may suggest that antibiofilm activity of *Z. oficinale* ethanolic extract under aerobic condition was equivalent to that of anaerobic condition.

Table 3: Antibiofilm activity of *Z. officinale* ethanolic extract measured as optical biofilm density at a wavelength of 570 nm with n=5

Concentration	Aerobic	Anaerobic	Significant difference ($p < 0.05$)
200mg/ml	0.064±0.005	0.060±0.003	No
100mg/ml	0.059±0.002	0.060±0.008	No
50mg/ml	0.063±0.004	0.065±0.007	No
25mg/ml	0.074±0.010	0.060±0.004	Yes
12.5mg/ml	0.080±0.008	0.066±0.005	Yes
+ve control	0.076±0.002	0.078±0.002	No
-ve control	0.082±0.011	0.109±0.028	Yes

In the last few years, medicinal plant and marine bacterial products have widely been investigated for their antibiofilm potential. The natural resources has become a new interest in developing antimicrobial agents because there are many resistance problems reported to be associated with the commonly used antibiotics. In 2011, Vacheva et al. [9] reported the antibiofilm activity of 14 plant extracts against three clinical isolates of *Escherichia coli*. In that study, it was observed that the growth percentage of the biofilm treated with plant extracts ranged from 34% to 367%. Some plant extracts were found to favor the *E. coli* biofilm growth which explained the growing resistance of *E. coli* isolates towards the selected plant extracts. We believe that the resistance of *P. aeruginosa* biofilm towards *Z. officinale* ethanolic extract was not observed in this study. A number of studies have attempted to explain the possible general antibiofilm mechanism using various experimental tools including electron microscopy. Recently, Chusry et al. [10] demonstrated that treatment of *Quercus infectoria G. Olivier* extract and tannic acid resulted in formation of clumps of

staphylococcal cells with thickened and slightly rough cell wall. This observation was supported by another finding from the same study where those treatments increased staphylococcal cell surface hydrophobicity (CSH). They concluded that changes in hydrophobicity index mediated the antibiofilm activity of *Quercus infectoria G. Olivier* extract and tannic acid. Furthermore, understanding of antibiofilm mechanism has been greater when accounts for aspect of cell-surface interaction as reported by [11]. In that study, it was revealed that a bacterial exopolysaccharide (A101) inhibited the cell-surface interaction and multicellular aggregates formation happened on *S. aureus* and *P. aeruginosa* which was detected by phase-contrast microscopy with 600 magnifications. However, the A101 was only able to disrupt the multicellular aggregates of *P. aeruginosa* FRD1, but not that of *S. aureus* RN6390. They proposed that the antibiofilm activity of A101 may involve irreversible binding or adsorption in *S. aureus* RN6390, and reversible in *P. aeruginosa* FRD1. Considering the fact that a particular antibiofilm agent can inhibit biofilm either by causing formation of multicellular clumps or interfering with the microbial cell-surface interaction, it was possible that the *Z. officinale* ethanolic extract exhibited the same antibiofilm mechanism.

d. *Extracellular biofilm DNA*

Cell death and lysis associate with release of most intracellular contents into extracellular environment including deoxyribonucleic acid (DNA). Also, microbial biofilms release their DNA into extracellular environment in order to make them incorporated into extracellular polysaccharide (EPS) matrix [12] which protects them from antibiotics treatment. Considering these facts, one of the possible ways to investigate further the antibiofilm effect of the plant extract is determining amount of extracellular DNA. In the present study, the extracellular biofilm DNA following treatment of *Z. officinale* ethanolic extract was measured spectrophotometrically. **Table 4** depicts amount of extracellular biofilm DNA at a wavelength of 260nm. It was noted that the amount of extracellular biofilm DNA of several test concentrations were lower than that of negative control under aerobic (200 mg/ml-0.6424±0.019; 50 mg/ml-0.6688±0.112) and anaerobic (50 mg/ml-0.6248±0.003) environments. At all test concentrations except 50 mg/ml, the amount of extracellular biofilm DNA under aerobic condition was significantly ($p < 0.05$) different from that of anaerobic environment.

A study of DNA release by microbial cells has been reported by [13] where the amount of extracellular DNA was measured using propidium iodide in order to determine effect of iron on DNA release. That experiment showed that a high level of iron (100 mM FeCl3) in the medium suppressed DNA release, structural biofilm development, and increased resistance of subpopulations towards antimicrobial compounds which might be due to suppression of pqs quorum-sensing systems. The extracellular DNA was suggested to be critical in the biofilm formation following treatment of DNAse I. However, in that study, planktonic cells were not separated from biofilm cells thereby the effect of iron was also accounting for the extracellular DNA of planktonic cells. According to [14] the extracellular DNA possess an antibacterial activity which contributed to cell lysis by chelating cations that stabilize lipopolysaccharide (LPS) and the outer membrane (OM). This DNA-mediated killing resulted in the release of cytoplasmic contents, including genomic DNA. Moreover, that study also demonstrated that the DNA release induced the PhoPQ- and PmrAB-regulated cationic

antimicrobial peptide resistance operon PA3552–PA3559 in *P. aeruginosa* which resulted in 640-fold increased resistance to aminoglycosides. They also concluded that the extracellular DNA caused development of cation gradients, genomic DNA release and antibiotic resistance. With regard to the cell lysis that associates with DNA release, Qin et al. [15] revealed that the release of genomic DNA into extracellular environment is mediated by autolysin. By using *Staphylococcus epidermidis* as a model of microbial biofilm, they suggested that the extracellular DNA is generated in bacterial populations through AtlE-mediated lysis of a subpopulation of the bacteria, and that the extracellular DNA promotes biofilm formation of the remaining population. The significance of extracellular DNA as a universal component of the biofilm matrices has also been supported by [16] which proposed that the changes of the biofilm biomass in the presence of DNase I may also be observed in the biofilms of different unrelated gram-positive and gram-negative bacteria. Our result was in agreement with that suggestion where the negative control also released a certain amount of genomic DNA into extracellular environment under both aerobic and anaerobic conditions. Considering the possible antibiofilm mechanism of *Z. officinale* ethanolic extract with regards to inhibition of cell-surface interaction, we believe that the treatment of the plant extract should result in low extracellular biofilm DNA. Meanwhile, inconsistency of our data (**Table 4**) should be further validated using propidium iodide as described by [13].

Table 4: Antibiofilm activity of *Z. officinale* ethanolic extract measured as amount of extracellular biofilm DNA at a wavelength of 260 nm with n=5.

Concentration	Aerobic	Anaerobic	Significant difference ($p < 0.05$)
200mg/ml	0.6424±0.019	0.9514±0.062	Yes
100mg/ml	0.9168±0.067	0.7152±0.177	Yes
50mg/ml	0.6688±0.112	0.6248±0.003	No
25mg/ml	0.9222±0.636	0.825±0.2489	Yes
12.5mg/ml	0.9414±0.554	0.7118±0.1357	Yes
+ve control	0.5842±0.0064	0.6078±0.0055	Yes
-ve control	0.713±0.0310	0.685±0.0066	No

CONCLUSION

We have demonstrated that *Z. officinale* ethanolic extract has antibacterial activity against *P. aeruginosa* under both aerobic and anaerobic conditions. Further investigation showed that it is also able to inhibit *P. aeruginosa* biofilm formation under both aerobic and anerobic conditions. With respect to extracellular biofilm DNA, it seems that treatment of *Z. officinale* ethanolic extract has also affected the DNA release by *P. aeruginosa* biofilm. We believe that the antibiofilm activity against *P. aeruginosa* observed in this study is due to the activity of zingiberene, the major compound in *Z. officinale* ethanolic extract.

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