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Antibiofilm Activity of Galangal (*Alpinia galanga*) Against *Staphylococcus aureus*

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ABSTRACT

A significant antibacterial effect of Galangal *(Alpinia galangal)* against foodborne bacteria *Staphylococcus aureus*, and *Listeria monocytogenes*, was reported with higher quantity of active biomolecule 1'-acetoxychavicolacetate. However, the effect on biofilm formation is yet unknown. Therefore, crude extract of Galangal rhizome was investigated for *in-vitro* antibiofilm activity against *Staphylococcus aureus*. Galangal extract was tested for antimicrobial activity against six strains of S. aureus including methicillin resisitant S. aureus. The antibiofilm activity was tested against *S. aureus SA113* using microtiter-plate assay and scanning electron microscopic imaging. Galangal extract showed antimicrobial activity with an inhibition zone ranging from 36 - 46 mm against for all tested strains of *S. aureus*. The minimum inhibitory concentration and the minimum bactericidal concentration of the extract were 1.25 mg/ml and 5 mg/ml, respectively. More than 50% reduction of biofilm adhered to the surface was observed in 2.5 mg/ml representing minimum biofilm resistant concentration $MBRC_{50}$ of the extract. The antibiofilm assay showed significant (80.68%) reduction at 20 mg/ml of extract. The 1´acetochavicol acetate (82.88%) was found to be the major chemical compound of galangal extract. Galangal rhizome extract possesses antibiofilm activity against *S. aureus.* Findings of the study could be useful for application of galangal crude extract as a disinfectant in food industry to eliminate the cross contaminations of *S. aureus*.

KEYWORDS

Crude Galangal extract; GC-MS Analysis; SEM imaging; 1'ÁCA; MIC

INTRODUCTION

Formation of bacterial biofilms on food processing surfaces leading to cross contaminations has been considered as a food safety issue worldwide. Around 80% of the bacterial infections including foodborne diseases in the United States have been associated with biofilms [1]. In particularly, biofilm associated infections are mostly caused by *Staphylococcus aureus* which has been recognized as a frequent cause of many foodborne contaminant outbreaks worldwide. In processed foods, *S. aureus* can cause adverse effects on consumers as it act

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as a vector in food poisoning. Biofilms is a microbiological sessile community where cells are irreversibly attached to a surface and are embedded in extracellular polymeric substances which are produced by the cells [2]. On food processing surfaces the bacterial resistivity increases as these extracellular polymeric substances can act as a barrier for sanitizers or antibacterial agents to penetrate through the biofilm [3]. Therefore, there is a need to investigate novel and safe biomolecules developed as antimicrobial agents to combat food-borne pathogens growing on food processing surfaces.

Galangal is a native plant in Southeast Asia and Southern China. The rhizome of Galangal is widely used as a spice and flavoring agent and an indigenous medicinal plant in traditional medicine to cure arthritis, diarrhoea, stomachache, asthma and bronchitis [4]. These have also reported the effect of galangal on antiprotozoal, antiplatelet, antiviral, antidiabetic, immunomodulatory, hypolipidemic activities [5]. Galangal rhizomes have exhibited strong antimicrobial activity against Gram-positive bacteria specifically S. aureus including methicillin resistant *S. aureus* [6,7]. Therefore, the use of crude Galangal extract as a natural antimicrobial agent to control biofilm formation would be a better option to eliminate cross contaminations in the food processing industry.

There has been no literature found on antibiofilm activity of Galangal against *S. aureus*. Therefore, this investigation for compounds with the potential to inhibit biofilm formation could be considered timely approach. The main objective of this study was to investigate antibiofilm activity of Galangal crude extract against *S. aureus*, phytochemical screening and its ability to eradicate biofilm formation on stainless steel surfaces. Exploitation of such plant derived compounds may lead to control biofilm formation upon treatment by Galangal extract of food industry utensils and surfaces.

MATERIALS AND METHODOLOGY

Hexane extraction of Galangal

Different plant parts of Galangal were collected from a herbarium in Nature's Secret (Pvt) Ltd. Horana and authenticated using a published key and the discription [8]. Galangal rhizomes were washed thoroughly under tap water and sliced into smaller pieces after skin removal. Galangal pieces were dried at 40°C in an oven (INC/75/55/DG, Genlab) for 3 days and subjected to grinding (MX-T110PN, National, Taiwan) to make a fine powder. Hexane extracts were prepared by adding 10 g of Galangal powder to 100 ml of hexane and agitated for 24 h at 28°C in a rotary shaker (Stuart orbital shaker SSLI, UK). Mixture was then centrifuged at 10000 rpm for 10 min and the supernatant was filtered using Watmann No 1 filter paper. The filtrate was filter sterilized and concentrated under vacuum at 40°C using a rotary evaporator (Bouchi Labortecnik RV 5, Switzerland). Concentrated extract was evaporated to dryness through nitrogen fluxing and re-dissolved in Di methyl Sulphur Oxide (DMSO) to make a 0.5 g/ml stock solution and stored at 4°C until use.

Test micro-organisms

The antimicrobial activity of Galangal extract was determined against six strains of *S. aureus* including Methicillin Resistant *Stapylococcus aureus* (MRSA). ATCC 25925, ATCC 29213 and ATCC 49476 stains of *S. aureus* were obtained from American Type Culture Collection (Manassa, USA) while, *S. aureus* SA113, MSSA 21D and MSSA 25D strains were obtained from Queensland University of Technology, Brisbane, Queensland, Australia. Bacterial growth media used in this study were from Oxoid (Basingstoke, UK). The antibiofilm activity was investigated using *S. aureus* SA113 strain particularly. Sub cultures were maintained using Tryptic Soy Agar (TSA) slants while main cultures of all *S. aureus* strains were maintained in glycerol stocks at -20°C.

Disk diffusion assay

The antimicrobial activity of Galangal extract was evaluated using a slightly modified agar disc diffusion method [9]. Bacterial cultures of six different strains of *S. aureus* were grown for 18 ± 1 hr at 37°C in 2 ml of Tryptic Soy Broth (TSB) and centrifuged to obtain an aliquot of 1 ml bacterial suspension in 0.85% saline solution. The suspension was then serially diluted three times in 9 ml of 0.85% saline solution to obtain 10^5 cfu/ml bacterial suspension. An aliquot of 100 µl from 10^6 cfu/ml bacterial suspension was spread on the surface of Mueller Hinton (MH) agar in petri plates. An aliquot (10 µl) of Galangal extract was then pipetted on to a sterile paper disc (Whatman No. 1, 5.5 mm diameter) on the agar surface. Two disks were impregnated with Streptomycin (10 µl) and DMSO (10 µl) on another plate to serve as a positive and negative controls. The plates were inverted and incubated for 18 ± 1 h at 37°C. Microbial inhibition was determined by measuring the diameter of the clear zone around each disc and recorded as diameter of inhibition zone (DIZ) of growth in millimeters.

Broth dilution assay

To determine the Minimum Inhibitory Concentrations (MIC), Galangal extract was tested against six strains of *S. aureus* with some modifications of the method described by Hufford and Clark, [10]. Twofold serial dilutions of extracts were obtained with MH broth. An aliquote of 40 µl of Galangal extract (10 mg/ml) was added to the first tube containing 2 ml of MH broth, two fold dilutions were carried out to the sixth tube to obtain five dilutions (5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml and 0.325 mg/ml). One drop of Tween 20 (29 mg) was added to each tube followed by vortexing and heating in a water bath at 50°C for 5 min to solubilize the extract. An aliquot of (10 µl) test microorganism was added to each tube. A tube containing MH broth and microorganism was considered as the positive control while a tube containing 10 µl of Streptomycin in MH broth and microorganism was considered as the negative control. The control with Streptomycin was used for the comparison of the growth of bacteria. Another control tube was prepared with MH broth and Galangal extract for further confirmation of zero contamination. Tubes were subsequently incubated at 37°C and visually examined for the lowest concentration of extract for the inhibition of microbial growth (indicated by a clear solution) after 24 h and 48 h. The lowest concentration of the crude Galangal extract where bacterial growth was not visible was recorded as the MIC. Further, the Minimum Bactericidal Concentration (MBC) was determined by adding an aliquot of 100 µl from the tubes showing no visible growth of bacteria to antimicrobial-free medium (TSA plates) and examined for no growth or < 0.01% growth after incubating for 18 ± 1 h at 37°C.

Biofilm assay

The antibiofilm activity of crude Galangal extract was tested against *S. aureus* SA113 using a method described by Kwasny and Opperman [11]. Biofilm adherence of *S. aureus* SA113 in standard microtiter assay plates was quantified using crystal violet staining method. Bacterial suspension of 10^6 cfu/ml was obtained after incubating *S. aureus* SA113 culture in 2 ml of TSB at 37°C for 18 ± 1 h (10^8 cfu/ml) and diluting 1:100 in a media containing TSB and 40% glucose where final glucose concentration was <1%. Sterile, flat bottom 96- plate (griener bio-one, Cellstar 655180) wells were inoculated with 200 µl of bacteria and incubated at 37°C for 24 h. The content was aspirated and each row of the plate was treated with 200 µl of previously prepared concentrations (20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml) of Galangal extract. The row treated with TSB and microorganism was served as the growth control whereas the row treated with TSB and the extract was served as the media control. After incubating the plate at 37°C for 24 h the content was aspirated and washed three times with distilled water. Then the biofilms were fixed using heat at 60°C for 60 min. staining of biofilms formed in each well

were carried out for 1 min using 0.06% crystal violet solution. The plate was then washed three times with 0.85% saline solution to remove excess crystal violet. Staining was removed using 95% ethanol solution to quantify the degree of biofilm adherence and the absorbance was measured using a microtiter well plate reader at 595 nm. These readings were used to calculate the Minimum Biofilm Inhibition Concentration ($MBIC_{50}$) which was considered as the concentration required for 50% reduction of the biofilm adhered to the surface.

Scanning Electron Microscopic (SEM) imaging

Biofilm formation of *S. aureus* SA113 was allowed on stainless steel surfaces using the method described in section 2.5. The SEM sample preparation was carried out according to a method described by Salimena et al., [12] with some modifications. Two identical stainless steel pieces with the dimentions of 5 mm× 5 mm× 1 mm were used to compare the biofilm growth on pieces treated with 5 mg/ml and 20 mg/ml of Galangal extract against the control**,** where complete biofilm growth was obtained without Galangal extract. Samples were then freeze dried at -20 ^{o}C for 24 h**r** after gradually dehydrating with a series of ethanol (25, 50, 70, 90, 95% for 2 min). Surface morphology of *S. aureus* SA113 biofilm sample treated with Galangal extract and the complete biofilm growth without the extract on stainless steel were observed seperately, using scanning electron microscope (LEO 1420VP), operated at an accelerated voltage of 18.00 kV and at a working distance of 12 mm. The samples were gold plated by using gold sputter.

GC-MS analysis for phytochemical screening of crude Galangal extract

The chemical composition of the galangal hexane crude extract was determined using GC-MS analysis in order to identify the major chemical compounds. The mass selective detector agilent 6890N series was used for the analysis. The capillary column of Rtx-wax crossbond carbowax polyethylene glycol (0.25 mm × 0.25 µm, 30 m) was used and the temperature was first held at 50°C for 2 min and then raised to 250°C at a rate of 10°C/min held at 250°C for 8 min. The carrier gas was helium at a flow rate of 0.9 ml/min. The injection volume was 1 µl. The components of the extract were recognized by the retention time of the chromatogram peaks and by their mass spectra. The percentage of each compound was calculated as the ratio of the peak area to the total chromatographic area. Wiley W9N08 database was used as the reference library.

Statistical analysis

Antibacterial and antibiofilm related to each experiment was independently replicated three times. The triplicate data were subjected to statistically analysis using ANOVA of the General Linear Model of SPSS statistical package (Version 16) and one way ANOVA of Minitab statistical package (version 14) respectively at 5% significance level. Means were compared using Dunnet simultaneously testing procedure at $P<0.05$.

RESULTS

Disk diffusion assay

All *S. aureus* strains tested showed higher DIZ ranging from 36 mm to 47 mm as presented in Table 1. However, there was no significant difference ($P < 0.05$) in mean DIZ between six *S. aureus* strains tested.

S. aureus strain	**Zone of growth inhibition/DIZ (mm) of crude Galangal extract**
S. aureus SA113	$43.08 \pm 1.26^{a*}$
S. aureus ATCC 25925	44.83 ± 1.53^{a}
S. aureus ATCC 29213	40.33 ± 1.53^{a}
S. aureus ATCC 49476	46.67 ± 1.70^{a}
S. aureus MSSA 25D	43.17 ± 1.61^{a}
S. aureus MSSA 21D	37.25 ± 1.25^{a}
Streptomycin	18.00±0.00
DMSO	6.00±0.00

Table 1: Antibacterial activity of crude Galangal extract against different strains of *S. aureus* using disk diffusion assay. **Note:** *Within the column, mean values followed by the same lowercase letter are not significantly different at $P < 0.05$ level.

Broth dilution assay

The MIC and MBC values obtained at 24 h and 48 h using broth dilution assay are presented in Table 2. The

MIC value of Galangal extract against all *S. aureus* strains tested showed 1.25 mg/ml except *S. aureus* MSSA 21D which showed MIC value of 2.5 mg/ml at 24 h. The MIC values did not change at 48 h except *S. aureus* ATCC 25925 strains. The MBC value for all tested strains of *S. aureus* was found to be 5 mg/ml irrespective of the strain and the value did not change at 48 h.

S. aureus strain	Time	Concentration of extract (mg/ml)	
	Period	MIC	MBC
S. aureus ATCC 25925	24 h	1.25	5
	48 h	2.5	5
S. aureus ATCC 29213	24 h	1.25	5
	48 h	1.25	5
S. aureus MSSA 25D	24 h	1.25	5
	48 h	1.25	5
S. aureus MSSA 21D	24 h	2.5	5
	48 h	2.5	5
S. aureus SA113	24 h	1.25	5
	48 h	1.25	5
S. aureus ATCC 49476	24 h	1.25	5
	48 h	1.25	5

Table 2: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of crude Galangal extract against *S. aureus*.

Biofilm assay

The biofilm assay results are shown in Table 3. The absorbance values of all tested concentrations were significantly different ($P < 0.05$) compared to the control. More than 50% reduction of biofilm adhered to the surface was observed in 2.5 mg/ml of extract.

Concentrations mg/ml	Absorbance ± SD nm	Inhibition %
20	$0.299 \pm 0.05^{a*}$	80.68
10	0.475 ± 0.05^{a}	69.38
5	0.618 ± 0.06^{a}	60.21
2.5	0.747 ± 0.09^{a}	51.94
1.25	0.880 ± 0.07^{a}	43.41
0.625	0.920 ± 0.06^{a}	40.82
Control	1.558 ± 0.11^{b}	
Media control	0.02 ± 0.00	

Table 3: Antibiofilm activities for different concentrations of crude Galangal extract against S. aureus SA113 and their percentage inhibition on biofilm adherence.
Note: *Within the column, mean values followed by the same lowercase letter are not significantly different at $P < 0.05$ level.

Therefore, the $MBRC_{50}$ of the extract could be considered as 2.5 mg/ml. Further, 80.68% biofilm reduction was observed at higher concentration 20 mg/ml of the extract. However, sub-inhibitory concentration (0.625 mg/ml) and MIC (1.25 mg/ml) did not show any considerable reduction in biofilm.

SEM imaging

The SEM view showed a considerable reduction of biofilm adherence at 5 mg/ml and a significant reduction at 20 mg/ml of crude Galangal extract compared to the control (Figure 1).

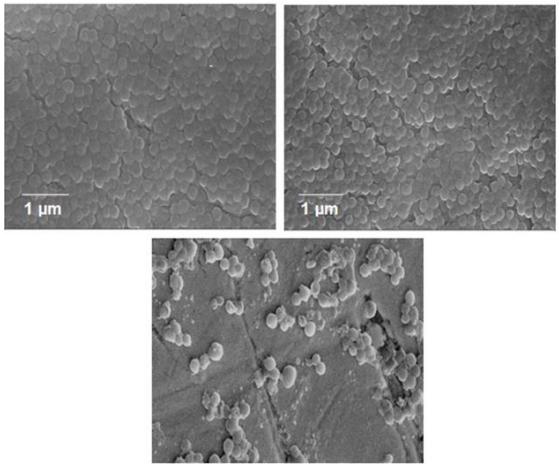

Figure 1: SEM view for the antibiofilm activity against S. aureus SA113 a) complete biofilm growth served as the control b) biofilm growth after treating with 5 mg/ml and c) 20mg/ml of Galangal extract.

GC-MS analysis

The chemical composition of crude Galangal extract is presented in Table 4. Nine compounds (>1%) were contributing to 97.03% of total chemical composition of the extract.

Peak time	Chemical compound	% of Total
8.863	1,8- cineole	1.16
14.474	Trans-.beta. –farnesene,	2.68
15.031	Beta.-Bisabolene	1.88
15.216	Beta-Sesquiphellandrene	1.31
15.402	Benzaldehyde, 2-methoxy-4-methyl	1.55
16.283	Unknown 01	82.88
18.091	Unknown 02	1.68
19.390	Hexadecanoic acid/ palmitic acid	1.26
20.920	9-octadecenoic acid	2.63
	Total	97.03

Table 4: Chemical compositions of crude Galangal extract using Gas chromatography- Mass spectrometry (GC-MS) analysis.

DISCUSSION

This study reveals strong antimicrobial activity of *A. galanga* due to high DIZ against all tested strains of *S.*

aureus. In a previous study, hexane and ethanol extracts of Australian grown Galangal showed a DIZ of 34.1 mm and 29.1 mm respectively against *S. aureus* ATCC 25925 [7], whereas in this study with the hexane extract of Galangal grown in Sri Lanka, a higher DIZ of 44.83 ± 1.53 mm obtained for the same strain. This could be mainly due to the differences in chemical composition of rhizome extracts due to geographical variations.

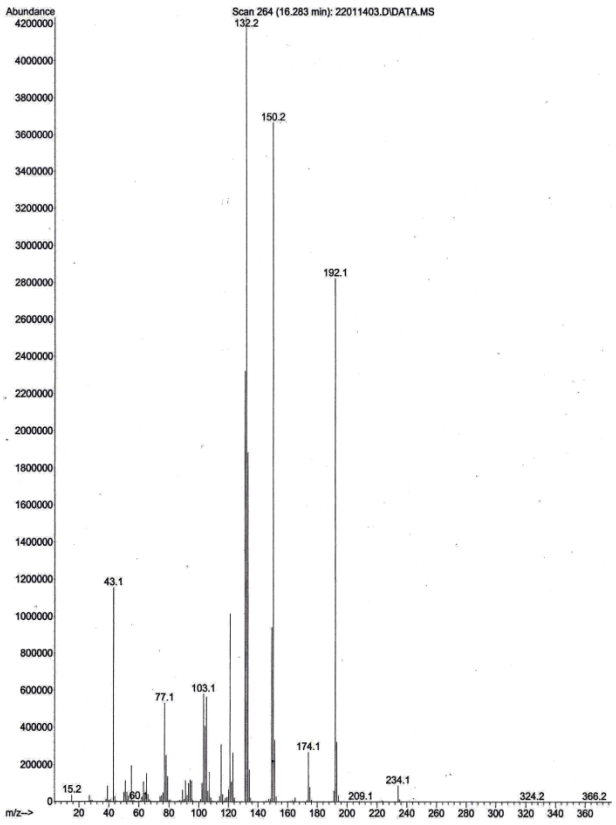

Figure 2: Mass spectrum of the major chemical compound (1'–Acetoxy-chavicol acetate) of crude Galangal extract.

According to Anbu Jeba Sunilson *et al.*, [13], methanol extract of Malaysian grown Galangal gave a DIZ value of 13.8 mm against *S. aureus*. In contrast our study hexane extract of Galangal gave a higher DIZ value ranging from 37.25 to 44.83 mm against six strains of *S. aureus.* The different DIZ values for tested *S. aureus* may be due to the variations in the extraction methodology, type of solvent used, and the thickness of agar on TSA plates. In addition, the broth dilution assay revealed that the MIC value was 1.25 mg/ml at 24h and 48h for the tested strains of *S. aureus* except the strain MSSA 21D. In a similar study Weerakkody *et al.*, [7], the MIC value was reported as 0.625 mg/ml against *S. aureus*. It could be argued that, personal errors in obtaining the MIC level may have some influence on the variability of the values. Determination of MIC level is difficult in broth dilution method as the oil and solvents tend to coagulate even in the presence of surfactant. Further, the dark color of the extract limits the clarity reading of MIC levels. Due to these reasons, we confirmed the MBC level as 5 mg/ml as the concentration of Galangal extract at which no bacterial colony growth was observed on agar plates. The MBC was four fold higher than the MIC value.

Crude Galangal extract exhibited a considerable reduction in biofilm adherence of *S. aureus*. In particularly, the antibiofilm activity was tested against *S. aureus* SA113, reported a biofilm forming strain of *S. aureus* [14]. The $MBRC_{50}$ was 2.5 mg/ml which is twice the MIC value of the extract of *S. aureus* SA113 strain. This could be due to sessile bacteria in biofilm having an elevated resistance towards antibiotics compared to the planktonic bacteria population [2]. However, >80% eradication of the *S. aureus* biofilm adhered to the surface was observed at a concentration of 20 mg/ml Galangal extract. Similar studies have reported that the minimum biofilm eradication concentrations are much higher than the MIC values for some antibiotics against certain biofilm positive bacteria such as *Enterococcus faecal*, *Proteus mirabili* and *S. aureus* including MRSA [15]. Therefore, the antibiofilm effect of the extract could be more promising during biofilm formation than controlling of mature biofilms adhered on to the surface [16]. This may be due to the delayed penetrability of antimicrobial agent through a diffusional barrier of extracellular matrix in mature biofilm and the slow growth of biofilm associated with slow cells take up of antimicrobial agent is slow [2]. In addition, the biofilm adherence surface material greatly influences the attachment of biofilm leading to difficulties in

quantification. We used treated polystyrene microtitre plate to allow better cell attachment and to avoid disruption and breaking off of biofilm layers during washing [17]. However, SEM view of *S. aureus* SA113 biofilm on stainless steel surface upon treatment of 20 mg/ml Galangal extract clearly showed a considerable reduction in biofilm adhered to the surface.

A previous study has investigated the biofilm formation of *S. aureus* on stainless steel which revealed better adherence ability of this bacterium on stainless steel surface. It is suggested that the adherence is better and easier when both the attaching surface and the bacterial cells are more hydrophobic [12]. However, biofilm formation of our study showed that this strong adhesion of *S. aureus* on stainless steel surface can be minimized using Galangal extract at concentrations higher than 20 mg/ml. Galangal at lower concentrations of less than 5 mg/ml may not be effective on removing *S. aureus* biofilm adhered to the surface.

In previous studies 1' acetoxychavicol acetate (1'ACA) was reported as the major chemical compound for ethanolic extract of Thailand grown Galangal (76.49%) [18] hexane extract of Australian grown Galangal (63.4%) [7] and 1.89 g of ACA was recovered from 500 g of *A. galanga* rhizome from HPLC [19]. There is a discrepancy in the literature on the major chemical constituents of Galangal, since 1,8-cineole was identified as the major chemical compound in ethanol and hexane extracts [20] or steam distilled extracts [21]. However, the mass spectra for the unknown major chemical compound obtained in this study (Figure 2) was in agreement with that of the mass spectra reported by Mitsui *et al.* [6], where the major chemical compound was identified as 1'ACA for the ethanol extract of Galangal. However, the major chemical compound present in hexane extract of Sri Lankan grown Galangal was identified as 1' ACA and, the amount of 1'ACA is much higher in Galangal grown in Sri Lanka (82.88%) compared to the other Galangal extracts. The composition of minor chemical compounds such as beta farnesene (2.68%), 1,8- cineole (1.16%) and palmitic acid (1.26%) discovered in this study were not similar to those reported in Weerakkody *et al.*, [7] and Oonmetta-aree *et al.*, [18] in the hexane and ethanol extracts. The chemical composition of plant oils and extracts are known to vary according to the variety of plant, agricultural method used, local climatic and environmental conditions [22,23]. Compared to the other studies, the climatic conditions, soil type and other environmental conditions of the location where the plant material was collected, may have contributed to the highest amount of 1'ACA recorded in this study.

CONCLUSION

The results of this study suggest that the hexane extract of Galangal rhizome could be used as a potential source of natural antibacterial and antibiofilm agents. The finding of this study could be useful in developing an antibiotic as an alternative to the antibiotic resistant *S. aureus* or as a disinfectant on stainless steel surfaces to control *S. aureus* cross contaminations in the food industry. Further, the assessment for potential toxic effects of a medicinal plant extract on human is crucial thus, evaluation of toxicological safety of galangal is important before human consumption or applications.

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CONFLICT OF INTEREST

The authors declare that there is no potential conflict of interest with respect to the research, authorship and/or publication of this article.

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