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Antimicrobial properties and action of galangal (*Alpinia galanga* Linn.) on *Staphylococcus aureus*

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Abstract

The ethanol extracts of the Zingiberaceae family (galangal, ginger, turmeric and krachai) were evaluated for antimicrobial action on *Staphylococcus aureus* 209P and *Escherichia coli* NIHJ JC-2 by using an agar disc diffusion assay. The galangal extract had the strongest inhibitory effect against *S. aureus*. The minimum inhibitory concentration (MIC) of the galangal extract was 0.325 mg/ml and the minimum bactericidal concentration (MBC) at 1.3 mg/ml using the broth dilution method. Transmission electron microscopy clearly demonstrated that the galangal extract caused both outer and inner membrane damage, and cytoplasm coagulation. The disruption of the cytoplasmic membrane properties was determined by the releasing of cell materials including nucleic acid which absorbed UV/VIS spectrophotometer at 260 nm. The major compound of the extract was D,L-1′-acetoxychavicol acetate which was identified by GC-MS and NMR.

Keywords: Galangal; Antimicrobial activity; Membrane damage; *Staphylococcus aureus*

1. Introduction

At present, food safety is a fundamental concern to both consumers and food industries in particular as there are an increasing number of reported cases of food associated infections. Most consumers prefer high quality, nutritious and long shelf-life food products with no preservative agents. Food preservation, however, is the basis of some of the largest and the most modern food industries in the world. Spices and their essential oils have been widely used as natural food preservatives to make the processed foodstuff safe for consumers.

Rhizomes of some members of the Zingiberaceae family such as galangal [*Alpinia galanga* (Linn.) Stuntz], ginger (*Zingiber officinalis* Roscoe), turmeric (*Curcuma longa* Linn.) and krachai [*Boesenbergia pandurata* (Roxb.) Schltr] have been extensively used as condiment for flavoring and local medicines for the stomachache, carminative and treating diarrhea. They are known to contain various antimicrobial agents. Galangal has characteristic fragrance as well as pungency; hence, its rhizomes are widely used as a condiment for foods in Thailand. Galangal is also used as a medicine for curing stomachache in China and Thailand (Yang & Eilerman, 1999). It has been shown that essential oils from both fresh and dried rhizomes of galangal have antimicrobial activities against bacteria, fungi, yeast and parasite (Farnsworth & Bunyapraphatsara, 1992). Janssen and Scheffer (1985) have reported that terpinen-4-ol, one of the monoterpenes in the essential oil from fresh galangal rhizomes, contains an antimicrobial activity against *Trichophyton mentagrophytes*. Acetoxychavicol acetate (ACA), a compound isolated from an *n*-pentane/diethyl ether-soluble extract of dried rhizomes, is active against

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some bacteria and many dermatophyte species (Janssen & Scheffer, 1985). In addition, the ability of ACA to act as an antiulcer and antitumor agents as well as an inhibitor of chemically induced carcinogenesis is event (Murakami, Toyota, Ohura, Koshimizu, & Ohigashi, 2000).

The staphylococcal food poisoning or food intoxication syndrome was first studied in 1894 (Jay, 2000). Staphylococcal gastroenteritis is caused by ingestion of enterotoxins produced by some staphylococcal species and strains. *Staphylococcus aureus* is nonspore forming Gram-positive cocci, which in some strains, are able to produce an enterotoxin (Vanderzant & Splittstoesser, 1992). In processed foods in which *S. aureus* should have been destroyed by processing, the reappearance of this particular bacterium can cause damages to food industries as it is a vector of food poisoning. It may be inferred that sanitation or temperature control or both were inadequate. There is no guarantee that foodstuff is safe enough for consumption, although only a trace amount of *S. aureus* is present. Natural preservatives such as spices and plant essential oils are used as additives, instead of chemical preservatives because food remains safe for consumers while *S. aureus* is eliminated.

The purpose of this study is to: (1) compare the antimicrobial effect of four genus in Zingiberaceae family against *S. aureus* and *Escherichia coli*; (2) to describe the primary mode of actions of galangal ethanol extract; and (3) to identify the main component of the galangal ethanol extract.

2. Materials and methods

2.1. Spice extracts preparation

Rhizomes of galangal, ginger, turmeric and root of krachai were purchased from a local market in Nakhon Ratchasima, Thailand. The fresh rhizomes were sliced and dried in a tray-dryer oven at 50 °C for 24 h, after which they were ground in a blender (National, MX-T2GN, Taiwan) to make powder.

2.2. Microorganisms preparation

Stock cultures of *S. aureus* 209P and *E. coli* NIHJ JC-2, used throughout this study, were kindly provided by the Laboratory of Ecological Circulation, Faculty of Bioresources, Mie University, Japan. *Staphylococcus epidermidis* TISTR No. 518, *Bacillus cereus* TISTR No. 687, *Bacillus megaterium*, *Streptococcus lactis*, *Salmonella* sp., *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, and *Saccharomyces cerevisiae* were used for antimicrobial galangal extract activity. They were maintained on Trypticase soy agar (TSA, Difco, USA) slants at 4 °C. For use in experiments, the bacteria were grown separately in Trypticase soy broth (TSB, Difco, USA) and incubated at 35 °C for 18–24 h. Each bacterial suspension was subsequently streaked on TSA plates and incubated at 35 °C for 48 h. A single colony was transferred to 10 ml of TSB and incubated at 35 °C for 18–24 h. This culture was used for the antibacterial assay.

2.3. Extraction of ethanol extracts from test spices

Ten grams powder of the spices were extracted with 100 ml of 100% ethanol and left at room temperature overnight. The extracts were dried by using a rotary evaporator (BUCHI Rotavapor R-114, Switzerland) and kept at 4 °C until use (Achararit, Panyayong, & Rachatagomut, 1983; Caichompoo, 1999; Harborne, Mabry, & Mabry, 1975 with some modifications).

2.4. Antimicrobial activities of spice ethanol extracts

2.4.1. Screening spice ethanol extracts

Antimicrobial activity was determined by modified agar disc diffusion method and broth dilution method using Kirby and Bauer (Lennette, Barilows, Hausler, & Shadony, 1991). Single colonies of the respective test bacteria were transferred into TSB and incubated overnight. Three milliliters of each culture were mixed with 100 ml of melted Mueller Hinton Agar (MHA, Difco, USA) at about 45 °C and poured onto the surfaces of an agar plate containing 2 g agar/100 ml distilled water.

Ethanol extracts (80 μl) were loaded onto sterile filter paper discs (∅ 8 mm, Advantec, Tokyo, Japan) twice with air-drying in between. Control discs were similarly prepared using distilled water and 100% ethanol. Each of the discs was placed on the aforementioned bacterial culture plates and incubated at 35 °C for 18–24 h. Inhibition zones (including the diameter of disc) were measured and recorded.

2.4.2. Antimicrobial activities of galangal ethanol extract against other microorganisms

Galangal ethanol extract was selected for determining antimicrobial activity against other microorganisms: *S. epidermidis* TISTR No. 518, *B. cereus* TISTR No. 687, *B. megaterium*, *S. lactis*, *Salmonella* sp., *E. aerogenes*, *P. aeruginosa* and *S. cerevisiae* by using modified agar disc diffusion method as previously described.

2.4.3. Minimum inhibitory concentration (MIC)

S. aureus was cultured into 5 ml of TSB, and incubated in a shaker incubator at 35 °C for 18–24 h. The optical density (OD) of the bacteria was adjusted to the standard of McFarland No. 0.5 with 0.85–0.9 g sodium chloride/100 ml sterile solution to achieve a concentration of approximately 10^8 cfu/ml. The final

concentration of the cell number of approximately 10^6 cfu/ml was obtained by diluting 100 times with sterile sodium chloride solution. One milliliter of the bacterial suspension at 10^6 cfu/ml was transferred into 1 ml of MHB.

The galangal ethanol extract was diluted in 100% ethanol to 1:1, 1:2, 1:4, 1:8, 1:16 (v/v), and 80 μl was loaded onto sterile filter paper discs (∅ 8 mm, Advantec, Tokyo, Japan) twice with air-drying in between. Infiltrated paper disc (160 μl) was submerged into 1 ml of MHB, after which 1 ml of *S. aureus* (ca. 10^6 cfu/ml) was added and incubated with shaking at 35 °C for 18–24 h. The MIC of the ethanol extracts was regarded as the lowest concentration of extracts that were not permitted for any turbidity of the tested microorganism (Lennette et al., 1991; Lorian, 1995).

2.4.4. Minimum bactericidal concentration (MBC)

All the tubes were used in the MIC studies that did not show any turbidity of the bacteria and the last tube with turbidity were determined for MBC. An Aliquot of 0.1 ml of the suspension was spread onto TSA and incubated at 35 °C, 18–24 h. The MBC was the lowest concentration which the initial inoculum was killed as 99.9% or more (Lennette et al., 1991; Lorian, 1995).

2.4.5. Effect of galangal ethanol extract on cell membrane of S. aureus

S. aureus was cultured into 100 ml TSB and incubated at 35 °C for 18–24 h. After incubation, the bacterial cells were separated by centrifugation at 10,000*g* for 5 min, and resuspended in sterile sodium chloride solution (0.85–0.9 g/100 ml). Suspensions were adjusted to achieve a concentration of approximately 10^{10} cfu/ml. Spice ethanol extracts at 0.8 mg/ml were loaded onto sterile filter paper discs. One disc was submerged into each test tube of 4 ml of the above bacterial suspension. This suspension was incubated at 35 °C for 0, 2, 6, 16 and 24 h. Additional extracts and bacterial pellets were removed after incubating as described previously by centrifugation at 10,000*g* for 5 min. Low molecular weight metabolites known to leak from cells include nucleotides and their component structures (purines, pyrimidines, pentose and inorganic phosphate), amino acids and inorganic ions. The levels of purines, pyrimidines and their derivatives in supernatant were determined by measuring the OD at 260 nm using UV/VIS spectrophotometer (GBC Scientific equipment Pty. Ltd, Australia) by the modified method used by Woo, Rhee, and Park (2000) and Carson, Mee, and Riley (2002).

2.4.6. Transmission electron microscopy (TEM)

The method of TEM was modified from Horiuchi et al. (2001).

S. aureus was cultured in TSB at 37 °C for 18–24 h. Twenty microliters of the bacterial suspension were transferred to 3 ml of TSB. Filter paper discs (∅ 8 mm, Advantec, Tokyo, Japan) were infiltrated with 160 μl of the extract and air-dried for 2 h. One paper disc was submerged into one test tube of the 2 and 4 h culture of bacterial suspension. This suspension was incubated at 37 °C for 18–24 h. Bacterial cells were collected by centrifugation at 10,000 *g* for 5 min. They were fixed in the mixture of 3 g glutaraldehyde/100 ml solution at 4 °C for 2 h. Cells were washed three times with 0.1 mol/l phosphate buffer solution, pH 7.2 and post-fixed with 2 g OsO_4/100 ml solution in 0.1 mol/l phosphate buffer solution at room temperature overnight. The pellets were embedded in 2 g/100 ml melted agar and then dehydrated through a serial concentration of ethanol (35, 50, 70, 95, 100 ml in 100 ml distilled water, respectively, for 15 min). They were infiltrated in a propylene oxide for 15 min twice, working mixture (propylene oxide: Spurr' resin, 1:1) for 30 min, followed by Spurr' resin for 60 min. They were kept in 100% Spurr' resin and polymerized in a hot air oven at 60 °C for 72 h. The polymerized samples were sliced with an ultramicrotome (MTX 75500, RMC, USA) and observed using a transmission electron microscope (JEOL, JEM 2010, Japan).

2.5. Identification of galangal ethanol extract compound

2.5.1. Gas chromatography-mass spectrometry (GC-MS)

The compound and structure of some ingredients from major galangal ethanol extract were analysed by GC-MS (Network GC System, 6890N, USA). A chromatographic column of 30.0 mm was used (Agilent 19091S-433), and a capillary column with a diameter 250.00 μm and a film thickness of 0.25 μm. Chromatographic conditions were as follows: split ratio 10:1; carrier gas:helium; initial oven temperature 100 °C; injection volume 1 μl. Mass spectrometer condition (Agilent 5973 Network Mass selective detector): ion source 230, threshold 100 eV.

The percentage of each compound was calculated as the ratio of the peak area to the total chromatographic area. The identification of the major peak was compared to the reference compound (Mitsui, Kobayashi, Nagahori, & Ogiso, 1976; Noro et al., 1988).

2.5.2. Nuclear magnetic resonance spectroscopy (NMR)

The structure of some active ingredients from major galangal ethanol extract was confirmed by ^{1}H-NMR, ^{13}C-NMR compared to standard reference (D,L-1′-ACA; LKT Laboratories, Inc., USA) (Mitsui et al., 1976; Noro et al., 1988).

3. Results and discussion

3.1. Antimicrobial activities of spice ethanol extracts

The growth inhibition zones measured by using agar disc diffusion assay are presented in Table 1. In general, *S. aureus* (Gram-positive bacteria) is more sensitive to extracts than *E. coli* (Gram-negative bacteria). These results are in substantial agreement with many previous studies (Burt, 2004; Maillard, 2002). Turmeric showed less inhibition in growth of both *S. aureus* and *E. coli*. Galangal extract exhibited the strongest inhibitory activity against *S. aureus*. Extract of galangal, krachai and ginger had no effect against *E. coli*. All of these results demonstrate that extract of galangal should be used for further studies about antimicrobial activity against other microorganisms, MIC and MBC of galangal extract against *S. aureus* and electron microscopy.

Antimicrobial activities of galangal extracts are present in Table 2. The results indicate that *S. epidermidis* and *S. cerevisiae* were susceptible to the extract. The extract was less antimicrobial activity against *B. cereus* and *B. megaterium* and there was no antimicrobial activity against some Gram-negative bacteria: *Salmonella* sp., *E. aerogenes* and *Pseudomonas aeruginosa*. *S. cerevisiae* was a primary representative of eukaryotic microorganisms that showed the susceptibility to the galangal extract as the same as the previous study; Janssen and Scheffer (1985) indicated that ACA had inhibitory effect against *T. mentagrophytes*.

Galangal extract was selected to study MIC and MBC by using the broth dilution method. MIC of galangal extract against *S. aureus* at a concentration of 0.325 mg/ml showed bacteriostatic compound and the MBC or bactericidal effects against *S. aureus* was 1.3 mg/ml.

Table 1
Antibacterial properties of ethanol spice extracts against various microorganisms using agar disc diffusion method

Spices	Zone of growth inhibition (mm)	
	S. aureus	*E. coli*
Galangal	22.33 ± 0.58^{a}	0
Krachai	11.00 ± 0.00^{a}	0
Ginger	11.00 ± 0.00^{a}	0
Turmeric	10.00 ± 0.00^{a}	10.00 ± 0.00^{a}
Ethanol	0	0
Distilled water	0	0

Interpretation into categories of susceptibility is achieved by comparing zone sizes of test organisms with appropriate control organisms on the same test plate.R, resistant: $\leqslant 9$ mm; I, intermediate: $\geqslant 10$–13 mm; S, susceptible: $\geqslant 14$ mm (Lorian, 1995).

[a]Values for zone of growth inhibition are presented as mean ± SD.

Table 2
Antibacterial properties of ethanol galangal extract against various microorganisms using agar disc diffusion method

Microorganisms	Galangal extract	Ethanol	Deionized water
B. cereus	11.00 ± 0.00^{a}	0	0
B. megaterium	11.50 ± 0.00^{a}	0	0
S. lactis	9.00 ± 0.00^{a}	0	0
S. epidermidis	19.67 ± 0.58^{a}	0	0
Salmonella sp.	0	0	0
E. aerogenes	0	0	0
P. aeruginosa	0	0	0
S. cerevisiae	21.67 ± 1.16^{a}	0	0

Interpretation into categories of susceptibility is achieved by comparing zone sizes of test organisms with appropriate control organisms on the same test plate.R, resistant: $\leqslant 9$ mm; I, intermediate: $\geqslant 10$–13 mm; S, susceptible: $\geqslant 14$ mm (Lorian, 1995).

[a]Values for zone of growth inhibition are presented as mean ± SD.

3.2. Identification of galangal ethanol extract compound

The major compound of galangal ethanol extract was analysed by GC-MS (Network GC System, 6890N, USA) and ^{1}H-NMR, ^{13}C-NMR was used to identify its chemical structure. The main compound of galangal extract were essential oil; D,L-1′-ACA (76.49%) which are defined as a group of odorous principles, of which the major constituent that derived from galangal rhizome and seed extract (Mitsui et al., 1976; Noro et al., 1988; Prasit, Chalermpol, Prasat, & Kamolchanok, 2002). The minor compounds of crude extract were also identified by GC-MS; *p*-coumaryl diacetate (7.96%), palmitic acid (3.19%), acetoxyeugenol acetate (3.06%), 9-octadecenoic acid (2.28%), eugenol, β-bisabolene, β-farnesene and sesquiphellandrene.

3.3. Effects of galangal ethanol extracts on growth of *S. aureus*

3.3.1. TEM

Galangal extract caused dramatic cytological modifications to *S. aureus* cell in Figs. 1C–E compare to normal cells (Figs. 1A and B). Galangal extract treated *S. aureus* showed alteration in outer membrane's integrity with cell membranes and cell walls being disrupted and damaged resulting in a release of cell materials in cytoplasm (Fig. 1C). Fig. 1D showed the integrity of the membrane and cytoplasmic material in cytoplasm was coagulated and aggregated near cell membrane. The intracellular components of cells, such as nucleic acids, proteins, ribosomes aggregated through the cell (Figs. 1C, E and F). Figs. 1E and F also showed that cell contents were coagulated. The results from TEM showed that the galangal extract might affect the cell osmolarity, ribosomes and cell coagulation compounds.

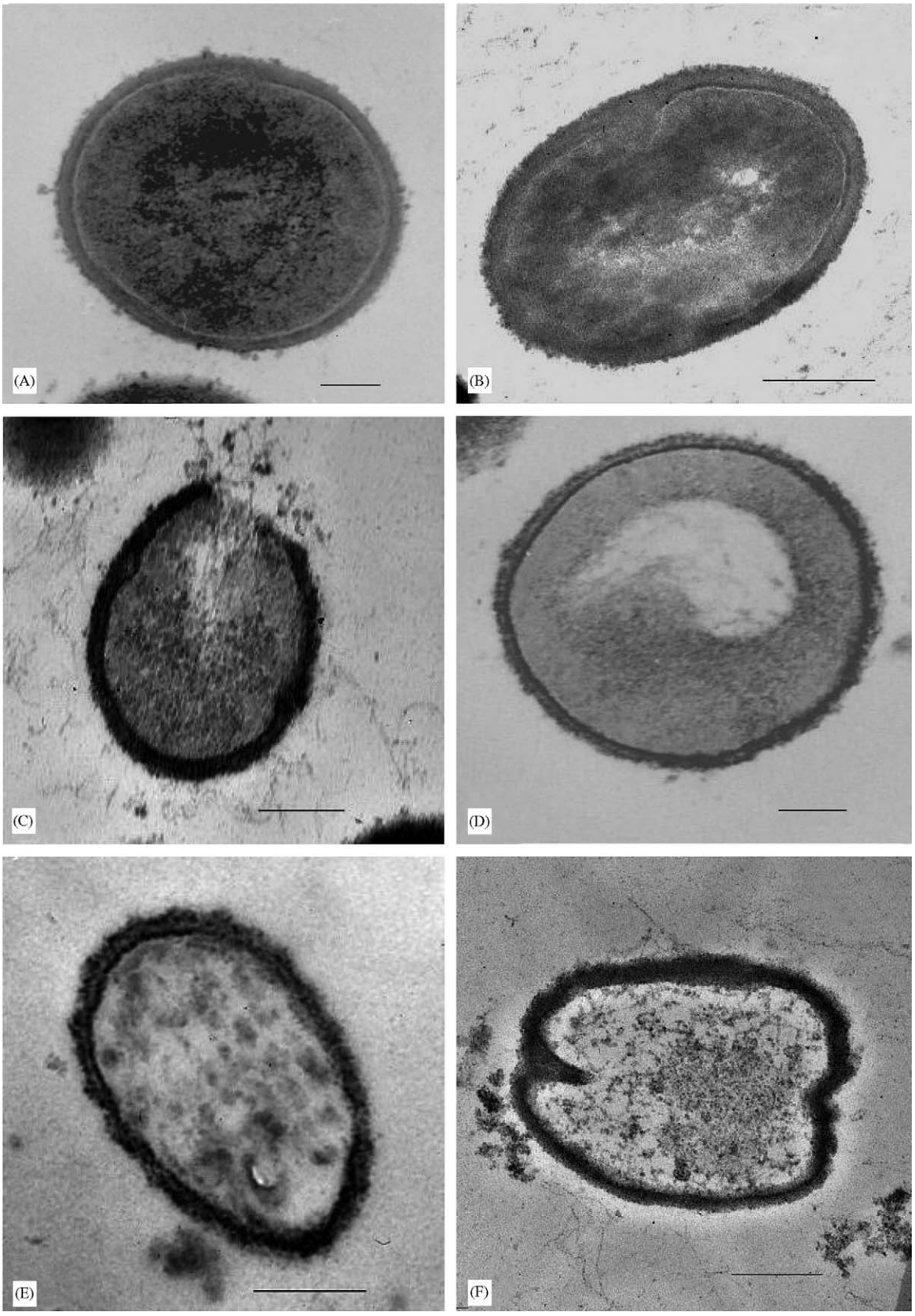

Fig. 1. Cytological effects of galangal ethanol extract on *S. aureus* cells (bar = 0.2 μm): (A) *S. aureus* after ethanol treatment; (B) *S. aureus* cells after distilled water treatment; and (C–F) *S. aureus* cells after galangal ethanol extract treatment.

3.3.2. Effect of galangal ethanol extract on cell membrane of S. aureus

The TEM results in Fig. 1 illustrated that the galangal extract ruptured the outer membrane of *S. aureus*. As a result, there was a loss of cell permeability properties which was correlated with the OD_{260} results. The leakage of cytoplasmic membrane was analysed by determining the release of cell materials including nucleic acid, metabolites and ions which was absorbed at 260 nm into the suspensions. Higher extract, the exposure times results in higher cell materials which show higher OD_{260}. Fig. 2 shows that the OD_{260} of bacterial cell materials were 1.09, 1.36 and 1.45 after *S. aureus* suspensions were treated with 0.27 mg/ml galangal extract at 2, 6, 16 h, respectively. The results from releasing of cellular material indicate that the higher exposure time leads to higher cell leakage that appears to lose nucleic acid, critical molecules and ions.

Crude extract of galangal was the lipophilic compounds, soluble in ethanol which is a property of essential oils. Most major and minor compounds were identified by GC-MS as ACA, *p*-coumaryl diacetate, palmitic acid, acetoxyeugenol acetate, eugenol, *β*-bisabolene, *β*-farnesene and sesquiphellandrene were the phenolic compounds, phenolic derivative compounds, the ester of weak acid, fatty acid, terpenes and others. Since the large number of different chemical compounds presented in this crude extract therefore its mechanism of action can affect multiple target sites against the bacterial cells (Figs. 1C–F). *β*-bisabolene, *β*-farnesene and sesquiphellandrene are terpenes in the essential oils of spices that mechanism of action should be similar to other terpenes and other phenolic compounds in this crude extract as indicated as involving disruption of the cytoplasmic membrane (Fig. 1C) and coagulation of cell contents (Figs. 1D–F) (Burt, 2004; Davidson & Naidu, 2000). The essential oils are hydrophobicity which enables them to a partition in the lipids of the bacterial cell membrane, disturbing the structures and rendering them more permeable. Besides, the essential oils are able to induce the leakage of ions and other cell contents. The results from TEM and the loss of 260 nm absorbing materials suggested that the extract affected the cytoplasmic membrane of *S. aureus* and induced the loss of nucleic acids and ions (Burt, 2004; Carson et al., 2002; Woo et al., 2000). ACA; main compound in this crude extract, is ester of acetic acid which have similar mechanisms in the bacterial cells (Freese, Sheu, & Galliers, 1973). Huang, Forsberg, and Gibbins (1986) and Freese et al. (1973) have been demonstrated that acetate acts as other organic acids, having membrane gradient neutralization and denaturing of proteins inside the cell. Antimicrobial activity of acetic acid is related to pH, and the undissociated form of the acid is primarily responsible for antimicrobial activity. When this ester is dissolved in solution, undissociated acetic acid can penetrate the cell membrane lipid bilayer of bacteria and yeasts and release protons into the cytoplasm because the cell interior has a higher pH than the exterior (Booth, 1985; Davidson, 2001; Marquis, Clock, & Mota-Meira, 2003). Excess intracellular protons can acidify the cytoplasm and cause protein denaturation and energy loss due to activation of ATP-dependent proton pumps located in the cell membrane (Booth, 1985; Marshall, Cotton, & Bal'a, 2000). The methyl ester is able to penetrate to the hydrophobic regions of the membranes and the carboxyl groups pass through the cell membrane, perturbing in the lowering of internal pH and denaturing of proteins inside the cell which coagulation of cell contents (Figs. 1E and F) (Huang et al., 1986; Marquis et al., 2003).

The galangal extract could not inhibit the growth of *E. coli* (Gram-negative bacteria) because the extract could not penetrate through the outer membrane which was composed of a lipopolysaccharide monolayer surrounding the cell wall that restricts diffusion of hydrophobic compounds (Burt, 2004; Vaara, 1992).

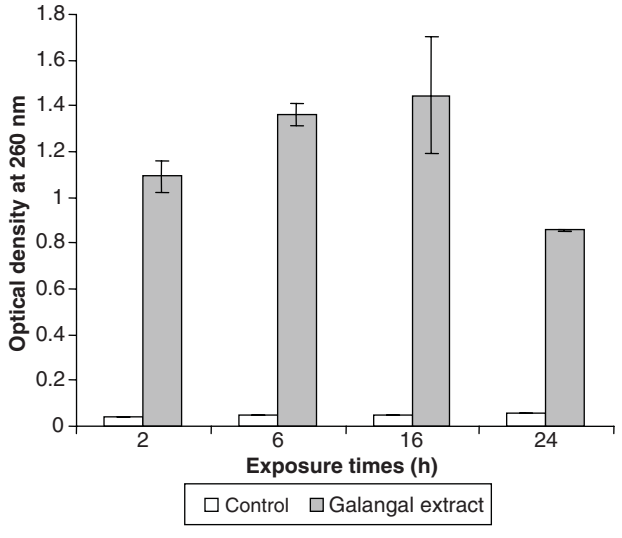

Fig. 2. Measuring absorbance of the cell materials contents at 260 nm releasing from *S. aureus* cells after treatment with galangal extract (gray bars) at 2, 6, 16 and 24 h, compared to *S. aureus* control suspension (white bars). The data are expressed as means ± standard deviations.

4. Conclusions

The major constituent of galangal ethanol extract was D,L-1′-ACA, which was found in some plants in the Zingiberaceae family, especially galangal. This galangal extract had the greatest inhibitory effect against *S. aureus* among ginger, turmeric and krachai. Some Gram-positive bacteria and yeast were susceptible to the extract, especially Staphylococcal species. The data

obtained from this study could be described as the antimicrobial effect of the extract which depended on the exposure time and the bacterial cell concentration. Galangal ethanol extract caused the internal pH changing and denaturing of proteins inside the cell and also disrupted the cytoplasmic membrane function of *S. aureus* cells which resulted in a loss of cytoplasmic constituents and ions. It leads to understanding the roles of the chemical compounds in galangal extract in microbial physiology and gain more information on their actions, including information on mechanisms. This should lead to effective application of the spice extracts as natural antimicrobial agents to control foodborne pathogens, food spoilage organisms in food industries.

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