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Full Length Research Paper

Killing kinetics and bactericidal mechanism of action of *Alpinia galanga* on food borne bacteria

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The aim of this study was to explore the killing kinetics and bactericidal mechanism of action of *A. galanga* against food borne bacteria in order to promote this plant as a source of natural preservative. The comparison on antibacterial power was firstly done among its essential oil and the crude extracts obtained from various extracting solvents. The essential oil showed the extremely strongest antibacterial activity and more effective killing activity against Gram negative than Gram positive food borne bacteria. The kinetic time of the oil for complete bactericidal action against *E. coli* was 40 min whereas that of gentamicin was 120 min. The scanning electron microscopy (SEM) of bacterial morphology after exposed to the oil showed bacterial membrane destruction. It was concluded that the essential oil is the highest potential part of *A. galanga* against food borne bacteria. Its killing kinetics against Gram negative bacteria was extremely faster than a broad spectrum antibiotic gentamicin. Its mechanism of bactericidal action was along with the bacterial membrane disruption and malfunction leading to cell death.

Key words: *Alpinia galanga*, antibacterial, mechanism of action, essential oil, killing kinetics.

INTRODUCTION

Certain food borne diseases are originated from contaminated food by different strains of bacteria. These bacteria are not only directly harmful as pathogens but also cause food deterioration and leaching of the toxic substances to human body. Food safety therefore becomes a highly important issue for both consumers and food industries due to the rising number of case reports on food associated infections. A good manufacturing practice has been introduced in food industries in order to control the pathogen level in food products (Fan et al., 2009; Grob et al., 2009). The most effective way to minimize food contaminated by microorganisms is to add an effective antimicrobial agent or a so-called preservative, into food products. The substances used as preservatives nowadays are both from chemical synthesis and natural source. The later, for example, the use of potential edible plants is having more increasing interest by consumer because of its less toxic and the feeling of natural sense.

Various plants have shown their potential on antimicrobial action (Akinpelu and Onakoya, 2006; Hernandez et al., 2007; Karaman et al., 2003; Vuddhakul et al., 2007). Some have been used for treatment of infectious diseases caused by pathogenic micro-organisms (Oke et al., 2009; Voravuthikunchai et al., 2004). *Alpinia galanga*, a plant in family Zingiberaceae is widely distributed in the tropical area. It has been used as a medicine for curing stomachache in China and Thailand (Yang and Eilerman, 1999). Under the names “Chewing John”, “Little John to Chew”, and “Court Case Root”, it is used in African-American folk medicine and hoodoo folk magic (Yronwode, 2002). *A. galanga* fresh rhizome has characteristic fragrance as well as pungency and used as essential component in many Asian food products. It was reported that the crude extracts of *A. galanga* have antioxidant and antimicrobial activities against certain microorganisms (Habsah et al., 2000; Mayachiew and Devahastin, 2008). Janssen and Scheffer (1985) reported that the monoterpenes in the essential oil from fresh galangal rhizomes contain an antimicrobial activity against *Trichophyton mentagrophytes.*

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The ethanol crude extract of *A. galanga* was reported to have inhibitory effect against *Staphylococcus aureus* (Oonmetta-aree et al., 2006). However, the antibacterial data of this plant against food borne bacteria was not enough to be ensured of using this plant as natural preservative. Moreover, the knowledge deeply on its antibacterial kinetics and mechanism of action against any bacteria is very rare. In order to promote *A. galanga* as a natural source of food preservative, various strains of common food borne Gram positive and Gram negative bacteria should be tested.

The purpose of this study was to investigate the antibacterial activity of the rhizome of *A. galanga* against food borne bacteria. We attempted to study in depth the antibacterial activity of the most active extract of *A. galanga* on minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), killing kinetics and the mechanism of action against the tested bacteria.

MATERIALS AND METHODS

Plant materials

Rhizomes of *A. galanga* (6 to 12 months age) cultured in the northern part of Thailand were collected. A voucher specimen was deposited at the Herbarium of Faculty of Pharmarcy, Chiang Mai University, Thailand. Fresh rhizomes were used for extraction of the essential oil. Dried rhizome powder was used for preparation of crude extracts. It was prepared by slicing the fresh rhizomes into small pieces and drying at 60°C for 48 h. The dried rhizome was ground into fine powder to be ready for solvent extraction.

Essential oil extraction

The fresh rhizomes were chopped and subjected to hydro-distillation for 6 h using a clevenger apparatus to obtain the essential oil fraction. The essential oil obtained was dried using anhydrous sodium sulphate and then stored in an airtight light resistant bottle at 4°C until further test.

Gas chromatography-mass spectrometry (GC-MS) analysis of the essential oil

The essential oil of *A. galanga* freshly obtained from hydro-distillation was subjected to gas chromatography-mass spectrometry (GC-MS) analysis performed on agilent 6890 gas chromatography coupled to electron impact (EI, 70 eV) with HP 5973 mass selective detector and fitted with a fused silica capillary column (HP-5MS) supplied by HP, USA (30.0 m × 250 ∝m, i.d. 0.25 ∝m film thickness). The oven temperature was programmed to increase from 100 to 280°C at a rate of 3°C/min and finally held isothermal for 10 min. The carrier gas was helium introduced at a rate of 1.0 ml/min. Diluted sample of 1.0 ∝l was injected manually and the split ratio was adjusted to 40:1. GC–MS analyses were performed using a Thermo Finnigan-TRACE GC (Waltham, Massachusetts, USA) coupled with a TRACE MS plus (Waltham, Massachusetts, USA) (EI 70 ev) of the same company.

Identification of essential oil constituents

The components of *A. galanga* essential oil were identified by comparison of their mass spectra with those of NIST98 library data of the GC–MS system and Adams libraries spectra, as well as by comparison with the compounds' elution order with their retention indices reported in the literature (Adams, 2001). Retention indices of the components were determined relative to the retention times of a series of n-alkanes with linear interpolation.

Preparation of the crude extracts

Dried powder of *A. galanga* was separately weighed and macerated in a different-polarity solvent, that is, hexane (non-polar), ethyl acetate (semi-polar), and ethanol (polar) for 4 cycles at room temperature. Each cycle lasted 7 days with 1 h mechanical stirred everyday. The filtrates of the same solvent from each macerated cycle were pooled. The solvent was removed under reduced pressure at 45°C by using a rotary evaporator. The weight of the resulting extracts was measured and stored in dark bottles at 4°C until use.

Microbial strains

The food borne microorganisms used in this study were composed of 7 reference strains, that is, *Escherichia coli* ATTCC 25922, *Staphylococcus aureus* ATCC 25923, *Salmonella enteritidis* ATCC 13076, *Salmonella typhimurium* ATCC 14028, *Salmonella typhi* DMST 5784, *Listeria monocytogenes* DMST 1730, *Shigella sonii* DMST 561 and three field strains of *E. coli*. Tryptic soy broth (TSB) or tryptic soy agar (TSA) from Merck, Darmstadt, Germany was used for culturing the bacteria. All strains were stored at -20°C in glycerol and regenerated twice before tested.

Screening for antimicrobial activity

A comparative antimicrobial potency of *A. galanga* essential oil and its three crude extracts was studied by using the disc diffusion method according to Najjaa et al. (2007) and Arias et al. (2004) with minor modification. Briefly, single colony of the test bacteria were transferred into TSB and incubated overnight. Three milliliters of each culture were mixed with 100 ml of melted TSA at about 45°C and poured onto the surfaces of an agar plate containing 2% agar. The sample test solution was loaded onto a sterile filter paper 8-mm disc (Advantec, Tokyo, Japan) to obtain a final sample deposition of 20 mg. Negative control discs were similarly prepared using distilled water and pure solvents. Each loaded disc was placed on the aforementioned bacterial culture plates and incubated at 37°C for 18 to 24 h. A disc of 0.8 mg gentamicin was used as a positive control. Inhibition zones (including the diameter of disc) were measured and recorded.

Minimum inhibitory and bactericidal concentrations

The determination of MIC and MBC of *A. galanga* essential oil was carried out by a broth dilution method (Yu et al., 2004). Tween 20 was used to solubilize the extracts. All tests were performed in TSB. Serial doubling dilutions of the oil was prepared in a 96-well microtiter plate ranged from 0.05 to 200 mg/ml. The final concentration of each strain was adjusted to 4×10^4 cfu/ml. Plates were incubated at 37°C for 24 h. The MIC is defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth. The microorganism growth was indicated by the turbidity. To determine MBC, broth was taken from each well and incubated in Tyramide Signal Amplification (TSA) at 37°C for 24 h. The MBC was defined as the lowest concentration of the essential oil at which incubated microorganism was completely

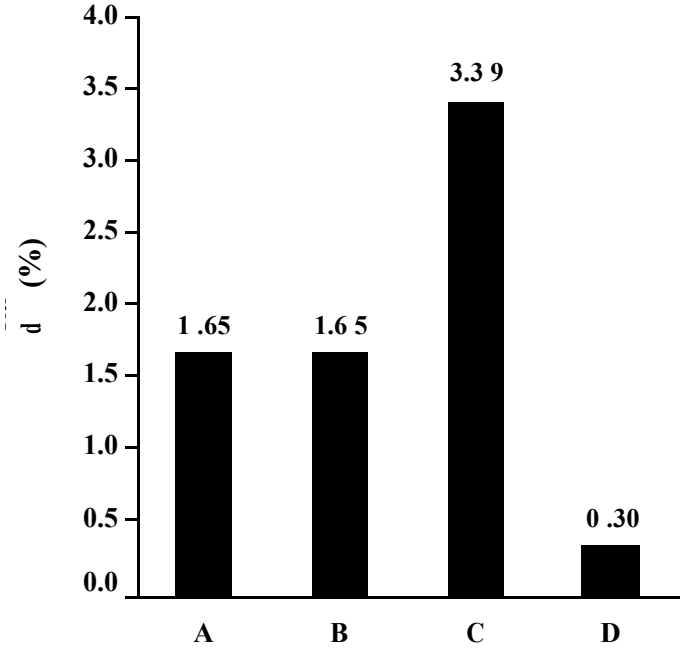

Figure 1. The yield of *A. galanga* rhizome extraction; hexane extract (A), ethyl acetate extract (B), ethanol extract (C) and essential oil (D).

killed. Each test was performed in three replicates. Gentamicin was used as a positive control.

Study of bacterial killing kinetics

In this study, 5 reference strains of bacteria; *L. monocytogenes* DMST 1730, *S. aureus* ATCC 25923, *S. typhi* DMST 5784, *S. sonii* DMST 561, *E. coli* ATCC 25922 and 3 field strains of *E. coli* were used as the test microorganisms. The killing kinetics of *A. galanga* essential oil were conducted at the oil concentrations equal to MBC of the bacterial strains. Bacterial cells were grown to logarithmic phase during 1 h pre-incubation in fresh broth prior to the addition of the essential oil solution. A bacterial concentration between 6 and 8 log cfu/ml was used. The cultures were then incubated in a shaker (Julabo, Allentown, PA) at 37°C for certain period of time or until no viable cells were found. Viable counts were determined by plating 50 µl of known dilutions of the culture samples on to TSA. Cell count plates were incubated for up to 48 h before any were considered as having no growth. Plates with 30 to 300 colonies were used for cfu counts. Log cfu was plotted against time for construction of the bactericidal kinetic curves. Gentamicin was used as a positive control.

Bacterial morphology

The bacterial morphology before and after exposed to *A. galanga* essential oil was examined by using scanning electron microscopy (SEM). Sample preparation for SEM was done as follows: The bacterial suspension before or after certain time of exposure to the oil was dropped into a filter membrane and air dried. Next, the bacteria were fixed with 2.5% glutaraldehyde in Phosphate buffered saline (PBS) and rinsed with the same buffer solution. Subsequently, the fixed bacteria were stained with 1% OsO_4 in PBS for 1 h and dehydrated with different mixtures of water and ethanol. The membrane was coated with gold and analyzed with SEM.

RESULTS

Yield of extraction

In hydro-distillation of the fresh *A. galanga* rhizomes yielded the essential oil of 0.30% v/w. For solvent extraction of the dried rhizome, it gave the crude extracts with various yields depending on the extracting solvents used. It was found that the most non-polar solvent hexane gave the crude extract with the highest yield of 3.39% w/w (Figure 1).

Chemical composition of the essential oil

By using chromatographic procedure, twenty seven compounds, representing 93.06% of *A. galanga* essential oil was identified. Quantitative and qualitative analytical results by GC–MS were shown in Table 1.

Antibacterial activity of the extracts

The growth inhibition zones of *A. galanga* essential oil in comparison with its crude extracts from different solvents against two common food borne bacteria, *E. coli* and *S. typhimurium* were presented in Table 2. The essential oil showed the highest antibacterial potency and was selected for further investigation on determination of MIC and MBC which more strains of food borne bacteria were used. The results were demonstrated in Table 3. It was found that *A. galanga* essential oil had strong bactericidal activity against *E. coli*, *S. aureus, S. sonii*, and *S. typhi* which MIC and MBC of the oil against each strain demonstrated the same value of 4.0, 8.0, 2.0, and 2.0 mg/ml respectively. The MIC of the oil against *L. monocytogenes* was 2.0 mg/ml demonstrating bacteriostatic effect whereas the bactericidal activity against this strain was 4.0 mg/ml.

Bacterial killing kinetics

The result in this study was comparative kinetic bactericidal action between *A. galanga* essential oil and a broad spectrum antibiotic gentamicin at the concentration of their MBC. The kinetic bactericidal action against Gram negative and Gram positive bacteria expressed as time killing curves was shown in Figures 2 and 3, respectively.

Bacterial morphology study

Under the SEM investigation of the bacterial cells morphology after various time of exposure to the essential oil, it was found that the bacterial cell was rapidly shrunk within 10 min. The morphology of normal bacteria in comparison with the shrink cells was shown in Figure 4(a-b). After shrinking, the cells were erupted and

Table 1. Main chemical composition of *A. galanga* essential oil.

Component	%Area	RT[a]	RI[b]
Limonene	29.64	3.32	1041
Gamma-terpinene	1.22	3.56	1058
Alpha-terpinolene	0.44	3.99	1087
1-Undecene	0.20	4.09	1093
(-)-Borneol	0.72	5.48	1166
Para-cymen-8-ol	3.06	6.03	1189
Alpha-terpineol	0.20	6.25	1198
Z-Citral	1.23	7.50	1248
(-)-Bornyl acetate	0.39	8.51	1282
Piperitenone	33.31	10.56	1349
Alpha-cubebene	0.15	10.78	1355
Decaoic acid	1.31	11.55	1377
Beta-elemene	1.91	12.30	1398
Alpha-gurjunene	0.20	12.83	1414
Trans-beta-caryophyllene	3.38	13.39	1431
Trans-beta-farnesene	0.42	15.44	1487
Beta-selinene	0.46	15.56	1490
Delta-selinene	0.31	15.71	1494
Pentadecane	5.62	15.95	1500
Alpha-amorphene	3.01	16.50	1517
7-Epi-alpha-Selinene	0.83	16.65	1521
Trans-gamma-Bisabolene	2.25	16.79	1525
Alpha-Cadinol	0.61	21.08	1661
Gamma-Selinene	0.40	21.62	1681
Beta-Bisabolene	0.84	22.21	1702
Apiol	0.65	22.45	1708
Alpha-trans-Bergamotol	0.30	27.46	1828

[a)] Retention times; [b)] Retention indices.

Table 2. Bacterial inhibitory zone of *A. galanga* crude extracts and its essential oil using the disc diffusion method (n=3).

Sample	Inhibition zone (mm)*	
	***E. coli*ATCC 25922**	***S. typhimurium*ATCC 14028**
Ethanol extract	NZ	NZ
Ethyl acetate extract	9.0±0.1	7.8 ±0.4
Hexane extract	NZ	NZ
Essential oil	10.2 ± 0.4	9.5 ± 1.0
Gentamicin	16.3 ± 0.9	10.4 ± 1.2

* NZ : no inhibition zone.

destroyed into pieces. Most of cell destruction occurred within 40 min.

DISCUSSION

Microbial contamination is a major cause of deterioration as well as the loss of quality and safety of food. Severity of pathogenic and spoilage microorganisms in foods is increasing due to the increase in outbreaks of food-borne diseases. The Gram-positive bacterium *Staphylococcus aureus* is mainly responsible for post-operative wound infections, toxic shock syndrome, endocarditis, osteomyelitis and food poisoning (Mylotte et al., 1987).

Table 3. MIC and MBC of *A. galanga* essential oil in comparison with gentamicin obtained by the broth dilution method (n=3).

Bacterial Strain	MIC (mg.mL^{-1})		MBC (mg.mL^{-1})	
	Essential oil	Gentamicin	Essential oil	Gentamicin
E. coli ATCC 25922	4	16	4	16
S. aureus ATCC25923	8	8	8	16
S. sonii DMST 561	2	8	2	16
S. typhi DMST5784	2	8	2	8
L. monocytogenes DMST 1730	2	4	4	8

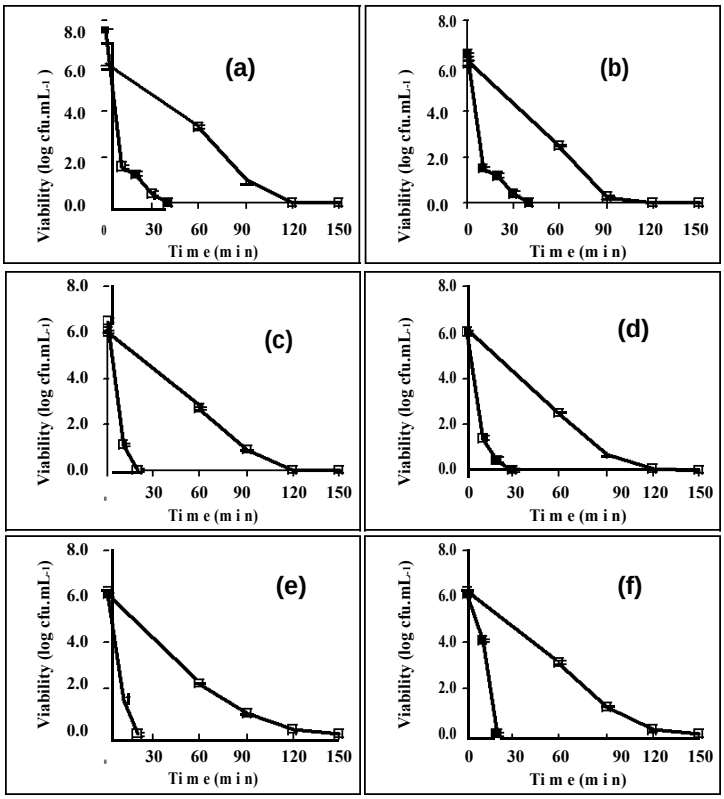

Figure 2. Killing kinetic time of *A. galanga* essential oil ( ) and gentamicin ( ) against *E. coli* ATCC 25922 (a), *E. coli* field strains (b-d), *S. typhi* DMST 5784 (e), and *S. sonii* DMST 561 (f) (n=3)

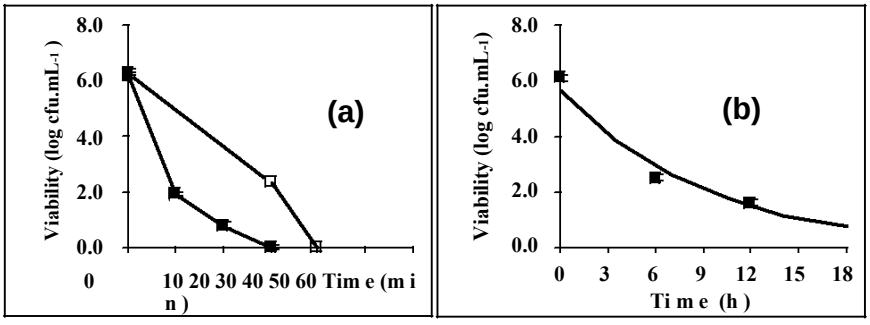

Figure 3 Figure 3. Killing kinetic time of *A. galanga* essential oil ( ) and gentamicin ( ) against *L. monocytogenes* DMST 1730 (a) and *S. aureus* ATCC 25923 (b) (n=3

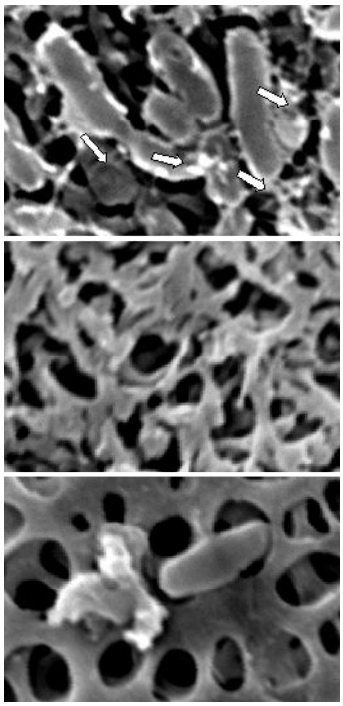

Figure 4. Morphology of *E. coli* ATCC 25922 after 5 min (a) and 40 min (b) exposed to *A. galanga* eseential oil (Arrows point to the lesions of cell membrane destruction) and the comparison of cell normal morphology (c) at the initial (right) and final (left) of exposure

Listeria monocytogenes is responsible for the severe food-borne illness, listeriosis, which has been recognized to be one of the emerging zoonotic diseases during the last two decades (Farber, 2000). The Gram-negative bacterium *Escherichia coli* is present in human intestines and causes urinary tract infection, coleocystitis or septicaemia (Singh et al., 2000). With the increase of bacterial resistance to antibiotics, the use of potential plant extracts represents a concrete alternative for infection control or for the preservation of food. *A. galanga* is one of the plants possesses high antimicrobial power and has been used by local people for a long time on the treatment of infectious diarrhea. However, such practice is in the lack of a sufficient scientific basis. This present study was attempted to provide more valuable scientific data base of this plant.

As seen, the yield of the extracts obtained by different solvent as well as different method of extraction was different. The amount of crude extract obtained by hexane was approximately 2-folds higher than those extracted by ethyl acetate and ethanol indicating that the rhizome of *A. galanga* contains large quantity of non-polar compounds. The yield of essential oil is the least when compared with the three crude extracts. It was reported previously by Ibrahim et al. (2009) that the yield of essential oil obtained from *A. conchigera*, a small galangal, was 0.16% v/w. From this present result, it demonstrated that *A. galanga*, which so called big galang -a, possesses higher essential oil content than a small galanga.

The essential oil consisted mainly of two cyclic terpenes; piperitenone (33.3%) and limonene (29.64%). As far as our literature survey could ascertain, there was only one report on the chemical composition of the essential oil of *A. galanga* grown in Malasia (De Pooter et al., 1985). Of the compounds identified previously which represented 83 to 93% depending on its method of preparation, the two main compounds were different from our results. This is possibly due to the fact that the rhizomes were grown in different regions, which may have caused the differences in their chemical composition.

The result of antibacterial activity demonstrated that for the crude extracts, *S. aureus* was more sensitive to the ethanol and hexane extracts than *E. coli* and *S. typhimurium*. These results were in substantial agreement with the previous study of Oonmetta-aree et al. (2006) which reported that ethanol extract of *A. galanga* had inhibitory effect against *S. aureus* but not be able to inhibit *E. coli.* The ethyl acetate extract showed less inhibitory effect against *E. coli* and *S. typhimurium* than the essential oil. This extract exhibited slightly higher inhibitory activity against the Gram positive *S. aureus* whereas the essential oil showed obviously the strongest inhibition against the Gram negative *E. coli* and *S. typhimurium*. These results demonstrated the extremely high power of *A. galanga* essential oil on both Gram negative and Gram positive bacterial tested strains. It has been reported that the essential oil of *Zingiber offinale*, a plant which belongs to the same family as *A. galanga*, showed no inhibitory action against *E. coli* (Singh et al., 2008). The result of our study presented the advantage of *A. galanga* oil that had ability to inhibit many Gram negative bacteria including *E. coli* and *S. typhimurium*. It is also obviously seen that *A. galanga* essential oil had stronger bactericidal activity than the broad spectrum antibiotic gentamicin demonstrating that the oil contained compounds with high antibacterial activities. Important to mention, *A. galanga* essential oil had a strong activity against Gram negative bacteria which are known for their insensitivity against by many antibacterial agents (Conejo et al., 2008; Jeon et al., 2008; Johnson et al., 2008). Moreover, all tested strains are concerning as food contaminations and are the causes of food borne diseases. Consequently, *A. galanga* essential oil showed a promising natural food preservative to minimize bacterial growth in food products. Our results also indicated the high potential of *A. galanga* essential oil to inhibit food borne pathogens of the field strains which tend to be antibiotic resistant strains.

It was seen that the essential oil of *A. galanga* killed all tested bacteria faster than gentamicin. The results revealed that within 10 min *A. galanga* essential oil killed

E. coli ATCC 25922 about 6 log cfu.mL^{-1} whereas in the same period of time gentamicin killed the bacteria less than 1 log cfu/ml (Figure 2 a). Further, the time for complete killing of *E. coli* ATCC 25922 by *A. galanga* essential oil was only 40 min whereas that of gentamicin was 120 min. The same fast kinetic killing results of *A. galanga* oil were observed in three field strains of *E. coli* as shown in Figure 2(b to d). It was reported that the crude extract of *Azadirachta indica* was the potent antibacterial; however it could not kill *E. coli* after 24 h exposure (Okemo et al., 2001). Therefore, it could be indicated that the essential oil of *A. galanga* had greater bactericidal activity than the other plants investigated previously. In addition, the other Gram negative bacteria included *S. typhi* DMST 5784 and *S. sonii* DMST 561 also showed highly sensitive to *A. galanga* oil.

This bacterial sensitivity expressed in a significantly decreased in numbers of bacteria after short exposure to the oil as shown in Figure 2(e-f) respectively. The kinetic bactericidal action of *A. galanga* essential oil to Gram positive bacteria demonstrated that within 10 min, *A. galanga* essential oil decreased about 5 log cfu/ml of *L. monocytogenes* DMST 1730 whereas approximately not more than 1 log cfu/ml were decreased by gentamicin. Moreover the time required for *A. galanga* oil on complete killing of the bacteria was only 30 min. Therefore, *A. galanga* essential oil was more potential for complete killing *L. monocytogenes* DMST 1730 than gentamicin. The earlier kinetic study of tea tree oil on pathogenic bacteria by LaPlante showed that tea tree oil could not completely kill the tested Gram positive bacteria within 24 h (LaPlante, 2007). Our results demonstrated the higher potency of *A. galanga* oil on killing Gram positive bacteria. The kinetic study in *S. aureus* ATCC 25923 revealed a slightly decrease in numbers of bacteria after exposed to the essential oil and the complete killing time was more than 24 h. These results indicated that *A. galanga* essential oil was more effective on killing Gram negative than Gram positive bacteria.

The SEM study was done to examine the change in bacterial morphology after different time exposure to the essential oil in order to indicate the possibility of mechanism of antibacterial action of *A. galanga* essential oil. As our results demonstrated that among the tested bacteria, *E. coli* showed the most sensitive to *A. galanga* oil. Therefore *E. coli* ATCC 25922 was selected to investigate its morphology under the SEM. *A. galanga* oil showed obviously cytological modification to *E. coli* cells. The appearance of the destroyed cells structure was obviously seen as shown in Figure 4(c). This could be considered that *A. galanga* oil had high affinity to interact with the lipopolysaccharide on bacterial cell membrane. The interaction altered the structure of cell membrane. The shrinkage of cell morphology was important evidence on cell membrane alteration caused by the oil. This result led to a malfunction of bacterial cell membrane on normal permeability. The leakage of bacterial essential intracellular components caused cell lyses and final death in a short period.

Conclusion

The use of natural antimicrobial agents is gaining interest due to consumer and producer awareness on health problems. This paper shows that the essential oil of *A. galanga* had strong bactericidal activity against both Gram negative and Gram positive bacteria but more potent in Gram negative strains. The MIC and MBC values of the oil against Gram negative bacterial were substantially lower which showed higher potency and the kinetic bactericidal action was extremely faster than gentamicin. SEM results suggested that the mechanism of *A. galanga* oil on antibacterial action was through a modification of bacterial cell membrane leading to a permeability malfunction of the membrane. The results from this study suggested that the essential oil of *A. galanga* is a promising natural potential food preservative.

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