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A study of antibacterial efficacy of *Alpinia galangal* extracts against *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Listeria monocytogenes*.

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Abstract

Aims: Plants are invaluable sources of pharmaceutical product that have drawn the attention of ethno-pharmacologists around the world. One of the invaluable plants that has many medicinal properties since from ancient time was *A. galangal.* The present study aimed at investigating the antimicrobial efficacies of *A. galangal* against gram-positive bacteria which were *S. aureus, S. epidermidis* and *L. monocytogenes* using disc diffusion method and minimum inhibitory concentration method. Besides, this study also aimed to identify the phytochemical properties such as phenolic, flavonoid and DPPH content and to evaluate cytotoxicity level of *A. galangal* extracts using brine shrimp lethality test.

Methodology and results: *A. galangal* extract exhibited antibacterial activity against all the three tested gram-positive bacterial strains. However, *L. monocytogenes* shows a large inhibition zone (20.62 mm) compared to *S. epidermidis* (18.06 mm) and *S. aureus* (17.25 mm). The minimum inhibitory concentration of *A. galangal* against *S. aureus, S. epidermidis* and *L. monocytogenes* was less than 1mg/ml. Based on the result obtained, *A. galangal* has higher total phenolic content (122 ± 2.6 mg GE/g), followed by total flavonoids content (110 ± 4.4 mg QE/g). However, the DPPH content in this plant extract was least with the value of 20 ± 1.0 mg TE/g. In cytotoxicity study, the extract caused 46.7% mortality of brine shrimp larvae after 24 hours at a concentration of 62.5 µg/mL which is considered moderate toxic. While, at a concentration of 1000µg/mL, the mortality rate of brine shrimp larvae was 100% which is considered extremely toxic.

Conclusion, significance and impact of study: *A. galangal* extract exhibited significant lethality against brine shrimp larvae which suggesting the presence of bioactive materials. The observed antibacterial efficacy demonstrated by *A. galangal* extracts presents some promising and beneficial aspects in treatment.

Keywords: *Alpinia galangal, S. aureus, S. epidermidis, L. monocytogenes*

INTRODUCTION

Various plants have been used as drug and exhibit medicinal properties since ancient time. The medicinal properties is contributed by the presence of secondary metabolism in the plant *Alpinia galangal* (L.) Wild syn. Languas galanga commonly known as greater galangal. *A. galangal* belongs to the kingdom Plantae, family Zingiberaceae, and genus *Alpinia*. *Alpinia galanga* (L.) also known as Galangal, a member of the ginger family and native to Southern China and Thailand. *A. galangal* is primarily used as a flavoring agent especially in the preparation of fresh Thai curry paste and Thai soup (Verma et al., 2011; Juntachote et al., 2006).

Besides, *A. galangal* is useful against various disease such as rheumatic pains, diabetes, tubercular glands, bronchitis and catarrhal infections (Mohiuudin et al., 2011). The rhizome of this plant is widely used as carminative, digestive tonic, anti-emetic, anti-fungal, antitumor, Anti-helmintic, anti-diuretic, anti-ulcerative, anti-dementia (Vanwyk and Wink, 2009). The seeds of *A. galangal* are also used as cardiotonic, diuretic, hypotonic, gastric lesions and antiplatelet (Mohiuudin et al., 2011). Moreover, a study by Zheng et al. (2002) mentioned that *A. galangal* also has been used as traditional medicine in Thailand, especially for antifungal and antibacterial treatment. Hence, this study mainly focused on effectiveness of *A. galangal* against antibacterial activity of selected gram-positive bacteria which are *S. aureus, S. epidermidis* and *L. Monocytogens*.

Gram-positive organisms are among the most common bacterial causes of clinical infection. This is primarily due to their association with a diverse spectrum of pathology, ranging from mild skin and soft tissue infections (SSTIs) to life-threatening systemic sepsis and meningitis (Eades et al., 2017). *S. epidermidis* is a nosocomial infection that have gain much attention as skin colonizer (Otto, 2010). Foodborne illness is a serious threat to public health around the world. Foodborne illness is caused by the consumption of harmful bacteria in form of contaminated food. The *L. monocytogenes* and *S. aureus* are pathogens that are widely found in ready-to-eat meat products (Khan et al., 2016).

Previous studies have been reported on the phytochemical properties of *A. galangal*. For example, a study by Ying and Boa An, (2006) isolated 1'S-1'-acetoxychavicol acetate (ACE) from the rhizomes of *A. galangal*. The 1'S-1'-acetoxychavicol acetate from *A. galangal* shows inhibition of Rev transport at a low concentration which result in a block in HIV-1 replication in peripheral blood mononuclear cells. Besides, Jaju et al. (2009) had found the presence of bioactive compound such as β-Sitosterol diglucoside (AG-7) and β-sitsteryl Arabinoside (AG-8) from the rhizome of *A. galangal*. As for this study, the presence of phytochemicals of *A. galangal* especially flavonoid, phenolic and DPPH will be finding out.

MATERIALS AND METHODS

Methanolic Extraction

Fresh sample of *A. galangal* were collected from Universiti Sains Malaysia (USM). Then, the samples were washed under running tap water and dried in oven to remove moisture. Later, the samples were grounded into a powder form and again it was dried inside the incubator at 37°C for 4 days. The dried samples were soaked in 70% methanol. Finally, the *A. galangal* extract was filtered and evaporated using a rotary evaporator.

Microorganisms

S. epidermidis, *S. aureus*, and *L. monocytogenes* were obtained from Department of Biology, University Sains Malaysia, USM Penang. Potato Dextrose Agar (PDA) was from Merck (Germany) and Luria Broth (LB) was from Sigma Life Science (USA). While, Mueller Hinton agar (MHA) was from Himedia Lab (India).

Disc Diffusion Assay

The antibacterial activity *of A. galangal* was tested by using disc-diffusion method which was modified from the method described by Daruliza et al. (2011). Firstly, the bacterial cultures were adjusted to 0.5 Mc Farland turbidity standard and 0.1 mL of the adjusted bacterial cultures were inoculated onto Mueller Hinton Agar (MHA). Then, the filter paper discs (5 mm in diameter) were impregnated with crude extracts (10 mg/disc) and were overlaid on Mueller Hinton agar (MHA), and incubated at 37ºC, for 24 hours. The inhibition zones were measured in millimetres and all the tests were performed in triplicate.

Minimum Inhibition Concentration

Minimum inhibitory concentrations (MIC) were determined by serial dilution method and was modified from the method described by Weigand et al. (2008). A single colony was picked from a PDA agar and dissolved in the LB broth. The culture was incubated overnight at 37°C, 220 rpm. In each well 50µl of LB broth was placed. Then, a serial dilution of *A. galangal* extract was carried out to achieve final concentration between 0.625-10 mg/ml was prepared in LB broth. After that, in each well 20µl of *S. aureus*, *S. epidermidis* and *L. monocytogenes* for each serial dilution were placed. Then, the plate was incubated at 37°C, for 20-24 hours. The next day, the absorbance of the blank (LB broth), each indicator strain and the incubated plate were measured at 600nm. Finally, a graph of MIC was plotted using Microsoft Excel. From the graph, MIC endpoint of the lowest concentration of *A. galangal* was recorded.

Total Flavonoid Content

Total flavonoid content (TFC) of the methanol extracts were determined by the method developed by Sakanaka et al. (2005) with slight modifications. Briefly, 250µl of *A. galangal* extract (1mg/ml) were mixed with 1250µL distilled water and 75µL of 5% $NaNO_2$ were added into the test tube. Then, the mixture was incubated for 6 minutes. After that, 150µL of 10% $AlCl_3$ solution was added to the mixture, and the mixture was incubated for 5 minutes. After 5 minutes, 500µL 1M NaOH was added and the mixture was brought to 2.5mL by adding distilled water. Then, the mixture was stirred well. An aliquot of 200µL of the mixture was transferred to a 96-well plate where the absorbance was measured immediately at 510nm. TFC of the samples was determined from the calibration curve of the quercetin standard in the range of 0.01 – 0.1 mg/ml. The results were expressed in mg or quercetin equivalents per gram of dry extract (mg QE/g).

Total Phenolic Content

The total phenolic content (TPC) was determined using spectroscopic as described by Anisworth and Gillespie (2007). The reaction mixture was prepared by mixing 1 mL of *A. galangal* extracts (mg/mL), 1mL of 10% Folin-Ciocalteu's reagent which was dissolved in 13ml of deionized water, followed by 5mL of 7% Na_2CO_3 solution. Then, the mixture was mixed thoroughly and kept in dark at room temperature for 2 hours. The blank solution was prepared, and the absorbance was recorded using UV-Vis spectrometer at 760nm. All the analysis was repeated three times and the mean value absorbance was obtained. TPC was determined by extrapolating calibration line which was conducted by gallic acid solution. Lastly, the TPC was expressed as gallic acid equivalent (mg GAE) per gram of the dried sample.

DPPH assay

The antioxidant activity of the extract was determined using the DPPH free radical scavenging assay described by Nithianantham et al, (2011) with some modifications. Firstly, 50 µL of *A. galangal* extracts in concentrations from 1 to 5 mg/mL were placed into the universal bottle. Then, 5 mL 0.004% (w/v) solution of DPPH was added into the mixture. After that, the mixture was vortexed and incubated for 30 min in room temperature in a relatively dark place. Lastly, the mixture was read using spectrophotometer at 517 nm. All the measurements were taken in triplicate.

Brine Shrimp Lethality Assay

The procedure for brine shrimp lethality test (BSLT) was modified from the assay described by Daruliza et al. (2011). Firstly, artificial seawater was prepared from commercial sea salt (38 g sea salt/liter deionized water). After that, the brine shrimp eggs were hatched in with constant light source and oxygen supply after 24 hours of incubation. Then, a serial dilution of *A. galangal* extract was carried out to achieve final concentration between 1.96 to 1000µg/mL was prepared in seawater respectively. After serial dilution, ten hatched nauplii were added into each concentration and adjusted to 3 ml sea water. Brine shrimp were then incubated for 24 hours under a constant light source and the number of living nauplii was counted the next day. Finally, the lethal concentration (LC50) for *Artemia nauplii* was determined using Microsoft Excel.

GC-MS analysis of A. galangal leaves extract

In order to investigate the volatile components of *A. galangal* leaves extract, 200 mg of ethanolic extract were dissolved in chloroform (20mL) and filtered to remove off the debris and precipitate. The obtained sample (10 mg/mL) was then subjected to GC-MS analysis. The procedure for GC-MS analysis was modified from the method described by Chear et al. (2016). The

phytochemical analysis was performed on an Agilent 6890N Network GC system coupled to an Agilent 5973i Mass Selective Detector (Agilent Technologies, Waldbronn, Germany). Separation of the volatile compounds was done on an HP-5MS column (30 m x 0.25 mm, 0.25µm: Agilent Technologies) with helium gas (carrier gas) flowing at 1.2 mL/min. For GC-MS detection, an electron ionization system was operated in electron impact mode with an ionization energy of 70eV. Then, a helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1mL/min, and an injection volume of 10µL was employed (a split ratio of 10:1). The injector temperature was maintained at 280°C, while the ion-source temperature was set at 250°C. The oven temperature was programmed from 70 °C (isothermal for 2 min), with an increase of 20 °C/min to 280°C, then isothermal at 280°C for 30 mins. Mass spectra were taken at 70eV, a scan interval of 0.5 s and fragments from 45 to 450 Da. The solvent delay was 0 to 2 min, and the total GC/MS running time was 32 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Identification of the phytochemicals was done by performing spectral match against the National Institute of Standards and Technology database (Gaithersburg, MD, USA) and the Wiley Registry (John Wiley and Sons, Hoboken, NJ, USA). Similarity between the MS spectrum of the analytes and those in the database was evaluated by comparing the mass of their molecular ions, base ions, fragment ions, as well as their peak intensities. Only those compounds with >90% spectral matching quality were considered acceptable.

RESULT AND DISCUSSION

More than 80% of the world's population relies on traditional medicine for their primary healthcare needs. The medicinal value of plants lies in certain chemical substances that produce a definite physiological action on the human body. Disc diffusion methods are extensively used to investigate the antibacterial activities of natural antimicrobial substances of plant extracts. These assays are based on the use of discs as reservoirs containing the solution of substances to be examined (Arullappan et al, 2009). In this study, different microorganisms were used to screen the possible antimicrobial activities of *A. galangal* extracts. Table 1 shows the zone of inhibition of *A. galangal* extracts against *S. aureus*, *S. epidermidis* and *L. monocytogenes*. Based on the result obtained, *A. galangal* extracts showed a good inhibition zone against *L. monocytogenes* (20.62 mm) compared to *S. aureus* (17.25 mm) and *S. epidermidis* (18.06 mm). As a result, it is sure that these *A. galangal* extract can surely inhibit the growth of these microorganisms thereby preventing various disease such as skin infections and foodborne diseases. All in all, the disc diffusion result of this study indicate promising baseline information for the potential uses of *A. galangal* in the treatment of infectious disease.

The MIC's values for antibacterial activity are presented in Figure 1. Fabry et al. (1998) defined that active crude extracts are those having MIC values less than 8 mg/mL. In this study, MIC values of less than 1 mg/mL were considered to have good activity. Overall, *A. galangal* extracts is effective with all three gram-positive bacterial strain. However, *Staphylococcus aureus* and *Listeria monocytogenes* being the most susceptible strain

Table 1: Zone of inhibition (mm) of crude extracts against pathogenic strains using disc diffusion assay.

Pathogenic strains	Zone of Inhibition (mm)			
	Trial 1	Trial 2	Trial 3	Average
Staphylococcus aureus	17.66	16.85	17.25	17.25
Staphylococcus epidermidis	17.63	18.14	18.42	18.06
Listeria monocytogenes	20.62	21.51	19.73	20.62

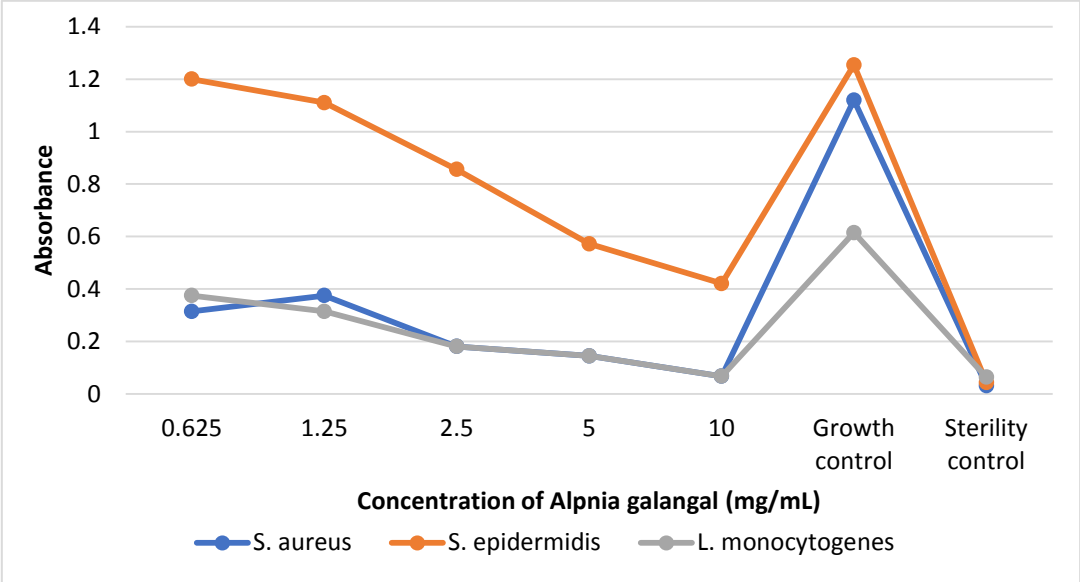

Figure 1: Antibacterial activity (MIC mg/ml) of *Alpinia galangal* against *S. aureus*, *S. epidermidis* and *L. monocytogenes*.

Table 2: Presence of phytochemicals in *Alpinia galangal* extract.

Phytochemicals	Result
Total phenolic assay	122 ± 2.6 mg GE/g
Total Flavonoid assay	110 ± 4.4 mg QE/g
DPPH Assay	20 ± 1.0 mg TE/g

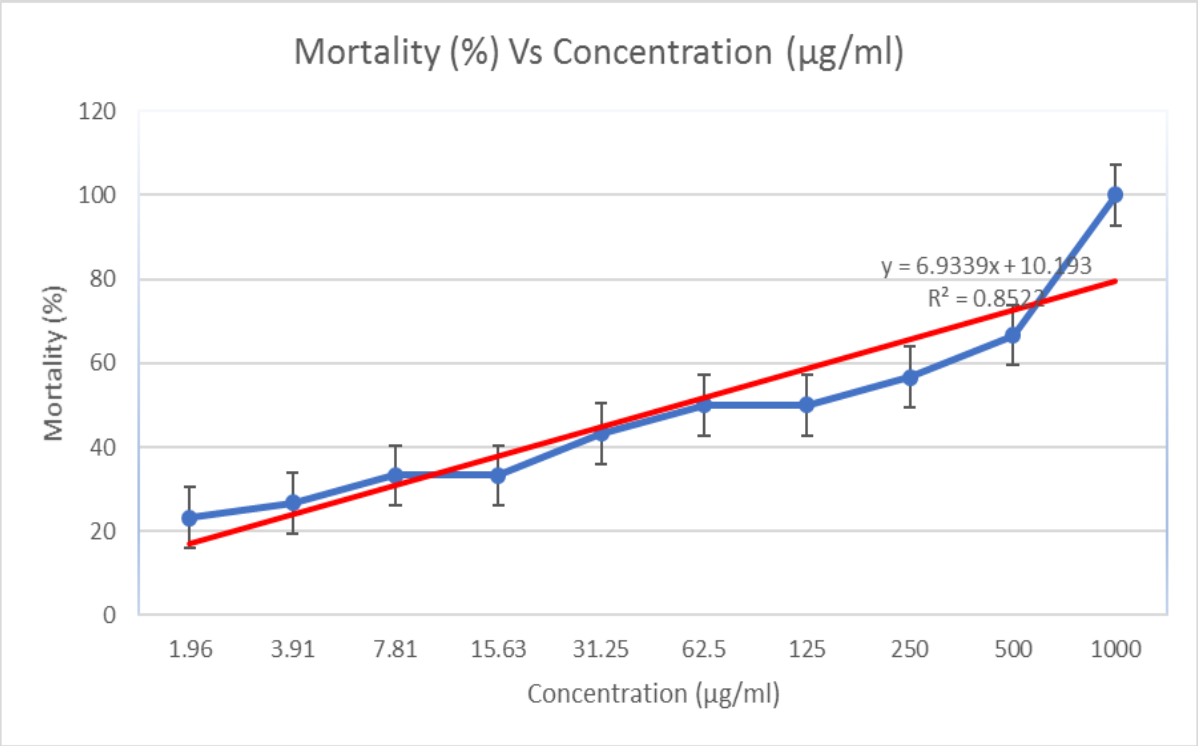

Figure 2: Result of brine shrimp lethality assay after 24 hours

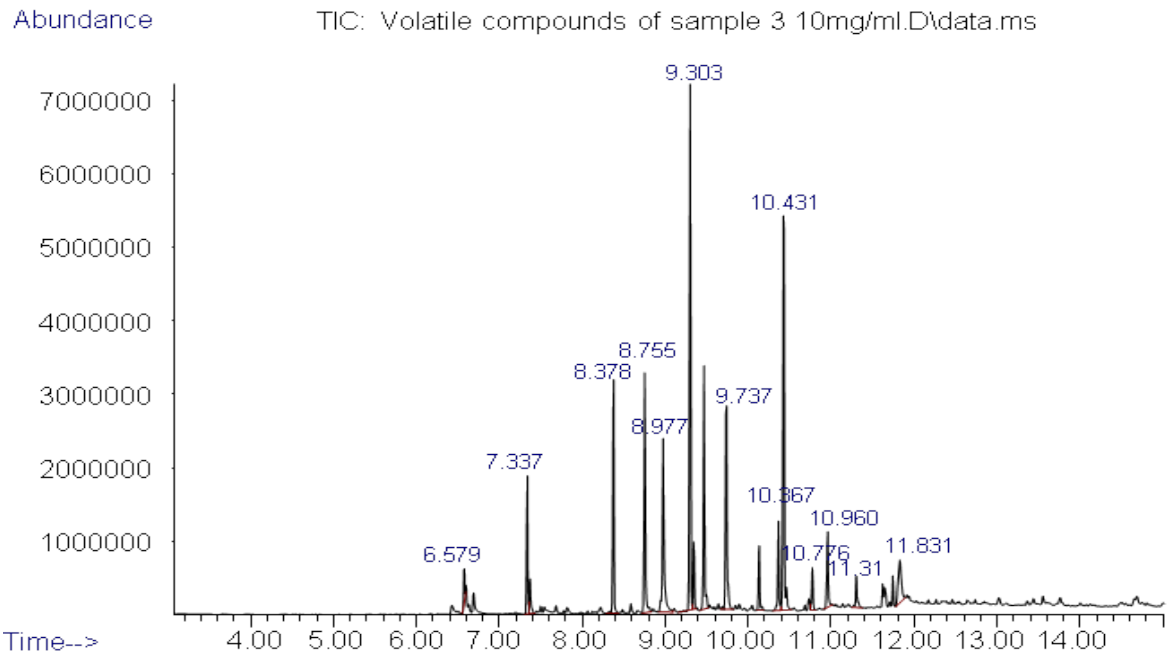

Figure 3: GC-MS chromatogram of *A. galangal* extract

Table 3: Identified volatile compounds in *Alpinia galangal* extract.

Sample No.	Retention time (RT, min)	Name of Compound	Chemical group	Area (%)
1	6.579	2-Cyclopenten-1-one, 2-hydroxy-1,3-Cyclopentanedione	aliphatic hydrocarbon / fatty acid	0.95
2	7.337	1,2,3-Propanetriol, 1-acetate	aliphatic hydrocarbon/ fatty acid	4.14
3	8.378	Benzyl alcohol	Aromatic compound	7.98
4	8.755	Benzenemethanol	Aromatic compound	8.87
5	8.977	Benzoic acid, 2,4-dimethyl-,methyl ester	Aromatic compound	8.20
6	9.303	Phenol, 2,4-bis(1,1-dimethylethyl)	Aromatic compound	17.76
7	9.737	Phenol, 4-butyl-	Aromatic compound	8.89
8	10.367	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, acetate, (E,E)	aliphatic hydrocarbon/fatty acid	3.11
9	10.431	Methyl Cinnamate	Aromatic compound	16.06
10	10.776	Hexadecanoic acid, methyl ester	aliphatic hydrocarbon/ fatty acid	1.50
11	10.960	Hexadecanoic acid	aliphatic hydrocarbon/ fatty acid	3.03
12	11.310	Phenol, 4-[3-(acetyloxy)-1-propenyl]-2-methoxy-, acetate	Aromatic compound	1.49
13	11.831	9-Octadecenoic acid (Z)- (Oleic acid)	aliphatic hydrocarbon/fatty acid	3.60

Phytochemical screening of natural products underpins the development of cosmetic and anti-aging products. Phenolic compounds, particularly flavonoids are known to enhance rapid skin regeneration and antimicrobial effects (Ghuman et al, 2016). In skin burns and wound healing processes, phenolic-protein complexes form a film that prevents dehydration and creates a physical barrier to damaged tissue thereby preventing microbial infection and chemical damage (Luseba et al., 2007). Total phenolic concentrations, flavonoid concentration, and DPPH content of *A. galangal* was represented in Table 2. Based on the result obtained, the total phenolic content of the methanol extract of *A. galangal* shows the highest total phenolic content with the value of 122 ± 2.6 mg GE/g. While the presence of DPPH in *A. galangal* extract shows the least with the value of 20 ± 1.0 mg TE/g. The presence of highest total phenolic content in *A. galangal* extracts indicates that the presence of most antioxidant properties and antimicrobial activity. Antioxidants are secondary metabolites, which form part of the plant's protective mechanism against free radicals. In Zingiberaceae, it is generally believed that antioxidants and other secondary metabolites are transported to the rhizomes where they are accumulated (Chan et al, 2007). Coincidentally, the *A. galangal* also demonstrated good antimicrobial activity against the tested microorganisms as shown in Table 1. Phenolic compounds in plants are purported to serve defence roles against invading pathogens and important pharmacological activities such as antioxidants, anticarcinogenic, antibacterial, and anti-inflammatory effects that have been recorded (Kuda et al., 2005).

In order to investigate the volatile compounds in the leaves extract of *A. galangal*, 10 mg of extract was analysed by GC-MS. In total, twelve volatile compounds, constituting 85.58% of the sample by peak area percentage, were identified in the dichloromethane fraction (Figure 3). Simple phenolic constituents was the dominant chemical class in this fraction, accounting for 69.25% of the fraction, and this figure was largely contributed by phenol, 2,4-bis(1,1-dimethylethyl) (17.76%), and methyl cinnamate (16.06%). On the other hand, aliphatic compounds (16.32%) was identified as the second major chemical group in the fraction. Identified phenolic compounds such as phenol, 2,4-bis(1,1-dimethylethyl), methyl cinnamate, benzenemethanol and benzyl alcohol (Table 3) were reported as plant antioxidants with good antibacterial activities (Saravanan et al., 2014; Rao et al., 2010; Lee & Shibamoto 2002). These compounds were abundantly found in the leaves and rhidzome of *A. galangal* and responsible for most of its antibacterial activities (Rao et al., 2010).

Brine shrimp lethality test has been used for toxicity screening of plant extracts, heavy metals, pesticides, food additives and pharmaceutical compound. Due to its simplicity, rapid, and inexpensive that has been received great attention to many researchers. Besides, the brine shrimp lethality assay represents a simple bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxic and anti-tumor properties (Krishnaraju et al, 2005, Mirazaei et al, 2013). The LC50 value of *A. galangal* was obtained by a plot of percentage of the shrimp nauplii killed against the concentrations of the *A. galangal* extract and the best-fit line was obtained from the data as shown in Figure 2. The data from brine shrimp lethality test were plotted using Microsoft Excel, from where the lethal concentration (LC50) for brine shrimp was determined. In the present study, the result of brine shrimp lethality assay after 24 hours, showed 100% mortality at 1000µg/ml concentration and LC50 value of the *A. galangal* extract was 5.74µg/ml. According to the classification by Meyer et al. (1982) and Parra et al. (2001) crude extracts and pure substances with LC50 value lower than 1000 µg/ml are considered bioactive in toxicity evaluation of plant extracts by brine shrimp lethality test (BSLT). Hence, *A. galangal* could be considered toxic against brine shrimp according to the results obtained from the BSLT. However, extracts from

this plant used as traditional medicines are unlikely to have any ill effects on patients.

CONCLUSION

As a conclusion, the results of in vitro antimicrobial screening of *Alpinia galangal* indicated that the methanol extract have good antimicrobial activity against *Listeria monocytogenes* (20.62 mm), followed by *Staphylococcus epidermidis* (18.06 mm) and *Staphylococcus aureus* (17.25 mm). The observed antibacterial efficacy demonstrated by *A. galangal* extracts presents some promising and beneficial aspects in treatment. Besides that, *A. galangal* contained highest total phenolic content with the value of 122 ± 2.6 mg GE/g which indicates the presence of most antioxidant properties and antimicrobial activity. In brine shrimp lethality assay, *A. galangal* extract exhibited significant lethality against *Artemia nauplii* which suggesting the presence of bioactive materials.

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