International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

International Journal of Pharmacology 5 (5): 285-297, 2009
ISSN 1811-7775

Antioxidative and Antibacterial Activities of *Pangium edule* Seed Extracts

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Abstract: Phenolic and alkaloid extracts of *Pangium edule* Reinw (Flacourtiaceae) seed were investigated for their antioxidative activities using DPPH radical scavenging and β-carotene bleaching assays. The extracts were evaluated for antibacterial activity against *Salmonella typhimurium* and *Listeria monocytogenes*. The acetone extract with higher phenolic content (22.22±0.05 mg GAE g^{-1}) showed the most potent antioxidative activity in both DPPH radical scavenging and β-carotene bleaching assays as compared to other extracts. The phenolic extract seems to have stronger inhibitory against *L. monocytogenes* than *S. typhimurium*. The free phenolic acid extract was found to have the highest Minimum Inhibition Concentration (MIC) among the seed extracts, indicates its weak antibacterial activity against both bacteria. Nevertheless, both tested pathogens were killed at the Minimum Bactericidal Concentration (MBC) of 30.3 and 55.5 mg mL^{-1}, respectively, for the phenolic extracts. Significant correlation ($p<0.05$) was observed between the total phenolic content and its antioxidative activity ($r = 0.878$) as well as antibacterial ($r = 0.840$) activity suggesting that phenolics of the seed extract could be potential sources of natural antioxidant and antibacterial.

Key words: Natural antioxidant, phenolics, alkaloids, *Listeria monocytogenes*, *Salmonella typhimurium*, minimum inhibition concentration

INTRODUCTION

Research has shown that oxygen is a toxic substance as it may release free radicals, which are unstable in normal conditions (Martinez-Cayuela, 1995). Oxygen derived free radicals or more generally known as reactive oxygen species (ROS), include superoxide (O_2^-), hydroxyl (OH^-), hydroperoxyl (HOO^-), peroxyl (ROO^-) and alkoxyl (RO^-) radicals. Other common reactive oxygen species produced in the body include nitric oxide (NO^-) and the peroxynitrite anion ($ONOO^-$) (Cao and Prior, 1999). Free radicals may initiate chain reaction by capturing an electron from other compounds in order to become stable. Thus, this may cause lipid peroxidation in food system and eventually spoil the related foods (Miller *et al*., 1995). Free radicals also induce oxidative damage on biomolecular components such as lipid, nucleic acid, protein and carbohydrate that may lead to aging and diseases including cancer and cardiovascular problems (Valko *et al*., 2006).

However, free radicals can be eliminated by the antioxidant protecting mechanism, as the antioxidant will de-activate the chain reaction that induced by free radical. Most of the synthetic antioxidants are used in food systems for preservation purposes. This includes butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ) and propyl gallete (PG). Synthetic antioxidants are widely used because they have low molecular weight and non-polar properties. Recent reports revealed that some synthetic antioxidants such as BHT and BHA could be toxic (Botterweek *et al*., 2000). Due to the lower efficiency of natural antioxidants (such as tocopherols) and the increasing awareness of consumers regarding the safety of food additives, this has created a need for identifying natural and alternative sources of food antioxidants and antimicrobials (Fernández-López *et al*., 2005).

Plant secondary metabolites are excellent sources of phenolic phytochemicals, especially as antioxidants and antimicrobials. In this regard, some phenolics have shown antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (Barik *et al*., 2007). The use of phenolic phytochemicals as antioxidants for human health and wellness is not surprising but, in fact, it is an important scientific agenda for the food and nutritional sciences studies. Natural antioxidants are able to protect the human body from free radicals and delay the progress of many chronic diseases (Behera *et al*., 2008; Papetti *et al*., 2006) as well as retard lipid oxidative rancidity in foods (Reddy *et al*., 2005). Many have reported that phenolic compounds are the

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most active antioxidants due to their structure-activity relationship and are responsible for the observed antimicrobial activity (Pereira *et al*., 2006; Tripoli *et al*., 2007).

Pangium edule Reinw (Flacourtiaceae), locally known as Pangi or Kepayang, grows wild in most South East Asia countries. Almost all parts of the plant are poisonous due to the presence of cyanogenic glycoside components in its leaves, barks and seed. It takes 10-15 years for the plant to bear fruits depending on location, climate and soil type. The fruit differs in size, ranging from 12-30 cm or the size of a football. The aril of this fruit is somewhat sweetish when ripe while the numerous seeds are the most prized part of the species. The Pangi seed is rich in fat and gynocardin, a glycoside that releases perussic acid during maturation. However, the Pangi seed can be consumed after certain treatments such as soaking and steaming. Fermented seeds are specialty in Indonesia and have been used as spices. In Northern parts of Borneo Island, East Malaysia, the dried seed is ground into powder form and used as an ingredient for some of the local delicacies and in some indigenous fermented foods. It is claimed that addition of this seed into the fermented foods helps in controlling the fermentation process as well as to provide the unique flavour of the products. The local people also use this indigenous plant as a folk medicine for wound healing or as a poison for fishing. However, their biological activities remain untapped and not well documented.

Previous studies have shown that many seed extracts possess antioxidative activity (Majhenic *et al*., 2007; Abdalla *et al*., 2007) and some also have antimicrobial properties (Gülçin *et al*., 2003; Chauhan *et al*., 2007). However, there is not much information about the antioxidative and antimicrobial activities of the seeds of this indigenous plant. Hence, the present study determined the *in vitro* antioxidative activity as well as its antimicrobial activity against selected foodborne bacterial pathogens. Furthermore, the relationship between the phenolic content and antioxidant and antibacterial activities was also investigated.

MATERIALS AND METHODS

Materials: Dried *Pangium edule* seed was purchased from local indigenous markets in Kota Kinabalu, Sabah from January 2006 to March 2007. The seeds were identified and authenticated by Mr. John Sugau from the Forest Reserve Centre of Sabah and voucher specimens were deposited at the Lumanis Forest Reserve Centre, Sandakan with a herbarium No. 28421. The samples were cleaned by a brush to remove sand and the fleshes before being ground into fine powder with a mechanical blender and kept at room temperature (28°C) in a sterile airtight container prior to extraction. All solvents and chemicals used for extraction were analytical grade and purchased from Merck, Germany. DPPH (1, 1-diphenyl-2-picrylhydrazyl), Folin-ciocalteu reagent, β-carotene and gallic acid were purchased from Fluka (Sigma-Aldrich, Singapore).

Two pathogenic bacterial cultures *Listeria monocytogenes* L55 and *Salmonella typhimurium* S1000 were obtained from Institute for Medical Research (IMR), Kuala Lumpur, Malaysia and used for the antibacterial activity determination. The cultures were revived from stocks stored at -80°C on Muller Hinton Broth (MHB, Oxoid) at 37°C for 24 h. They were subsequently checked for purity by grown at 37°C for 18 h on Muller Hinton Agar (MHA, Oxoid). The antibiotic susceptibility test was used to verify that the selected bacteria cultures were native/normal isolates and would not showed an abnormal susceptibility or resistance (mutant).

Extraction of polar phenolic group: Approximately 100 g of powdered pangi seeds were placed in a Soxhlet extractor and treated with petroleum ether at 60°C for 6 h to remove the fatty materials. The defatted pangi seed was retreated with 150 mL acetone: water: acetic acid (90:9.5:0.5) in Soxhlet apparatus for 8 h at 60°C, as described by Jayaprakasha *et al*. (2003). Another method of phenolic extraction was carried out according to the method described by Baydar *et al*. (2004) using ethyl acetate: methanol: water mixture (60:30:10) at 60°C. The extracts were concentrated using a rotary evaporator (Buchi Rotavapor R-114) at 45°C and the extraction yields were determined.

Extraction of free and glycoside bound phenolic acid: The phenolic acid extraction was carried out as described by Krygier *et al*. (1982). For free phenolic acid extract, 30 g of pangi seed powder was soaked in 100 mL of a mixed solvent containing methanol, acetone and distilled water at a ratio of 6:3:1. The solution was then centrifuged at 2000 x g for 30 min. The supernatant collected was concentrated to approximately 2 mL using rotary evaporator. HCl solution (3 mL) was added to the extract to acidify the suspension before further extracted by adding 15 mL of diethyl ether and concentrated by using rotary evaporator at 45°C. Finally, 50% methanol (1 mL) was added to the extract in order to liberate the bound phenolic acid to free phenolic acid.

For the glycoside-bound phenolic acid extraction, the above steps were followed as usual except during the addition of 3 mL of HCl solution to the extract, the

suspension was mildly heated at 96°C for 1 h before concentrated to approximately 2 mL using rotary evaporator at 45°C for 10 min. Fifty percent Methanol (1 mL) was added to the concentrated extract to re-suspend the glycoside-bound phenolic acid.

Alkaloid extraction: Alkaloid in the seed samples was extracted according to the method described by Zaches-Hanrot *et al.* (1995). A total of 30 g of defatted pangi seed was weighted and soaked in methanol for overnight. The methanol solution was then filtered with Whatman paper No. 1 and the filtrate was concentrated using rotary evaporator at 45°C. The extract was then dissolved in 100 mL chloroform before mixed with petroleum ether to form a two-layer solution. The upper layer (petroleum ether) was discarded and the lower layer (chloroform layer) was mixed with 10 mL 20% sulphuric acid to form a white layer. The white layer solution was removed and the remaining extract was then mixed with 10 mL 10% aqueous ammonia. Once precipitation completely formed, the extract was then filtered through a Whatman Filter paper No 1. The filtrate was mixed with 2 g anhydrous sodium sulphate to remove excessive moisture in the extract and kept for 1 h. The extract was finally concentrated using rotary evaporator at 50°C. The extract was kept in a sterile universal bottle wrapped with aluminium foil to avoid any lost of essential properties prior to further analysis.

Determination of total phenolic content: The determination of total phenolic content in Pangi seed extract was based on the method described by Kahkonen *et al.* (1999). A standard calibration curve at 0.01-0.1 mg mL^{-1} Gallic Acid Equivalent (GAE) was first established. Pangi seed extracts with different concentration was added to Folin-ciocalteu reagent and the absorption was read through a UV spectrophotometer (Perkin-Elmer) at λ = 725nm. The total phenolic contents of the pangi seed extracts were calculated based on the following formula:

$$C = c \times (V/m)$$

where, C is the total phenolic content in mg g^{-1}, c is the concentration of gallic acid from calibration curve in mg mL^{-1}; V is the volume of the sample in mL and m is the weight of sample extract in gram.

The unit for the total phenolic content is GAE mg g^{-1} extract.

Determination of antioxidative activity: Free radical scavenging activity (1,1-diphenyl-2-picrylhydrazyl DPPH).

The determination of antioxidative activity of the extracts was based on the scavenging activity against stable free radical (DPPH) according to the method described by Brand-Williams *et al.* (1995) with some modifications. The radical scavenging activity of Pangi seed extracts against stable DPPH was determined spectrophotometrically. Pangi seed extract (1 mL) was added to 0.5 mL of methanol solution containing 1,1-diphenyl-2-picrylhydryl (DPPH, Sigma) radicals, resulting in a final concentration of 0.1 mM DPPH. The mixture was shaken vigorously and left to stand for 30 min in dark at room temperature and the resultant absorbance was recorded at 517 nm using UV spectrophotometer. Different concentrations of test samples (0, 5, 10, 15, 20, 25 and 30 mg mL^{-1}) were tested. Distilled water was used as a blank and the controls contained all the reaction reagents except the extract. The percentage of Radical Scavenging Activity (RSA) was calculated using the following equation:

$$\text{DPPH radical-scavenging activity (\%)} = [(A_0-A_1)/A_0]\times 100$$

where, A_0 is the absorbance of the control and A_1 is the absorbance of the samples at 517 nm.

TBHQ was used as a standard for comparison. EC_{50} values denote the concentration of sample required to scavenge 50% DPPH free radicals.

β-Carotene-linoleic acid assay: The total antioxidative activity of *Pangi* seed extracts were evaluated by the β-carotene-linoleate bleaching method as described by Ismail *et al.* (2004). One millilitre of β-carotene solution (0.2 mg mL^{-1} chloroform) was pipetted into a round-bottom flask (50 mL) containing 0.02 mL of linoleic acid and 0.2 mL of 100% Tween 20. The mixture was then evaporated at 40°C for 10 min using a rotary evaporator to remove chloroform. After evaporation, the mixture was immediately diluted with 100 mL of distilled water and agitated vigorously to form an emulsion.

Aliquots of the emulsion (5 mL) were transferred into different test tubes containing 0.2 mL of samples in 70% ethanol at a final concentration of 1 mg mL^{-1}. The tubes were gently shaken and placed at 45°C in a water bath for 2 h. The absorbance of the samples was measured at 470 nm using UV spectrophotometer at initial time (t = 0) against a blank, consisting of an emulsion without β-carotene. TBHQ at the same concentration was used as the standard for comparison and 0.2 mL of 70% ethanol in 5 mL of the above emulsion was used as the control. The measurement was carried out for 120 min at 15 min intervals. All samples were assayed in triplicate.

The Antioxidative Activity (AA) was calculated in terms of successful bleaching of β-carotene using the following equation:

$$\text{Antioxidative activity} = 1-(A_0-A_t)/(A^0_0-A^0_t)\times 100$$

Where:

A_0: Absorbance value obtained at initial incubation time for sample

A^0_0: Absorbance value obtained at initial incubation time for control

A_t: Absorbance value obtained at 120 min for sample (or standard)

A^0_t: Absorbance value obtained at 120 min for control

Antibacterial activities of Pangi seed extracts against *S. typhimurium* and *L. monocytogenes* Screening of antibacterial activity using paper discs: The screening of antibacterial activity was performed by the paper discs assay. Pangi seed extracts were tested on two pathogenic bacteria, *Salmonella typhimurium* (ST) and *Listeria monocytogenes* (LM) using inhibition zone assay on Tryptic Soy Agar (TSA). Bacterial concentration at 107 CFU mL^{-1} of a 24 h cultures in Tryptic Soy Broth (TSB) was prepared based on the standard optical density growth curves for each strain. A 100 µL aliquot of culture was evenly spread onto agar plates using a sterile glass rod spreader. The plates were left at room temperature for 20 min to allow the agar surface to dry. Sterilized paper discs (6 mm) which were added with 10 µL aliquot of seed extracts were arranged on the inoculated plates (four discs per plate). After the plates were incubated at 35°C for 24 h, the diameter of the distinctly clear zones surrounded the disc that contained seed extracts were measured using a metric ruler in millimeters. The solvents that used in the extractions were served as negative control.

Pre-determination of the inhibitory ranges for Pangi seed extracts: After the paper disc assay, extracts with inhibitory activity on the selected bacteria were further screened to determine the smaller range of inhibitory concentrations using broth microdilution method as described by Chandrasekaran and Venkatesalu (2004). Each Pangi seed extract (1 mL) was pipetted into eight sterile bijou bottles, which contained 1 mL of Muller Hinton Broth (MHB), respectively. Twofold dilution method was used to obtain seven concentrations of seed extract including the original extract (1000, 500, 250, 125, 62.5, 31.25 and 15.63 mg mL^{-1}). Then, 0.1 mL of bacterial inoculums of 10^7 CFU mL was pipetted into bijou bottles, which contained the extracts and MHB before they were incubated at 37°C for 24 h. A bijou bottle without extract was used as a control. After 24 h, 0.1 mL of the inoculated MHBs were transferred and spread onto Muller Hinton Agar (MHA, Oxoid) to determine the range of inhibitory concentration for each extract based on the visible growth on the agar.

Further dilution of the extracts was done after the preliminary range was obtained in order to get the more specific inhibitory concentrations that showed antibacterial activity against selected bacteria. The selection of the more specific inhibitory range of concentration was based on the ability of the selected bacteria to form colonies on MHA in 24 h. These narrow concentrations of extracts were subjected to time-kill assay to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) against ST and LM.

Kinetic time-kill assay: Kinetic time-kill assay was carried out according to the method described by Kim *et al.* (2004). Five-selected concentrations of extract from the screening test were transferred into sterilized tubes, which contained 10 mL of MHB. Then 0.1 mL of bacterial inoculums of 10^7 cfu mL^{-1} were pipetted into the tubes and incubated at 37°C for 36 h. At each time interval (0, 6, 12, 18, 24, 30 and 36 h), 1 mL of the inoculated broth was serially diluted with 0.1% peptone water and plated to determine the growth of the tested bacteria. All plates were incubated at 37°C for 24 h and the growth was reported as log cfu mL^{-1}. A survival growth curve was plotted by connecting the log CFU mL^{-1} for the time intervals against the specific inhibitory concentration of the Pangi seed extracts. The MIC is defined as the lowest concentration of the Pangi seed extracts causing 90% reduction in growth within 24 h. However, the MBC is defined as the lowest concentration that caused 99% reduction in growth within 24 h.

Determination of decimal reduction time: Decimal reduction time (DRT) is defined, as the time resulting in a $\log_{10}$ reduction (90% killing) of cfu mL^{-1} of the tested strains. The decimal reduction time for Pangi extracts were determined from the negative reciprocal of the slopes of the regression lines, using the linear portions of the survivor curves ($\log_{10}$ cfu mL^{-1} versus time of exposure to the extract, at constant temperature done by plotting the change in $\log_{10}$ cfu at each point from the initial inoculum versus time).

Statistical analysis: Results were expressed as Mean±SD for three parallel measurements. One way Analysis of Variance (ANOVA) and Duncan's multiple range test (SPSS version 15) were applied on the values/data obtained in the experiments. Correlation was carried out to determine the relationship between the antioxidant and antibacterial properties. Statistical significance was defined at $p<0.05$.

RESULTS AND DISCUSSION

Extraction yields and phenolic content of Pangi seed extracts: Phenolics of acetone were shown to have the highest extraction yield (5.48±0.01 g/100 g) indicating that Pangi seed may contains higher amount of water-soluble compounds (Table 1). The alkaloid extract gave the lowest extraction yield (1.83±0.10 g/100 g) among the Pangi seed extracts. The results are consistent with those of Jayaprakasha *et al.* (2003), who found that the use of acetone: water: acetic acid solvent mixture with a high polarity gave higher extraction yields of phenolic compounds especially procyanidins groups of the grape seed. Extraction efficiency is a common function of process conditions. Higher phenolic concentration of extracts was obtained by increasing the temperature and lowering the solvent-to-solid ratio of samples (Pinelo *et al.*, 2005). Previous findings have reported the influence of some variables (e.g., temperature, time contact, solvent-to-solid ratio, etc.) on the phenolic yields capable of being extracted from diverse natural products such as lemon grass, rosemary, pine sawdust, or apple by-products (Pinelo *et al.*, 2004; Juntachote *et al.*, 2006). The chemical characteristics of the solvent and the diverse structure and composition of the natural products ensure that each material-solvent system shows different behaviour, which is difficult to be predicted.

The content of phenolics in Pangi seed extracts varied between 0.71 and 16.39 mg Gallic Acid Equivalents (GAE) g^{-1} with the phenolic of acetone extract was the highest (Table 1). This was related to the ability of the acetone to extract greater polar compounds including phenolic acids and their derivatives (condensed tannins), coumaric acid, flavonoid and gallic acid that may be present in the Pangi seed. Acetone and methanol seem to have distinct specificities in the extraction of polyphenolic substances. This is in accordance with polarity of the solvent used for the extraction and solubility of phenolic compounds (Turkmen *et al.*, 2006). On the other hand, the free and glycoside-bound phenolic acid extracts exhibited lowest phenolic contents compared to other phenolic extracts probably due to the losses of hydroxycinnamic acid derivatives after hydrolysis. The total phenolic contents in the acetone and ethyl acetate extracts in this study were slightly higher than the result reported by Andarwulan *et al.* (1999) on germinated Pangi seed. This may be due to the conversion of phenolic compounds to polymerized derivatives like lignans and lignins by guaiacol peroxidase during germination of the seed. Alkaloid extract had the lowest phenolic content indicates the present of non-phenolic alkaloids which may not possess any aromatic benzene ring with less hydroxyl group (C-OH). Many alkaloids are plant metabolic by-products derived from amino acids and some alkaloids have remarkable structural similarities with neurotransmitters in nervous system of human, thus plant-containing alkaloid has been used as medicinally useful agents due to their valuable pharmaceutical properties.

Table 1: The extraction yields (g/100 g), total phenolic content and EC_{50} values of different Pangi seed extracts

Extracts	Yields (g/100 g)	Total phenolic content (mg GAE g^{-1} extract)	EC_{50} (mg extract mL^{-1}) DPPH	EC_{50} (mg extract mL^{-1}) BCT
Phenolic (Ace)	5.48±0.01[a]	16.39±0.04[a]	7.3±0.1	6.1±0.1
Phenolic (EtOAc)	4.05±0.02[b]	12.65±0.02[b]	10.5±0.5	8.2±0.2
FPA	2.07±0.01[d]	2.57±0.02[d]	15.6±0.8	12.2±0.1
GBPA	2.26±0.01[c]	4.67±0.02[c]	20.9±0.1	15.1±0.2
Al	1.83±0.10[e]	0.71±0.01[e]	30.5±0.5	41.3±0.3

[1]Phenolic (Ace): Phenolic extracted by acetone, Phenolic (EtOAc): Phenolic extracted by ethyl acetate, FPA: Free phenolic acid, GBPA: Glycoside-bound phenolic acid, Al: Alkaloid. [2]Means followed by different letters in the same column indicate a significant difference ($p<0.05$), [3]EC_{50} value: The effective concentration (mg mL^{-1}) at which the antioxidant activity was reduced by 50%. [4]DPPH radical scavenging activity. [5]BCT- β-carotene bleaching method

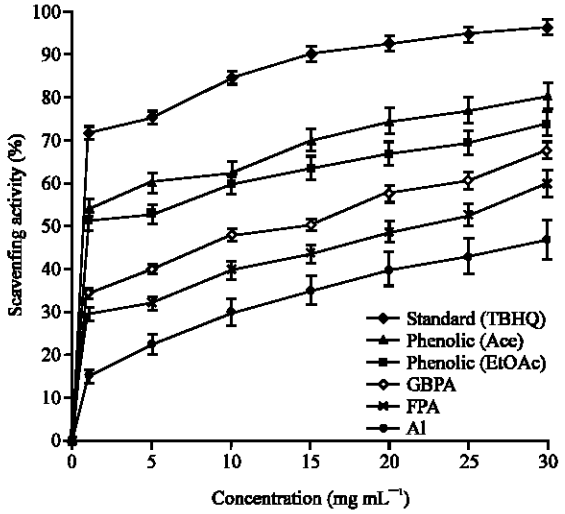

Fig. 1: DPPH radical scavenging activity (%) of Pangi seed extracts at different concentrations

Antioxidative activity

DPPH radical scavenging activity: The scavenging activity of each Pangi seed extract is shown in Fig. 1 where different concentration of extracts gave different scavenging activity. As an antioxidant donate protons to DPPH (radicals), the absorbance decreases. Thus, the decrease in absorbance is taken as a measure of the extent of radical scavenging. The phenolic of acetone extract was a good radical scavenger with an activity of 80.14% at 30 mg mL^{-1}, significantly ($p<0.05$) higher than other

extracts. This was supported by Miliquskas *et al.* (2004) who discovered that the acetone extract of *Echinacea purpurea* was the most effective radical scavenger because many polyphenolic compounds were present in acetone extract as compared to other extracts. The antioxidative activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators or even singlet oxygen quenchers. The major role of phenolics compounds as scavengers of free radicals was highlighted in several reports (Moller *et al.*, 1999; Adedapo *et al.*, 2008).

It was observed that at 10 mg mL^{-1} of the Pangi seed extract, the scavenging activity was fluctuated from 29.9% (alkaloid extract) to 62.3% (phenolic of acetone extract), whereas, at 30 mg mL^{-1}, the activity increased to 46.8 and 80.1% for the respective extracts. Synthetic antioxidant (TBHQ) used in this test always possessed higher scavenging activity over the sample extracts except for the phenolic of acetone extract which was almost comparable (80.1%). This indicates the potential of phenolic extract as a source of natural antioxidant replacing synthetic antioxidants (TBHQ, BHT and BHA), which were believed to cause severe health problems such as tumour promotion, stomach cancer and cell death to kidney and bladder (Botterweek *et al.*, 2000). Polyphenolics of ethyl acetate or acetone extract showed higher scavenging activity at any concentrations compared to monophenol extracts (free phenolic acid, glycoside-bound phenolic acid or alkaloid extract). This may be due to the antioxidative activity of a phenol is affected by its degree of polymerization. Phenols with a second hydroxyl group in the *ortho* and *para* positions possess stronger antioxidant activity than those with the *meta* position (Rice-Evans *et al.*, 1996).

Total antioxidative activity: The total antioxidative activity, reflected by the ability of the Pangi seed extracts to inhibit the bleaching of β-carotene was measured and compared with that of the control. The phenolic extracts of the Pangi seed always had higher antioxidative activity than other extracts for all concentrations tested (Fig. 2). This can be explained by the presence of greater phenolic compounds due to the breakdown of conjugated phenolics by the β-glycosidase at higher concentrations, which allowed it to react with β-carotene and inhibit the bleaching activity. According to Jayaprakasha *et al.* (2001), the bleaching mechanism of β-carotene is a free radical mediated phenomenon resulting from the formation of hydroperoxides from linoleic acid. In the absence of antioxidant, β-carotene undergoes rapid discoloration indicated by the decrease in absorbance. For a typical case at the concentration of 20 mg mL^{-1} extracts across time, higher absorbance values indicate that the seed extracts possessed greater antioxidative activity by prohibiting the bleaching of β-carotene (Fig. 3). However, the absorbance for the standard (synthetic antioxidant) seems to be always higher than the seed extracts at all time intervals. The inhibition of the β-carotene activity by the Pangi extract at 1 mg mL^{-1} was 58% and this result is slightly lower than the guarana [*Paullinia cupana* K. (Sapindaceae)] seed extract (73%) (Majhenic *et al.*, 2007). This may be due to the higher temperature (75-100°C) was used in the guarana seed extraction, which increases the amount of extractable phenolic compounds as compared to the Pangi seed extraction at room temperature. However, the inhibition of β-carotene bleaching activity of the *Pangi* seed extract was higher than the nori

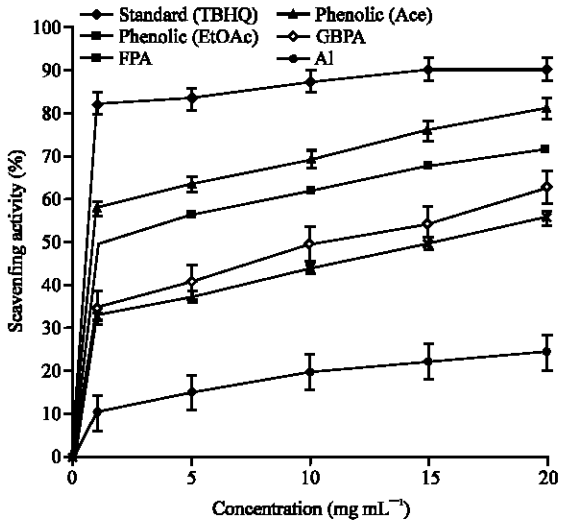

Fig. 2: Total antioxidative activity of Pangi seed extracts evaluated by ß-carotene bleaching method at different concentrations

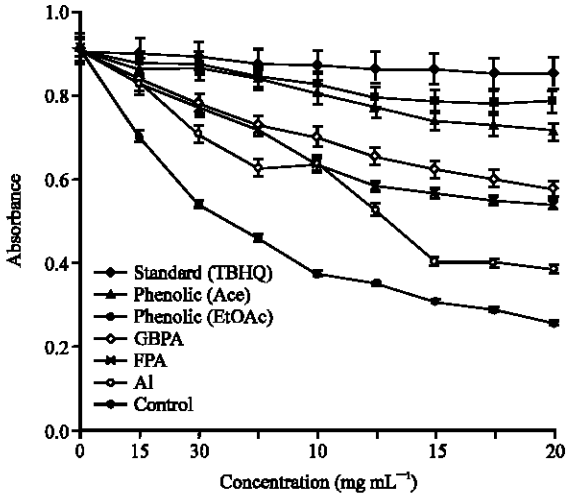

Fig. 3: Antioxidative activity of Pangi seed extracts at 20 mg mL^{-1} measured by β-carotene bleaching method

[*Porphyra* sp. (Bangiaceae)] (20%) and kale extracts [*Brassica alboglabra* L. (Brassicaceae)] (50%) (Ismail *et al*., 2004). This could be due to the used of mixed solvents (acetone/ethyl acetate, water, acetic acid) in the extraction of Pangi seed which gave a higher inhibition of β-carotene bleaching activity. This is in accordance to the findings by Jayaprakasha *et al*. (2001) on grape [*Vitis vinifera* L. (Vitaceae)] seed extract.

EC_{50} value of the antioxidative activities: The antioxidative property of the Pangi seed extracts is expressed as EC_{50} value, which is defined as the amount of antioxidant necessary to decrease the initial activity by 50%. The EC_{50} for the DPPH radical scavenging activity of the phenolics of acetone extract was 7.3±0.1 mg mL^{-1}, indicating the effective concentration of the extract to act as DPPH scavenger (Table 1). This may be related to the presence of hydroxyl group in the aromatic rings of polyphenols, which donate a hydrogen atom and converting free radicals to more stable products during the radical chain reaction. On the other hand, the EC_{50} for the phenolic extract to bleach β-carotene was the lowest (6.1±0.1 mg mL^{-1}) as compared to other extracts. It was observed that the effectiveness of antioxidative properties is inversely related to the EC_{50} values. Generally, EC_{50} of lower than 10 mg mL^{-1} indicates the particular extract tested is an effective antioxidant (Lee *et al*., 2007).

Antibacterial activity of Pangi seed extracts against *Listeria monocytogenes* and *Salmonella typhimurium*

Preliminary screening of antibacterial activity of Pangi seed extracts: The Pangi seed extracts showed inhibition zones ranged from 8.67-24.07 mm against LM and ST. The phenolics exhibited the strongest inhibitory against both LM and ST with mean values of 24.07±0.17 mm and 22.24±0.05 mm respectively (Table 2). This may be due to the greater availability of bioactive phenolic compounds in the acetone extract such as flavonoids, anthocyanins, catechin and flavan (Chirinos *et al*., 2007). However, the free phenolic acid extract showed the lowest inhibitory activity against LM and ST. Free phenolic acid such as protocatechuic acid, vamillic acid, ferullic acid, anisic acid, p-coumaric acid and p-hydroxybenzoic acid was generally identified as weak antibacterial compounds against selected microorganisms. Alkaloid extract also showed fairy good inhibitory against both LM and ST although they have low phenolic contents (0.71±0.01 mg GAE g^{-1} extract). Some of the alkaloid compounds, such as quinine and indole have shown great antimicrobial effects against selected Gram positive and Gram-negative bacteria (Tanaka *et al*., 2006).

Table 2: Antibacterial activity (Inhibition zones) of Pangi seed extracts against *Listeria monocytogenes* (LM) and *Salmonella typhimurium* (ST)

	Inhibition zones (mm)	
Pangi seed extracts	LM	ST
Phenolic (Ace)	24.07±0.17[a]	22.24±0.05[a]
Phenolic (EtOAc)	12.52±0.12[b]	11.73±0.06[b]
FPA	9.77±0.02[c]	8.67±0.05[c]
GBPA	9.87±0.06[d]	9.36±0.05[d]
Al	13.44±0.05[e]	11.69±0.04[e]

[1]Phenolic (Ace): Phenolic extracted by acetone, Phenolic (EtOAc): Phenolic extracted by ethyl acetate, FPA: Free phenolic acid, GBPA: Glycoside-bound phenolic acid, Al: Alkaloid. [2]Inhibition zone determined from a 6 mm paper disc on Tryptic Soy Agar (TSA). [3]Means followed by different letters in the same column indicate a significant difference ($p<0.05$)

The crude phenolic extract showed higher inhibitory effect against LM and ST as compared to other Pangi extracts in the present study. This is supported Rauha *et al*. (2000), who found that crude phenolic extract usually contains a substantial quantity of phenolic compounds such as phenolic acid derivates, flavonoids, tannins, essential oils, gallic acid and terpenoid that are effectively react as antimicrobial agent. Rodriguez-Vaquero *et al*. (2007) confirmed that phenolic compounds especially gallic acid is the most active against food-borne bacteria. The result also showed that Gram-positive bacteria (LM) were more susceptible as compared to Gram-negative bacteria (ST) with larger inhibition zone. This result may be explained by the fact that Gram-negative bacteria possess an outer layer surrounding the cell wall, which restricts diffusion of active compounds through its lipopolysaccharide covering.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC): The *in vitro* antimicrobial activity of Pangi seed extracts against the LM and ST was assessed quantitatively by broth dilution method and time-kill assay. From the two-fold dilution method, precise inhibitory concentration ranges were pre-determined in order to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the potent extracts. The inhibitory ranges of phenolic of acetone extract against LM and ST were found at 25.3-30.3 and 52.5-57.5 mg mL^{-1} respectively. On the other hand, higher concentration of free phenolic acid (75-250 mg mL^{-1}) and glycoside-bound phenolic acid (75-200 mg mL^{-1}) extracts were required to show inhibitory activity against the tested strains. Alkaloid extract inhibited the growth of LM and ST at concentration range of 37.5-42.5 and 75.0-100.0 mg mL^{-1}, respectively. From the results, phenolic extract seems to require lower concentration to inhibit both tested strains as determined by the micro dilution method.

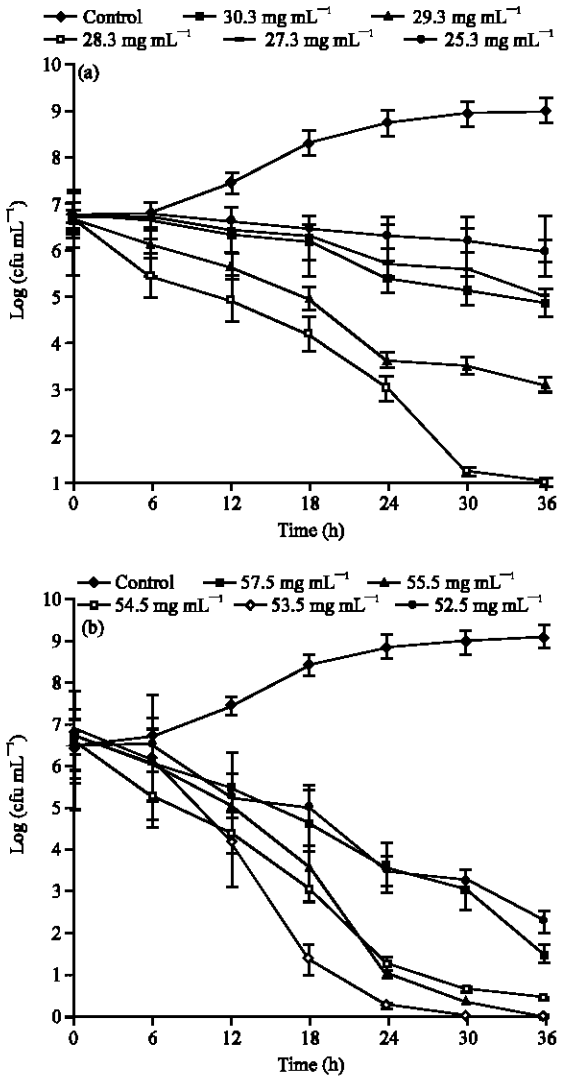

Fig. 4: Effect of phenolics of acetone extract on the growth of *Listeria monocytogenes* (a) and *Salmonella typhimurium* (b) at pre-selected concentrations

Further determination of the inhibitory concentration on phenolic extract by time-kill assay against both LM and ST was depicted in Fig. 4a and b. With the initial load of LM at 6.7 log cfu g^{-1}, all levels of the phenolic extract were able to reduce more than one log cfu of the tested strain after 24 h of exposure except at 25.3 mg mL^{-1} extract. According to Moody and Knapp (2004), the lowest concentration of an extract to reduce the bacterial load for one log (90% reduction) and three-log cycle (99.9% reduction) was defined as MIC and MBC, respectively. Therefore, the lowest concentration of phenolics required to exhibit inhibitory effect (MIC) against LM was 27.3 mg mL^{-1}, while the MBC was 29.3 mg mL^{-1} (Fig. 4a). The lethal effects of phenolic extract on ST were generally permanent at concentration above 52.5 mg mL^{-1} (Fig. 4b). The numbers of ST were reduced to non-detectable levels after 30 h of exposure to 57.5 mg mL^{-1} extract. An acute dropdown of the ST was found when treated with phenolic extract at 54.5 mg mL^{-1}, which may be due to leakage of membrane cell and eventually lead to cell death. This is supported by Lambert *et al.* (2001), who found that the phenolic compounds are able to induce permeability alteration in the microorganisms' membranes with a consequent leakage of protons, phosphates and potassium. Phenolic extract at all concentrations, inhibited the growth of ST for at least two log cfu and the lowest concentration (MIC) was at 52.5 mg mL^{-1}, with 2.3 log cfu reduction within 24 h of exposure.

In the time-kill assay, the alkaloid extract at various concentrations reduced the growth of LM and ST by 3 and 2 log cfu, respectively (Fig. 5a, b). The MICs for the alkaloid extract against LM and ST were 37.5±0.1 and 75.0±0.30 mg mL^{-1}, respectively, whereas the MBCs were 38.5±0.3 mg mL^{-1} and 80.00±0.2 mg mL^{-1}, respectively. Alkaloid is one of the most diverse groups of secondary metabolites found in living organisms with a wide array of biosynthetic pathways, structural types and even pharmacological activities (Roberts and Wink, 1998). They are part of the systemic chemical defence response in plants. Crude alkaloid extract may contain non-phenolic compounds such as quindine, indole, terpenoid, quinolizidine, dopamine and tropane, which were shown as good antimicrobial agents (Penna *et al.*, 2001). The LM does not possess specific receptor molecules or permeases to assist or block the antimicrobial agents' penetration effectively, thus the extracts are able to show bacteriostatic or bactericidal effect after a short period of exposure. At 40.5 mg mL^{-1}, the viable count of LM was not detected after 18 h of exposure to the alkaloid extract. On the other hand, the ST cell envelope had evolved to regulate the passage of substances into and out of the cell to a remarkable specificity and create barrier mechanisms against the phenolic compounds at the initial stage of action before they were inhibited (Cloete, 2003).

The decimal reduction time (DRT) of the Pangi seed extracts ranged from 6.0 to 17.7 h (Table 3). At the MIC level, the DRT were 17.7±0.5 and 11.1±0.5 h against LM and ST respectively. However, at the MBC of the same extract, the DRT values were much lower, 12.0±0.1 and 6.1±0.6 h respectively. This could be due to the ability of the extract to exhibit greater bactericidal effect (high concentration), thus it required significant shorter time ($p<0.05$) to reduce the growth for both tested bacteria as compared to phenolic of ethyl acetate extract. However, a

lower MIC or MBC values do not necessarily demonstrate a lower DRT as indicated in Table 3. Ben Arfa *et al.* (2006) discovered that the hydrophobicity of the phenolic compounds could be accumulated in the cell membrane, which induces conformational change of the membrane resulting in the death of the bacterial cell. Free phenolic acid extract was shown to have lower DRT values even it is considered as weak antimicrobial due to its MIC values (75.1±0.36 and 224.9±0.15 mg mL^{-1}) and MBC values (90.1±0.10 and 230.2±0.10 mg mL^{-1}) against LM and ST. This probably due to the diffusion of free phenolic acid compounds especially protocatechuic acid, vanillic acid, ferullic acid, ellagic acid and coumaric acid into the bacterial membrane easily and alter its ion stability leading to the inhibition of the bacterial growth within a shorter time before the bacteria can actually respond to them (Vattem *et al.*, 2004).

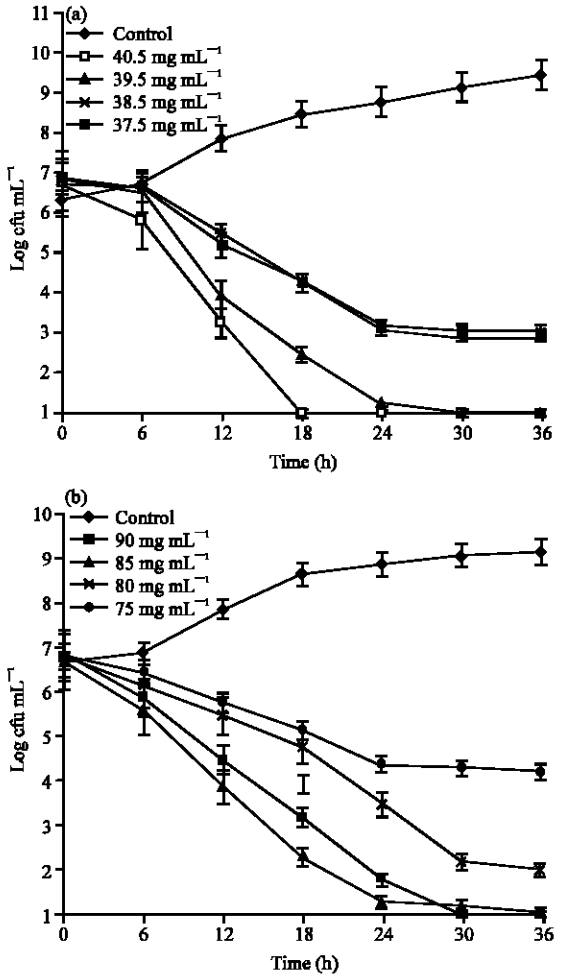

Fig. 5: Effect of alkaloid extract on the growth of *Listeria monocytogenes* (a) and *Salmonella typhimurium* (b) at pre-selected concentrations

Relationship between the phenolic content, antioxidative activities and antimicrobial activities of the Pangi seed extracts: Phenolic content of the Pangi seed extracts was significantly correlated ($p<0.05$) with the antioxidative activities, especially DPPH radical scavenging activity ($r = 0.878$) and the inhibition of β-carotene bleaching activity ($r = 0.849$) (Table 4). These results indicate that the phenolics contribute significantly to the antioxidative activity of the Pangi seed, which is in accordance with many studies worked on different plants and vegetables (Yu *et al.*, 2005; Tawaha *et al.*, 2007). However, a negative correlation ($p<0.05$, $r = -0.785$) was observed between the phenolic content and the DPPH radical scavenging activity for free phenolic acid extract. This inconsistent relationship between the phenolic content and antioxidative activity of the Free Phenolic Extract (FPA) could be due to the structure and interaction among the antioxidants found in the extract with the radical scavenging capability. The DPPH radical scavenging activity involves electron transfer mechanism between the phenolics and the DPPH radical. However, the mechanism occurs slowly in strongly hydrogen-bond solvents such as methanol and ethanol, thus it may affect the radical scavenging activity. On the other hand, the phenolic content of free phenolic acid extract showed positive relationship ($p<0.05$, $r = 0.872$) with the β-carotene bleaching activity. This might be due to the presence of

Table 3: Minimum Inhibition Concentration (MIC), Minimum Bactericidal Concentration (MBC) and decimal reduction time of various seed extracts against *Listeria monocytogenes* (LM) and *Salmonella typhimurium* (ST) determined from time-kill assay

	Inhibitory ranges (mg mL^{-1})		MIC		MBC	
Extracts	LM	ST	LM	ST	LM	ST
Phenolic (Ace)	25.3-30.3	52.5-57.5	27.3±0.1^{d} (17.7±0.5^{a})	52.5±0.1^{c} (11.1±0.5^{a})	29.3±0.1^{c} (12.0±0.1^{a})	55.5±0.1^{c} (6.1±0.6^{c})
Phenolic (EtOAc)	42.5-47.5	70.0-75.0	42.6±0.2^{b} (10.0±0.2^{d})	70.2±0.2^{d} (10.1±0.4^{c})	47.6±0.2^{e} (6.1±0.1^{d})	75.0±0.2^{e} (6.0±0.5^{d})
FPA	75.0-100.0	225.0-250.0	75.1±0.4^{a} (6.2±0.3^{a})	224.9±0.2^{a} (8.1±0.2^{c})	90.1±0.1^{b} (5.7±0.6^{c})	230.2±0.1^{b} (4.9±0.6^{c})
GBPA	75.0-100.0	175.0-200.0	75.1±0.3^{a} (12.1±0.2^{c})	174.9±0.3^{b} (10.6±0.2^{b})	100.2±0.3^{a} (10.0±0.2^{b})	190.1±0.3^{a} (7.2±0.1^{a})
Al	37.5-42.5	75.0-100.0	37.5±0.1^{c} (13.1±0.4^{d})	75.0±0.3^{c} (8.2±0.2^{d})	38.5±0.3^{d} (8.1±0.1^{c})	80.17±0.2^{d} (7.1±0.1^{b})

[1]Phenolic (Ace): Phenolic extracted by acetone, Phenolic (EtOAc): Phenolic extracted by ethyl acetate, FPA: Free phenolic acid, GBPA: Glycoside-bound phenolic acid, Al: Alkaloid, [2]Lower value of the range indicates less antibacterial activity. [3]Values in bracket are the Decimal reduction time for the respective extracts. [4]Means followed by different letters in the same column indicate a significant difference ($p<0.05$)

Table 4: Interrelationship (r values) between phenolic content, antioxidant and antibacterial activity of Pangi seed extracts

Correlation coefficient (r)	Antioxidant activity			Antimicrobial activity		
	TPC	DPPH	BCT	DIZ	MIC	MBC
Phenolic (Ace)						
TPC	-	0.878	0.849	0.84	-0.695	-0.515
DPPH	0.878	-	0.879	0.858	0.626	-0.896
BCT	0.849	0.879	-	0.439	-0.977	-0.956
DIZ	0.84	0.858	0.439	-	-0.863	-0.900
MIC	-0.695	0.626	-0.977	-0.863	-	0.985*
MBC	-0.515	-0.896	-0.956	-0.900	0.985*	-
Phenolic (EtOAc)						
TPC	-	0.768	0.809	0.808	-0.638	-0.407
DPPH	0.768	-	0.997	0.913	-0.988*	-0.974
BCT	0.809	0.997	-	0.878	-0.964	-0.630
DIZ	0.808	0.913	0.878	-	-0.839	-0.793
MIC	-0.638	-0.988*	-0.964	-0.839	-	0.965*
MBC	-0.407	-0.974	-0.630	-0.793	0.965*	-
FPA						
TPC	-0.785	-	0.872	0.832	-0.686	-0.981*
DPPH	0.972	-	0.641	-0.827	0.818	-0.926
BCT	0.832	0.641	-	0.965	-0.652	-0.587
DIZ	-0.686	-0.827	0.965	-	-0.893	-0.593
MIC	-0.981*	0.818	-0.652	-0.893	-	0.924
MBC		-0.926	-0.587	-0.593	0.924	-
GBPA						
TPC	0.819	0.819	0.776	0.652	-0.597	-0.957
DPPH	0.776	-	0.441	0.914	-0.946	-0.894
BCT	0.652	0.441	-	0.786	-0.665	-0.640
DIZ	-0.597	0.914	0.786	-	-0.828	-0.619
MIC	-0.957	-0.946	-0.665	-0.828	-	0.764
MBC	-	-0.894	-0.640	-0.619	0.764	-
Al						
TPC	-	0.880	0.501	0.728	-0.583	-0.888
DPPH	0.800	-	0.968	0.915	-0.989*	-0.948
BCT	0.501	0.968	-	0.808	-0.932	-0.996
DIZ	0.728	0.915	0.808	-	0.829	-0.367
MIC	-0.583	-0.989*	-	0.829	-	0.785
MBC	-0.888	-0.948	-0.932	-0.367	0.785	-

[1]Phenolic (Ace): Phenolic extracted by acetone, Phenolic (EtOAc): Phenolic extracted by ethyl acetate, FPA: Free phenolic acid, GBPA: Glycoside-bound phenolic acid extract, Al: Alkaloid extract, DPPH: DPPH radical scavenging activity, BCT: B-carotene bleaching activity, [b]DIZ: Diameter inhibition zone, MIC: Minimum inhibition concentration, MBC: Minimum bactericidal concentration. [2]All values are significant at $p<0.05$ unless otherwise stated at ($p<0.01$)

free phenolic acid that minimize the oxidation of β-carotene by hydroperoxides (free radical) in an emulsion phase during incubation.

Studies have shown that phenolic compounds found in plants, vegetables, spices and herbs possessed antioxidative and antimicrobial activities (Shan *et al*., 2007; Wu *et al*., 2006). Therefore, it has become an interest to understand the relationship between the phenolic content and its biological activity especially the antioxidative and antimicrobial activities. There was a strong correlation ($p<0.05$, $r = 0.840$) between the phenolics content and the antimicrobial activity (inhibition zone) of the Pangi seed extracts. The result of the present study is in accordance with the finding of Baydar *et al*. (2004), who discovered that the antibacterial activity of the grape (*Vitis vinisfera* L.) extracts was positively correlated with the phenolic content of the extracts. The inhibition zone was negatively correlated with MIC values but not with the MBC values except for phenolic extracts (both extracted by acetone and ethyl acetate), indicating that the higher the inhibition zone, the lower the concentration of Pangi seed extract required to exert bacteriostatic activity, but not necessarily for bactericidal activity. This finding is in agreement with Kim *et al*. (1995), who showed that most of the highly active compounds in essential oils with a large zone of inhibition would have low MIC and MBC values against selected bacteria. In contrast to the earlier findings by Cimanga *et al*. (2002), who found no correlation in between the amount of major constituent and the antibacterial activity. This suggests that the compounds present in the greatest proportion may not necessarily responsible for the total antibacterial activity. Thus, the synergistic involvement of the less abundant constituents should be considered.

The results showed that the antimicrobial activity (inhibition zone) of the extracts correlated strongly ($p<0.05$) with the DPPH activity ($r = 0.915$) and BCT activity ($r = 0.965$). This may be due to the availability of the antioxidative compounds to exert different inhibitory effect against LM and ST indicating that the antibacterial activity was closely related to the phenolics as well as the antioxidative activity of the extracts. These findings further support the study by Vattem *et al*. (2004), who found that the antimicrobial activity of cranberry pomace extracts were correlated strongly with both DPPH radical inhibition and the antioxidative protection ability. A similar relationship was also reported by Shan *et al*. (2007) on various dietary spices and medical herb extracts.

Current study demonstrates that phenolic and alkaloid extracts of pangi seed displayed good antioxidant and antibacterial activities. Therefore, Pangi seed extracts could be a promising source of natural preservatives for foodstuffs against foodborne pathogens as well as used as an ingredients for the pharmaceutical industry.

ACKNOWLEDGMENTS

The authors wish to thank Universiti Malaysia Sabah and Ministry of Higher Education Malaysia for their financial support on this project [B-0101-03-ER/U023]. We wish to thank Mr. John Sugau from the Forest Reserve Centre of Sabah for the assistance in authentication of the species. The provision of bacteria cultures from Institute for Medical Research, Malaysia is gratefully acknowledged.

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