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Bioactivity Screening of Thai Spice Extracts for Applying as Natural Food Preservatives

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Absract Herbs and spices have been used to enhance the flavour and aroma in Thai food for thousands of years and are also considered as a medicinal plant. This study was to investigate the antioxidant and antimicrobial activities of selected Thai spices (sweet basil, holy basil, finger root, kaffir lime leaf, black pepper, galangal, and lemongrass), using different extraction solvents, including aqueous and ethanol. The results of antimicrobial activity against four foodborne pathogens (*Escherichia coli*, *Salmonella Typhimurium*, *Staphylococcus aureus,* and *Listeria monocytogenes*), using agar disc diffusion and broth dilution assays, showed that the galangal ethanol extracts had the highest antimicrobial activity with the minimum inhibitory concentration and minimum bactericidal concentration of ≤12.5 and ≤50 mg/ml, respectively. In antioxidant activity, the sweet basil ethanol extracts showed the highest antioxidant activity in terms of total phenolic content (2.65 mg GAE/g), DPPH radical scavenging (90.30%) and thiobarbituric acid reactive substances. This study has demonstrated that galangal and sweet basil extracts could be used as natural antioxidant and antibacterial agents in food preservation with low side effects and could also promote human health.

Keywords: Thai spice extracts, solvent extraction, antimicrobial activity, antioxidant activity

Introduction

Consumers and the food industry must still maintain a high awareness of food poisoning due to the high and growing numbers of illness outbreaks. The treatment of this concern is mainly based on the use of artificial food preservatives (Shan *et al.*, 2007), such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and sodium benzoate which have some toxic and carcinogenic effects with continuous consumption. Therefore, consumers prefer using natural preservatives, such as the extract of plant materials which have no side effects on human health.

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Herbs and spices have been well known as a medicine and food additive since ancient time (Neilsen and Rios, 2000), and they are becoming more popular in recent years for extending shelf life of food products by preventing lipid oxidation and microbial spoilage (Shan *et al.*, 2007). Therefore, many researchers have confirmed that herb and spice extracts are rich in natural bioactive compounds called "phytochemicals" consisting of alkaloids, essential oils, phenolic compound, tannins, terpenoids, saponins, and many more (Zhao *et al*., 2014). These compounds play an important role in inhibition the oxidative mechanisms by free radical scavenging properties, chelating metal ions, and preventing radical formation (Al-Azzawie and Mohamed-Saiel, 2006); especially flavonoids have been proved to be more effective than vitamin C, E, and carotenoids (Dai and Mumper, 2010). In addition, phenolic compounds also have activity against pathogens (Negi, 2012). Furthermore, crude extracts from herbs and spices have been reported to be more effective than the purified individual components (Delaquis *et al*., 2002) possibly due to containing about 85% of several phytochemicals as the major bioactive compounds (Burt, 2004).

Thailand is located in a tropical area that has a wide variety of herbs and spices (Nugboon and Intarapichet, 2015) which are used as cooking ingredients and traditional medicines to treat some illnesses. Thus, there has been research conducted on antioxidant and antimicrobial activities of several herbs and spices grown domestically in Thailand which have demonstrated inhibitory efficiency against some microbial strains, such as *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella spp*. (Panutat and Vatanyoopaisarn, 2002). They also provide powerful antioxidants which can improve storage quality of meat products (Nugboon and Intarapichet, 2015).

The objective of this study was to determine the antimicrobial activities against selected foodborne pathogens and antioxidant activities of the crude extracts from Thai spices, including sweet basil (*Ocimum basilicum*), holy basil (*Ocimum sanctum*), finger root (*Boesenbergia rotunda*), kaffir lime leaf (*Citrus hystrix*), black pepper (*Piper nigrum*), galangal (*Alpinia galangal*), and lemongrass (*Cymbopogon citratus*), and also compare different solvent types in order to select solvents and crude extracts for further application in meat products.

Materials and methods

Spice extracts preparation

The extracts were prepared according to the methods of Weerakkody *et al*. (2010) with some modifications. Fresh Thai spices, including sweet basil,

holy basil, finger root, kaffir lime leaf, black pepper, galangal, and lemongrass were purchased from a local market (Phitsanuloke, Thailand), washed, cut and dried at 40 °C for 24 h, ground into a fine powder, and sieved (24-mesh). The spice powder was extracted with two different solvents (aqueous and 95% ethanol) by adding 10 g of spices to 100 ml of solvent and stirring for 24 h at ambient temperature. Mixtures were filtered under a vacuum through Whatman filter paper no 1 and centrifuged at 10,000 g for 10 min. The extraction process was repeated three times by using the residue after filtration and centrifugation. Solvents were evaporated under vacuum at 70 °C, using a rotary evaporator. The crude extracts were lyophilized and kept in sterilized cape vials at 4 °C until use.

Microbial strain

The antibacterial activities of all extracts were determined against four foodborne bacteria, including *Escherichia coli*, *Salmonella Typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes*; a single colony of each strain was cultivated in tryptic soy broth for 18 h at 37 ◦C. Finally, all bacteria were suspended in sterile water and diluted to approximately 10^6 CFU/ml (Shan *et al*., 2007).

Disc diffusion assay

The extract powder was re-suspended by using dimethyl sulphoxide (DMSO) at a concentration of 100 mg/ml before it was tested for antibacterial activity. The sterile paper discs (Whatman No. 1, 6 mm in diameter) were impregnated with 20 µl of each diluted extract and fully dried before being placed on the inoculated agar. Then, 100 µl of each suspension bacteria was spread on the surface of nutrient agar plates. All inoculated plates were incubated at 37 ◦C for 24 h. Microbial inhibition was evaluated by measuring the diameter (mm) of the clear zone around the discs.

Minimum inhibitory concentration and Minimum bactericidal concentration

Minimum inhibitory concentrations (MIC) were determined by a broth micro-dilution method as described by Zarai *et al.* (2013). A serial two-fold dilution was prepared in a 96 well plate with a concentration from 100 to 0 mg/ml with nutrient broth. Each well was inoculated with 10 µl of bacterial suspension and incubated at 37℃ for 24 h. The lowest concentrations of extracts, designated as MIC, were without a white pellet on the well bottom by visual reading.

Minimum bactericidal concentration (MBC) was determined by selecting the wells that did not have any turbidity on the MIC determination. First, 10 µl amounts of each well were taken and inoculated in Tryptone Soy agar (TSA) plates. Plates were incubated at 37^{o}C for 24 h, and then bacterial growth was evaluated. The agar plates with no grown colonies were expressed as MBC and recorded as mg/ml.

Total phenolic content

Total phenolic content was determined by using the Folin-Ciocalteu colourimetric method described by Ozsoy *et al*. (2008); 0.1 ml of each sample extract was mixed with 0.1 ml of Foline-Ciocalteu reagent and then allowed to oxidize for 3 min. The mixture was neutralized with 0.3 ml of 2% sodium carbonate (Na_2CO_3) solution. After incubation for 2 h at ambient temperature and in darkness, the absorbance of the resulting blue color was measured at 760 nm with a spectrophotometer. The total phenolic content was quantified using a standard curve of gallic acid, and results expressed as milligram of gallic acid equivalent (GAE) per gram of the dry sample (mg/g).

DPPH radical scavenging activity

The free radical scavenging activity of the extracts was quantitatively assessed using the DPPH radical method which was developed by Brand-Williams *et al*. (1995); 0.1 mM DPPH solution was mixed with 1.0 ml of each sample extract and incubated at room temperature in darkness for 30 min. The absorbance was measured using a UV–Vis spectrophotometer at 517 nm. DPPH radical scavenging activity was expressed as inhibition percentage calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{[(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})]}{(\text{Abs}_{\text{control}})]} \times 100$$

Where $\text{Abs}_{\text{control}}$ and $\text{Abs}_{\text{sample}}$ are the absorbance of the control and the sample extract, respectively.

Thiobarbituric acid reactive substances (TBARS)

TBARS assay was used to determine the reactions of peroxides using egg yolk homogenates according to Ruberto *et al.* (2000). Briefly, 0.5 ml of 10% egg yolk homogenate (prepared in distilled aqueous, v/v) and 0.1 ml of extract sample were mixed in a test tube and made up to 1.0 ml with distilled water. Thereafter, 1.5 ml of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5

ml of 0.8% thiobarbituric acid (TBA) (prepared in 1.1% sodium dodecyl sulphate) were added and vortexed thoroughly. The mixture was heated at 95 ℃ for 60 min and after cooling, 5.0 ml of butanol was added and centrifuged at 3,000 rpm for 10 min. The absorbance activity of the supernatant was measured using a UV–Vis spectrophotometer at 532 nm against the blank which used 0.1 ml of distilled aqueous to replace the extract sample.

Statistical analysis

All data were subjected to variance analyses and differences between means of Thai spice extracts were evaluated by Duncan's multiple range test, and means of solvent extraction were evaluated by t-test using Statistical Analysis System's Procedures (Version 9.1, SAS Institute Inc., Cary, NC) with a 5% level of significance.

Results

Antimicrobial activity

The mean diameters of the inhibition zones of all spice extracts against four food-borne pathogens demonstrated that the galangal extract showed a significantly ($p < 0.05$) higher inhibition against all bacteria tested than other spice extracts, and the ethanol extracts of galangal displayed strong antimicrobial activity with the highest inhibition zone diameter of 27.33 mm against *S. aureus*, followed by *E. coli* (12 mm), *S. typhimurium* (8.67 mm), and *L. monocytogenes* (8 mm) (Table 1). Similarly, the lemongrass and kaffir lime leaf ethanol extract showed a significantly higher inhibition against *S. aureus* and *E. coli* than the aqueous extract. The black pepper and holy basil extracts were observed only against *S. aureus*, an interesting result in that the aqueous extract generated a significantly larger inhibition zone diameter than ethanol extract ($p < 0.05$).

The spice extract concentrations ranged from 0-100 µg/ml against different bacterial strains, including *E. coli*, *S. typhimurium*, *S. aureus*, and *L. monocytogenes* (Table 2). The data demonstrated that MIC values for the spice extracts ranged from 3.12-50 mg/ml and 0.78-50 mg/ml for aqueous and ethanol extract, respectively. Ethanol extracts were more effective against bacteria tested, especially the galangal extract which showed the lowest MIC value in all bacteria whereas the highest MIC value, which showed weak inhibitory effects, was observed for aqueous extracts from lemongrass. Among the four foodborne pathogens, *S. aureus* was the most sensitive microorganism with a MIC value in the range of 0.08 mg/ml to 12.5 mg/ml, and *L.*

monocytogenes was the least sensitive bacterium to the spice extracts with a MIC value in the range of 12.5 mg/ml to 50 mg/ml.

Table 1. Antimicrobial activity of Thai spice extracts against several bacteria using disc diffusion method.

Bacterial	Solvent	Diameter of the zones of inhibition (mm)*						
		Sweet basil	Holy basil	Finger root	Kaffir lime leaf	Black pepper	Galangal	Lemon grass
E. coli	Ethanol	6^{C}	6^{C}	6^{C}	9^{B}	6^{C}	$12^{A,X}$	$9.33^{B,X}$
	Aqueous	6^{C}	6^{C}	6^{C}	8^{B}	6^{C}	$10^{A,B}$	$6^{C,Y}$
S. Typhimurium	Ethanol	6^{B}	6^{B}	6^{B}	6^{B}	6^{B}	$8.67^{A,X}$	6^{B}
	Aqueous	6^{A}	6^{A}	6^{A}	6^{A}	6^{A}	$6^{A,Y}$	6^{A}
S. aureus	Ethanol	6^{D}	8^{C}	6^{D}	12^{B}	$8^{C,Y}$	$27.33^{A,X}$	$8^{C,X}$
	Aqueous	6^{D}	7.33^{C}	6^{D}	9.33^{B}	$12^{A,X}$	$12^{A,Y}$	$6^{D,Y}$
L. monocytogenes	Ethanol	6^{B}	6^{B}	6^{B}	6^{B}	6^{B}	$8^{A,X}$	6^{B}
	Aqueous	6^{A}	6^{A}	6^{A}	6^{A}	6^{A}	$6^{A,Y}$	6^{A}

$^{X-Y}$ Means within the same column for the same spice extract and bacterial test with different superscripts are significantly different ($p < 0.05$).
$^{A–D}$Means within the same row for the same solvent and bacterial with different superscripts are significantly different ($p < 0.05$).
* The paper disc diameter is 6 mm; if the DIZ value is 6 mm, it means the spice extract had no activity against bacteria.

Table 2. Minimum inhibitory concentration of Thai spice extracts with aqueous and ethanol extraction against several bacteria.

Bacterial	Solvent	Minimum Inhibitory Concentration (mg/ml)						
		Sweet basil	Holy basil	Finger root	Kaffir lime leaf	Black pepper	Galangal	Lemon grass
E. coli	Ethanol	25	25	12.5	12.5	12.5	3.12	12.5
	Aqueous	25	25	25	12.5	25	25	25
S. Typhimurium	Ethanol	6.25	3.12	25	25	25	3.12	25
	Aqueous	25	12.5	25	25	25	25	50
S. aureus	Ethanol	6.25	6.25	3.12	3.12	0.78	0.08	0.78
	Aqueous	6.25	6.25	6.25	6.25	6.25	3.12	12.5
L. monocytogenes	Ethanol	12.5	12.5	12.5	50	50	12.5	50
	Aqueous	25	25	50	50	50	25	50

Results from the MBC assays demonstrate that the galangal ethanol extract had the strongest bactericidal effect among all of the spice extracts with MBC values ranging from 0.39-50 mg/ml. The MBC values for the galangal ethanol extract were 25 mg/ml, 6.25 mg/ml, 0.39 mg/ml, and 25 mg/ml for *E. coli*, *S. Typhimurium*, *S. aureus*, and *L. monocytogenes*, respectively (Table 3). These MBC values are consistent with the disc diffusion and MIC determination assay, which further confirmed that the galangal ethanol extract was the most potent to prevent foodborne bacteria.

Table 3. Minimum bactericidal concentration of Thai spice extracts with aqueous and ethanol extraction against several bacteria.

Bacterial	Solvent	Minimum Bactericidal Concentration (mg/ml)						
		Sweet basil	Holy basil	Finger root	Kaffir lime leaf	Black pepper	Galangal	Lemon grass
E. coli	Ethanol	100	50	50	50	50	25	50
	Aqueous	100	50	50	50	50	50	100
S. Typhimurium	Ethanol	25	12.5	50	50	50	6.25	50
	Aqueous	100	25	-	-	100	25	-
S. aureus	Ethanol	25	12.5	6.25	6.25	1.56	0.39	3.12
	Aqueous	50	50	50	12.5	12.5	25	100
L. monocytogenes	Ethanol	50	50	50	50	100	25	-
	Aqueous	50	50	-	-	100	50	-

- No antibacterial activity

Antioxidant activity

The results given in Figure 1 showed that the total phenolic contents of the aqueous and ethanol extracts of Thai spices ranged from 0.29 to 1.07 mg GAE/g and 0.72 to 2.65 mg GAE/g, respectively. The total phenolic content in the different spice ethanol extracts was in the ascending order of finger root, holy basil, kaffir lime leaf, black pepper, lemongrass, galangal and sweet basil with values of 0.72, 0.81, 0.85, 1.24, 1.75, 1.92 and 2.65 mg GAE/g, respectively. These results indicated that sweet basil ethanol extract exhibited the highest total phenolic content. Figure 2 showed that the ethanol extracts exhibited a DPPH scavenging activity of 71.80 to 90.30% whereas the aqueous extracts were found to be 33.82 to 56.52%. The sweet basil ethanol extract had the highest DPPH scavenging activity (90.30%).

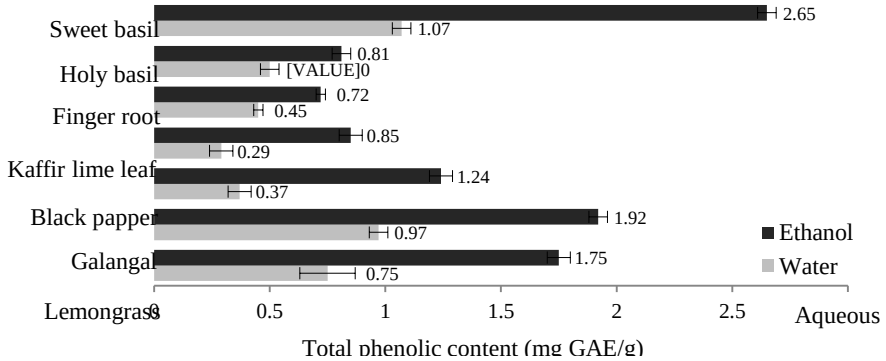

Figure 1. Total phenolic contents of Thai spice extracts with aqueous and ethanol extraction.

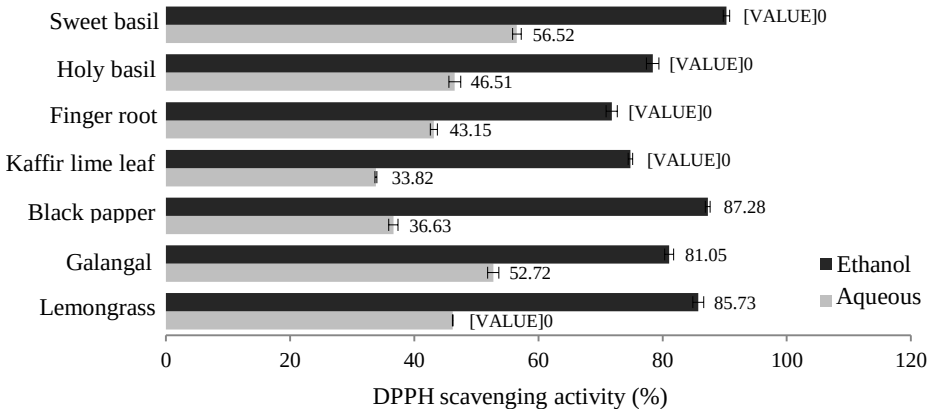

Figure 2. DPPH radical scavenging activity of Thai spice extracts with aqueous and ethanol extraction

The antioxidative activities of Thai spice extracts determined by the thiobarbituric acid method are shown in Figure 3. Results from this assay indicated that the antioxidant activities of ethanol extracts were higher than aqueous extracts. All the ethanol extracts were at 2.12, 2.38, 2.62, 2.73, 3.22, 3.42 and 3.73 mg MDA/Kg for sweet basil, galangal, black pepper, lemongrass, finger root, kaffir lime leaf and holy basil, respectively.

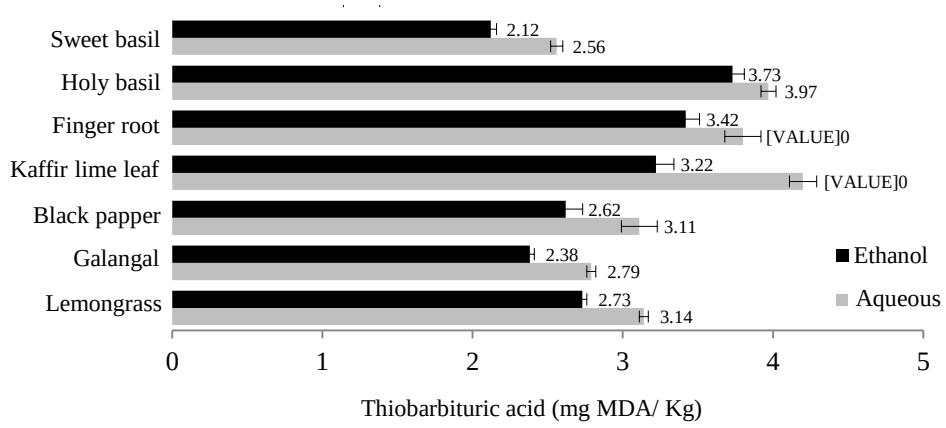

Figure 3. Thiobarbituric acid of Thai spice extracts with aqueous and ethanol extraction

Discussion

Our study showed that there were significant differences in the antimicrobial activity between the solvents. The ethanol extracts were more efficient antimicrobial agents than aqueous extracts. Dupont *et al*. (2006) found that for Australian herbs, ethanol and hexane extracts more effectively inhibited bacteria than the aqueous extracts. Many studies reported that the antimicrobial activity of herbs and spices extracts are related to the concentration of phenolic substances (Kim *et al.*, 1995; Shan *et al*. 2007), including the presence of tannins, saponins, essential oils, and flavonoids (Aboaba and Efuwape, 2001). In order to extract phytochemical compounds, Areias *et al.* (2001) compared different solvent extracts, including petroleum ether, chloroform, ethyl ether, ethyl acetate, acetone, ethanol, and methanol in fresh peppermint and found that ethanol produced significantly higher phenolic content. Shan *et al*. (2007) also reported that the extraction of rosemary was significantly higher in the amount of total phenols when using ethanol rather than hexane. Additionally, the ethanol extract also showed high anounts of other compounds, including terpenoids and alkaloids (John *et al*., 2015). This may be due to the polarity of the solvent, which is believed to be one of the major factors in the variability of quantity and phytochemical profiles of the herb extracts (Zarai *et al.*, 2013). It has been reported that aqueous solvent is effective in extracting some phytochemical compounds with strong polarity while the ethanol solvent is effective in extracting a broad range polarity of compounds (Sun *et al*., 2015). However, the selection of solvents for the extraction process should be made while considering interactions between efficiency, environment, and safety. Ethanol is widely used as an organic solvent by herbal medicine manufacturers because it is safe for consumers and environmentally friendly (Yi and Wetzstein, 2011). Additionally, the ethanol extract has shown excellent antimicrobial activity against both Gram-positive and Gram-negative bacteria. In the current study, *S. aureus* was the most sensitive to the extracts and was in agreement with several studies that indicated that Gram-positive bacteria were more sensitive than Gram-negative ones towards the plant extracts studied. Zarai *et al.* (2013) studied the antibacterial effects of black pepper extracts and also indicated that *S. aureus* and *B. subtilus* (Gram-positive) were the most sensitive bacteria with an MIC value of 156.25 mg/ml, whereas the highest MIC value (1,250 mg/ml) showed in *E. coli* and *K. pneumonia* (Gram negative). The difference of antimicrobial resistance between Gram-positive and Gram-negative bacteria can be explained by the fact that the cell membrane of Gram positive bacteria is a single layer which is sensitive to the extracts, whereas the Gram-negative cell wall is a multilayer structure (Yao and

Moellering, 1995) which acts as an important barrier to the entry of antibiotic molecules, and the periplasmic space allows the production of β-lactamases enzymes which are able to destroy exogenous molecules (Munita and Arias, 2016).

However, it is interesting to note that the crude extracts of plants showed good activity against antibiotic resistant strains (Khan *et al*., 2009) due to the fact that crude extracts have a large number of different bioactive compounds and these compounds are found to possess multiple functions for antibacterial activity, such as degradation of the cell wall, damage to cytoplasmic membrane and membrane proteins, leakage of intracellular contents, coagulation of cytoplasm, and reduction in the proton motive force (Burt, 2004).

For the spice extracts evaluated in the current study, galangal displayed strong antimicrobial activity against all tested strains. This is in general agreement with previous reports. Oonmetta-Aree *et al*. (2006) compared the antimicrobial effect of the Zingiberaceae genus (galangal, ginger, turmeric, and krachai) and reported that the ethanol extracts of galangal exhibit the strongest inhibitory effect against *S. aureus* with MIC and MBC values of 0.325 and 1.3 mg/ml, respectively. In our study, the ethanol extract of galangal showed 0.08 and 0.39 mg/ml for MIC and MBC values, respectively. However, several previous studies have also reported that the galangal extract had strongest antimicrobial activity against *S. aureus* (Mayachiew and Devahastin, 2008). This antimicrobial activity is related to their chemical compounds; galangal ethanol extracts produced lipophilic compounds that demonstrated high antibacterial activity (Natta *et al*., 2008). Youssef *et al*. (2015) found that the ethanol extracts of galangal contain flavonoids, alkaloids, terpenoids, glycosides, coumarins, and tannins while alkaloids and tannins were absented in their aqueous extract. However, the ability to inhibit bacteria growth might not be due to only one active compound, but to the combined action of different compounds originally in the plant (Sunayana, 2003).

It was observed that the antioxidant activity of the extracts was positively correlated with their total phenolic contents (Aksoy *et al*., 2013; Juliani and Simon, 2002). The phenolic compounds are secondary metabolites derived from plants, and they have miscellaneous antioxidant properties, including proton loss, chelate formation, and dismutation of radicals. The same correlation was also observed between total phenolics content and the DPPH free radical scavenging effect (Aksoy *et al*., 2013). In this study, the results of analysis of total phenolic content and DPPH scavenging activity indicated that the ethanol extract of sweet basil had the highest antioxidant activity. Among the basil cultivars, the purple basils exhibited a higher total phenolic than the green cultivars (Juliani and Simon, 2002). This is in agreement with the

chemical composition of sweet basil extract, which consists of linalool (36-69%) as the dominant component followed by eugenol (13.66-18.20%) (Gurbuz *et al*., 2006; Juliani and Simon, 2002; Keita *et al*., 2001; Prideevech *et al.*, 2010). It was reported that the strong antioxidant of sweet basil is in accordance with the high level of linalool and eugenol (Jiliani and Simon, 2002). Moreover, eucalyptol, methyl eugenol, and methyl chavicol (estragole) were also detected as the main components in Thai sweet basil leaf oil (Lawtrakul *et al*., 2014). In this study, galangal and lemongrass possessed a similar total phenolic content with a previous study by Tangkanakul *et al.* (2009), who reported that Thai galangal and lemongrass provided a total phenolic content of 2.17 and 1.53 mg GAE/g, respectively. This variation might be due to genetic factors, location, and seasonal conditions (Burt, 2004; Grayer *et al.*, 2001; Prideevech *et al*., 2010). From the antioxidant activities using different solvents in this study, it can be concluded that the ethanol extract of the seven Thai spices possessed the highest antioxidant activity in-vitro as seen in total phenolic content, DPPH scavenging activity, and Thiobarbituric acid assay. This result was in line with previous study which reported that the ethanol extract of herbs and spices had higher antioxidant activities than the aqueous extract (Do *et al*., 2014; Zarai *et al*., 2013).

Conclusion

The antimicrobial and antioxidant activities of seven Thai spices, including sweet basil, holy basil, finger root, kaffir lime leaf, black pepper, galangal, and lemongrass were evaluated. The results from these in vitro tests demonstrated that the ethanol extract of galangal exhibits the highest antibacterial activity against all the bacteria tested, especially S. aureus. It was also found to possess antioxidant activities with high total phenolic content. However, sweet basil ethanol extract exhibited the highest antioxidant activity and free radical scavenging activities. These results provide data that support the use of galangal and sweet basil extracts as a natural food preservative, which might release antimicrobial and antioxidant agents to helpfully extend the food shelf life.

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