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Antimicrobial Activity of Thai Herb Extracts Against Coconut Milk Spoilage Microorganisms

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ABSTRACT

Twenty-five Thai herb extracts were determined for their antimicrobial activities against 11 coconut milk spoilage microorganisms by an agar-well diffusion method. The results showed *Piper betle* (Betle vine) could inhibit all strains of the test bacteria. However, *Phyllanthus emblica* (Malacca tree), *Senna siamea* (cassod tree) and *Punica granatum* (pomegranate) exhibited greater significant ($P \leq 0.05$) antimicrobial activity when compared with other herb extracts, with the zone of inhibition ranging from 12.33±0.58 to 25.00±1.73 mm. The ethanol extracts of the three herbs (Malacca tree, cassod tree, pomegranate) were the most efficient antimicrobial compounds. The values of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the herb extracts were 0.3-2.4, >3 and 1.2-2.4% (w/v), respectively.

Key words: antimicrobial activity, Thai herbs, coconut milk, spoilage microorganism, agar-well diffusion.

INTRODUCTION

Coconut milk is the liquid obtained by manual or mechanical extraction of the coconut endosperm. It is widely used in food products of South East Asia countries including Indonesia, Malaysia, Philippines and Thailand. Fresh coconut milk is prone to rapid microbiological spoilage because it supports the growth of microorganisms (Seow and Gwee 1997; Simuang *et al.,* 2004). Therefore, synthetic additives have been used in the food industry to inhibit microbial growth but the consumer concern about the harm associated with synthetic additives. Natural additives from herbs are interesting to be used in food products. Consequently, search for natural additives has notably increased in recent years. Several researchers have reported a potential of spice and herb extracts of Australian native herbs, Turkish spices, Indian medicinal plants, Finnish plants and south-indian spices as an antimicrobial agents to prevent food product (Rauha *et al.,* 2000; Ahmad and Beg, 2001; Sağd1ç and Özcan, 2003; Dupont *et al*., 2006; Indu *et al.,* 2006).

In Thailand, there are many herbs and spices that exhibit antimicrobial activity. Previous studies showed the ethanol extracts of galangal exhibited the strongest inhibitory effect against *Staphylococcus aureus* as same as medicinal plants tthe heptaro oil and sweet flag the (Phongpaichit *et al.,* 2005; Oonmetta-aree *et al.,* 2006; Phongpaichit *et al.,* 2007). The objective of this study was to screen for antimicrobial activity of Thai herb extracts against coconut milk spoilage microorganisms. Effect of solvent types on the growth inhibition of the extracts was also investigated.

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MATERIALS AND METHODS

Plant materials

Twenty five Thai herbs listed in Table 1 were purchased from a local market and drugstore in Bangkok, Thailand. Betel vine leave were washed with tap water, cut into small pieces and dried in incubator at 50°C for 36 h. Then, the material was pulverized into fine powder. Other Thai herbs were purchased in powder. All powder materials were stored at 4°C.

Microorganisms

Spoilage microorganisms were isolated from coconut milk by our laboratory and identified by laboratory of the National Institute of Health, Department of Medical Science, Bangkok, Thailand as *Bacillus licheniformis* KUB1, KUB2, KUB3, KUB4 and KUB5; *Klebsiella pneumoniae* KUK1 and KUK2; *Enterobacter cloacae*; *Trichosporon mucoides*; *Candida lusitaniae* and *C. tropicalis*. Bacteria and yeast were grown in nutrients broth (NB) at 37°C and in yeast malt extract (YM) medium at 30°C for 24 h, respectively. The stock cultures of microorganisms were maintained at 4°C until used.

Preparation of crude extracts

Each herb powder (10 g) was individually extracted with 100 ml of 95% ethanol (1:10 w/v)

Table 1 List of Thai herbs used in the experiment.

Scientific name	Common name	Plant part
Syzygium aromaticum Linn.	Clove	Stem
Piper betle Linn.	Betle Vine	Leaf
Curcuma longa Linn.	Turmeric	Tuber
Punica granatum Linn.	Pomegranate	Fruit Peel
Garcinia mangostana Linn.	Mangosteen	Fruit Peel
Andrographis paniculata (Burrn. f.) Nees.	The creat	Stem, Leaf, Flower
Senna alata (Linn.) Roxb	Seven Golden Candle stick	Seed
Boesenbergia pandurata Holtt.	Kachai	Tuber
Cassia angustifolia Vahl	Senna	Leaf
Cinnamomum zeylanicum	Cinnamon	Bark
Caesalpinia sappan Linn.	Sappan Tree	Core
Curcuma xanthorrhiza Roxb	Wan-chak-mot-luk	Tuber
Carthamus tinctorius Linn.	Saffron	Flower
Derris scandens Benth	Fabaceae	Vine
Cyperus rotundus Linn.	Nutgrass	Tuber
Acanthus ebracteatus Vahl	Sea holly	Stem, Leaf
Tinospora crispa (L.) Miersex Hook. F and Thoms	Boraphet	Vine
Eclipta prostate	Trailing Eclipta	Stem, Leaf, Flower
Phyllanthus emblica Linn.	Malacca Tree	Fruit
Azadirachta indica A. Juss	Neem Tree	Leaf
Morinda citrifolia	Noni Indian mulberry	Fruit
Senna siamea (Lam) lrwin et Barneby	Cassod Tree	Core
Morus alba Linn.	Mulberry Tree	Leaf
Citrus aurantifolia	Lime	Fruit Peel
Piper retrofractum Vahl	Java long Pepper	Flower

at 60°C for 24 h. The extract solution was filtered using Whatman filter paper (No. 4). The solvent was removed from the sample by using a rotary vacuum evaporator (Büchi Rotavapor, R200, Switzerland). The sample was rotary vacuum evaporated at 40°C, with until it reached $^1/_4$ its volume. Distilled water (10 ml) was added to the sample and the content was continuously rotary vacuum evaporated until it reached 10 ml yielding concentration of 100 mg of dried plant material/ml and kept at 4°C until used (Jaturapronchai, 2003).

Screening for antimicrobial activity

Single colony of the test bacteria and yeast were transferred into NB and YM medium, and the cultures were incubated overnight at 37° C and 30°C, respectively. Each culture (250 µl) with a cell concentration of approximately 10^8 CFU/ml for bacteria and yeast was mixed with 25 ml of melt nutrient agar/YM agar medium at about 45°C and poured onto sterile Petri-dishes. Wells (8 mm-diameter) were punched out of the solid agar using a sterile cork borer. The crude extracts (50 µl) were introduced into the wells. Ethanol (95%) was used as a control (Fazeli *et al*., 2007). The plates were incubated at 37°C and 30°C for bacteria and yeast, respectively, for 24 h. The diameters of the inhibition zones were measured in millimeters (mm). Each experiment was repeated in triplicate.

Effect of solvent types on antimicrobial activity

Various solvents (water, methanol, ethyl acetate, chloroform and hexane) were used for the extraction of each Thai herb. Extraction and antimicrobial activity assays were carried out in the same manner as described above.

Determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

The crude extracts were prepared at concentrations of 3, 2.4, 1.2, 0.6, 0.3, 0.15, 0.075 and 0.0375% (w/v) in distilled water. The MIC and MBC were determined by a broth dilution method (Davidson and Parish, 1989). Nutrient broth samples (10 ml) were inoculated with different concentrations of the crude extracts and with 100 µl of active inoculums of microorganisms (approximately 10^8 CFU/ml) for 24 h at 37°C for bacteria and at 30°C for yeast. The viable plate counts were determined by spreading a 0.1 ml sample of each treatment on the surface of NA for bacteria and on YM agar for yeast and the colonies were counted after incubation. The MIC was defined as the minimum level of the extract that produced a 90% reduction in growth of the test microorganism. The MBC/MFC was the lowest concentration that killed at least 99.9% of the initial inoculum (Ponce *et al*., 2003).

RESULTS AND DISCUSION

Screening for antimicrobial activity

The antimicrobial activities of the Thai herb extracts by an agar-diffusion method are presented in Table 2. The results indicated that the extracts from the plants studied showed varying degrees of growth inhibition of the test microorganisms. Of the 25 plants, crude ethanol extracts of three, Malacca tree (*Phyllanthus emblica* Linn.), cassod tree (*Senna siamea* (Lam) Irwin et Barneby) and Sappan tree (*Caesalpinia sappan* Linn.) exhibited an inhibitory effect against all test bacterial strains except *Enterobacter cloacae*. The Malacca tree, cassod tree and pomegranate (*Punica granatum* Linn.) showed high antimicrobial activity against *Bacillus licheniformis,* with zones of inhibition ranging from 12.33±0.58 to 25.00±1.73 mm. Moreover, the ethanol extracts could inhibit *Trichosporon mucoides* with zones of inhibition ranging from 16.17±0.29 to 17.50±0.50 mm. A comparison between the antibacterial activity of Gram-

Table 2 Antimicrobial activity of ethanol extracts from Thai herbs against spoilage microorganisms using an agar-well diffusion method.

Herbs	Zone of Inhibition (mm)										
	Bacteria									Yeast	
	Bacillus licheniformis KUB1	*Bacillus licheniformis* KUB2	*Bacillus licheniformis* KUB3	*Bacillus licheniformis* KUB4	*Bacillus licheniformis* KUB5	*Klebsiella pneumoniae* KUK1	*Klebsiella pneumoniae* KUK2	*Enterobacter cloacae*	*Trichosporon mucoides*	*Candida lusitaniae*	*Candida tropicalis*
Betle Vine	11.33 ± 0.58a	14.33 ± 1.15bcd	20.00 ± 1.00bc	14.17 ± 0.29bc	20.00 ± 0.00d	12.67 ± 0.58b	15.33 ± 4.16b	13.83 ± 0.03	14.67 ± 1.15c	-	-
Malacca Tree	12.33 ± 0.58ab	14.00 ± 1.73bcd	21.00 ± 2.00cd	16.67 ± 2.52de	21.00 ± 1.73de	16.00 ± 0.00c	14.00 ± 0.00b	-	17.50 ± 0.50e	-	-
Neem Tree	-[a]	-	22.67 ± 1.53de	-	12.00 ± 0.00a	-	-	-	-	-	-
Lime	-	-	14.00 ± 0.00a	11.00 ± 0.00a	13.33 ± 0.58ab	-	-	-	11.17 ± 0.29a	-	-
Cassod Tree	23.00 ± 1.00f	22.67 ± 0.58e	25.00 ± 1.00e	16.67 ± 0.58de	23.00 ± 1.00ef	18.33 ± 0.58d	23.00 ± 0.00c	-	16.17 ± 0.29d	-	-
Pomegranate	20.00 ± 0.00e	16.33 ± 2.89d	25.00 ± 1.73e	15.67 ± 1.53cd	22.33 ± 2.08def	-	-	-	16.33 ± 0.58d	-	-
Mulbeery Tree	-	-	18.00 ± 1.00b	-	12.00 ± 0.00a	-	-	-	-	-	-
Noni Indian mulbeery	-	11.33 ± 1.15a	19.67 ± 1.53bc	-	15.33 ± 0.58bc	-	-	-	13.00 ± 0.00b	-	-
Clove	14.67 ± 1.53bc	16.00 ± 0.00cd	14.33 ± 0.58a	10.67 ± 1.15a	-	-	13.00 ± 1.73ab	-	14.33 ± 0.58c	-	-
Sappan Tree	16.00 ± 1.00cd	15.67 ± 0.58cd	20.67 ± 2.08cd	15.67 ± 1.53cd	22.33 ± 2.52def	11.00 ± 0.00a	16.33 ± 2.87b	-	12.17 ± 1.04b	-	-
Kachai	-	-	-	-	-	-	-	-	-	-	-
Wan-Chak-Mot-Luk	-	-	-	-	-	-	-	-	-	-	-
Java long Pepper	-	-	-	-	17.00 ± 3.46c	-	-	-	-	-	-
Saffron	-	-	-	-	25.00 ± 0.00f	-	-	-	-	-	-
Cassia angustifolia Vahl	13.33 ± 1.15ab	14.33 ± 0.58bcd	-	18.33 ± 0.58e	21.33 ± 2.89de	-	16.33 ± 1.53b	-	16.67 ± 0.29de	-	-
Derris scandens Benth	-	-	-	-	-	-	-	-	10.67 ± 0.29a	-	-
Mangosteen	16.33 ± 0.58cd	-	-	13.33 ± 0.58b	15.00 ± 0.00bc	-	13.00 ± 0.00ab	-	-	-	-
Cinnamon	17.67 ± 3.21d	13.67 ± 0.58bc	-	-	11.67 ± 0.58a	-	10.00 ± 0.00a	-	-	-	-
Nutgrass	13.33 ± 0.58ab	12.00 ± 1.00ab	-	-	25.00 ± 0.00f	-	-	-	10.83 ± 0.29a	-	-
Sea holly	-	-	-	-	-	-	-	-	15.67 ± 0.58d	-	-
Turmeric	-	-	-	-	-	-	-	-	-	-	-
Seven Golden Candle stick	-	-	20.00 ± 0.00bc	-	-	-	-	-	10.67 ± 0.29a	-	-
Boraphet	-	-	-	-	-	-	-	-	-	-	-
Trailing Eclipta	-	-	-	-	-	-	-	-	-	-	-
The creat	-	-	-	-	-	-	-	-	-	-	-

[a] - = not detected (diameter of wells was 8 mm).

Values expressed are mean ± SD of three experiments.

Mean values with a different letter in a column are significantly different ($P \leq 0.05$).

negative and Gram-positive bacteria indicated that the Gram-positive bacteria were more sensitive to many of the medicinal plants. This may be related to a difference in the structure of their cell wall. Gram-positive bacteria do not have an outer membrane and their cell walls are made up of twenty times as much peptidoglycan than the walls of Gram-negative bacteria. In addition, antibacterial substances can easily destroy the bacterial cell wall and cytoplasmic membrane resulting in a leakage of the cytoplasm (Shan *et al.,* 2007). Based on these results, the Malacca tree, cassod tree and pomegranate species were selected for further study.

Effect of solvent types on antimicrobial activity

Table 3 shows the effect of the extracts of the selected herbs (cassod tree, Malacca tree and pomegranate) using various solvent types on the inhibition of coconut milk spoilage microorganisms. Ethanol extracts of the herbs showed higher antimicrobial activity than water extracts, methanol and ethyl acetate extracts, while chloroform and hexane extracts could not inhibit all test microorganisms. This was probably due to phenolic compounds in the plants that had medium hydrophilic properties, which easily dissolved in a polar solvent (Zang and Liu, 2007). Similarly, the ethanol extracts from the artichoke (*Cynara scolymus* L.) leaf had higher antimicrobial activity against 15 microbial species (7 food-borne bacterial pathogens, 4 yeasts and 4 molds) (Zhu *et al.,* 2005). The methanol and water extracts from starfish (*Asterina pectinifera*) were found to be the most active against *Aspergillus* spp. and *Cryptococcus reoformans* (Choi *et al.,* 1999). Furthermore, the ethyl acetate and water extracts were the most efficient antimicrobial compounds (Nostro *et al.,* 2000; Springfield *et al.,* 2003).

Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

Table 4 shows the MIC and MBC/MFC of selected herb (cassod tree, Malacca tree and pomegranate) extracts on the inhibition of nine test strains. There is a wide range of MIC and MBC/MFC values that are dependent on the different microbial strains. An ethanol extract of the cassod tree showed the highest antimicrobial activity with an MIC of 0.3-1.2% (w/v). The MIC of the Malacca tree and pomegranate ranged from 1.2 to 2.4% (w/v). The results for the MBC values were similar to the MIC values, except for *B. licheniformis* KUB3. In another study, garlic extract and nutmeg extract at 25% (v/v) concentration could inhibit *Salmonella* spp. (Indu *et al.,* 2006). Ponce *et al.* (2003) reported that a clove oil concentration of 0.049 ml/100 ml was needed to inhibit the growth of native microflora of organic Swiss chard. The essential oil of clove at 0.125 and 0.25% could inhibit *V. parahaemolyticus* and *E. coli* ATCC 25158 (Moreira *et al.,* 2005; Yano *et al.,* 2006) and galangal extract of 0.325 mg/ml showed an inhibitory effect against *S. aureus* (Oonmetta-aree *et al.,* 2006).

CONCLUSION

The study results suggested that some Thai herbs have potential to act as natural antimicrobial agents for food preservatives and to prevent the growth of coconut milk spoilage microorganisms. The extracts may be further explored to isolate and characterize the active compounds to provide a new food preservative agent in the near future.

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Table 3 Effect of solvent types used in Thai herbs extraction against spoilage microorganisms using an agar-well diffusion method.

Solvent	Zone of inhibition (mm)								
	Bacillus licheniformis KUB1	*Bacillus licheniformis* KUB2	*Bacillus licheniformis* KUB3	*Bacillus licheniformis* KUB4	*Bacillus licheniformis* KUB5	*Klebsiella pneumoniae* KUK1	*Klebsiella pneumoniae* KUK2	*Enterobacter cloacae*	*Trichosporon mucoides*
Cassod Tree									
Ethanol	23.00 ± 1.00b	22.67 ± 0.58d	25.00 ± 1.00d	16.67 ± 0.58d	23.00 ± 1.00c	18.33 ± 0.58	23.00 ± 0.00c	-	16.17 ± 0.29c
Water	12.83 ± 1.15a	11.83 ± 0.58a	12.00 ± 0.00b	12.00 ± 0.00b	16.33 ± 0.58b	-	-	-	11.33 ± 0.58a
Methanol	24.00 ± 0.87b	21.33 ± 0.29c	15.67 ± 0.29c	15.83 ± 0.29c	-	-	15.50 ± 0.00b	12.33 ± 0.29	14.50 ± 0.50b
Ethyl acetate	13.33 ± 0.58a	16.17 ± 0.29b	11.00 ± 0.00a	10.83 ± 0.29a	10.50 ± 0.50a	-	11.17 ± 0.29a	-	-
Chloroform	-[a]	-	-	-	-	-	-	-	-
Hexane	-	-	-	-	-	-	-	-	-
Pomegranate									
Ethanol	20.00 ± 0.00c	16.33 ± 2.89a	25.00 ± 1.73c	15.67 ± 1.53a	22.33 ± 2.08c	-	-	-	16.33 ± 0.58b
Water	18.50 ± 0.50b	19.00 ± 0.00ab	17.33 ± 0.29b	16.33 ± 0.58a	18.00 ± 0.00b	-	20.83 ± 0.29b	-	14.17 ± 0.28a
Methanol	17.67 ± 0.29a	19.83 ± 0.29b	14.67 ± 0.29a	15.33 ± 0.58a	14.50 ± 0.50a	-	15.00 ± 0.00a	-	14.00 ± 0.00a
Ethyl acetate	-	-	-	-	-	-	-	-	-
Chloroform	-	-	-	-	-	-	-	-	-
Hexane	-	-	-	-	-	-	-	-	-
Malacca Tree									
Ethanol	12.33 ± 0.58a	14.00 ± 1.73a	21.00 ± 2.00c	16.67 ± 2.52b	21.00 ± 1.73c	16.00 ± 0.00b	14.00 ± 0.00b	-	17.50 ± 0.50b
Water	17.00 ± 0.00c	17.67 ± 0.58b	16.83 ± 0.29b	15.00 ± 0.00b	16.00 ± 1.00b	-	-	-	15.83 ± 2.02b
Methanol	15.33 ± 0.29b	17.50 ± 0.87b	16.50 ± 0.00b	16.00 ± 0.00b	15.00 ± 0.50b	11.00 ± 0.00a	15.00 ± 0.00c	11.50 ± 0.50	16.50 ± 0.50b
Ethyl acetate	-	-	11.00 ± 0.00a	11.17 ± 0.29a	10.00 ± 0.00a	-	10.83 ± 0.58a	-	12.17 ± 0.29a
Chloroform	-	-	-	-	-	-	-	-	-
Hexane	-	-	-	-	-	-	-	-	-

[a] - : not detected (diameter of wells were 8 mm).

Values expressed are mean ± SD of three experiments.

Mean values with different letter in a column are significantly different ($P \leq 0.05$).

Table 4 Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of ethanol extracts from Thai herbs against spoilage microorganisms.

Microorganisms	Parameters (% w/v)	Herb extracts		
		Malacca Tree	Pomegranate	Cassod Tree
Bacteria				
Bacillus licheniformis KUB1	MIC	1.2	1.2	0.6
	MBC	1.2	1.2	0.6
Bacillus licheniformis KUB2	MIC	2.4	2.4	0.3
	MBC	2.4	2.4	0.3
Bacillus licheniformis KUB3	MIC	2.4	2.4	1.2
	MBC	>3	>3	3
Bacillus licheniformis KUB4	MIC	1.2	2.4	0.6
	MBC	3	2.4	0.6
Bacillus licheniformis KUB5	MIC	1.2	2.4	0.6
	MBC	1.2	2.4	0.6
Klebsiella pneumoniae KUK1	MIC	2.4	-[a]	-
	MBC	2.4	-	-
Klebsiella pneumoniae KUK2	MIC	2.4	-	0.6
	MBC	2.4	-	0.6
Enterobacter cloacae	MIC	2.4	-	-
	MBC	2.4	-	-
Yeast				
Trichosporon mucoides	MIC	1.2	2.4	1.2
	MFC	1.2	2.4	1.2

[a] - = not detected.

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