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Communication

Antilisterial effects of ethanolic extracts of some edible Thai plants on refrigerated cooked pork

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Abstract: *Listeria monocytogenes* is a major foodborne pathogen responsible for the disease listeriosis. Effective methods for reducing *L. monocytogenes* in foods would reduce the likelihood of foodborne outbreaks of listeriosis and decrease economic losses to the food industry. Crude ethanolic extracts from 50 edible Thai plants were screened for inhibitory effects on isolated strains and type strains of *L. monocytogenes* by the well assay technique. Ethanolic extracts of *Micromelum minutum*, *Artocarpus heterophyllus*, *Piper retrofractum* and *Cucurbita moschata*, which showed listerial growth inhibition, were applied to cooked pork to determine their antimicrobial activities against *L. monocytogenes*. Pork was cooked to an internal temperature of 85°C, allowed to cool to 8°C and then treated by surface application with the plant extracts. Low (10^2 cfu g^{-1}) or high (10^5 cfu g^{-1}) population of *L. monocytogenes* were applied and samples were stored at 4°C for up to 7 days. *M. minutum* and *A. heterophyllus* extracts were most effective in inhibiting the growth of the pathogen. These results suggested that some edible Thai plant extracts might be useful as antimicrobials in cooked, ready-to-eat pork.

Keywords: antilisterial activity, edible Thai plants, *Listeria Monocytogenes*, *Micromelum minutum*, *Artocarpus heterophyllus*

INTRODUCTION

Listeria monocytogenes is a gram-positive foodborne pathogen which has been involved in several outbreaks in the past two decades. This psychrotrophic bacterium is able to grow at a wide temperature range from 2.5°C to 44°C [1] and is widespread. It has been isolated from various foods including poultry, meat, milk, milk products, seafoods and vegetables. The bacterium causes listeriosis, a severe foodborne illness with a mortality rate as high as 30-40% [2].

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Refrigerated, ready-to-eat foods have become increasingly popular in recent years because of their convenience. Unfortunately, this type of food may have a potential microbiological safety problem. Most ready-to-eat foods receive little or no final heat treatment before being consumed because they are assumed to be, and are often labelled as, fully cooked. The addition of antimicrobial ingredients in combination with refrigeration may compensate for the lack of a terminal heating step and provide protection in addition to low temperature alone. However, consumers are sometimes suspicious of food additives and prefer additive-free foods. Thus, the challenge of ensuring the safety of refrigerated, ready-to-eat foods and at the same time satisfying consumer preference is an important issue for the food industry.

Herbs and spices are widely used components in food preparation and are classified as GRAS (generally recognised as safe) products. The use of such naturally occurring plant products may provide an additional barrier to the growth of foodborne pathogens as well as satisty both the consumers and the regulatory agencies. Testing of natural products for their antilisterial properties and their potential use as natural preservatives in food has been performed [3-6]. In Thailand, many plants are utilised as food and medicine. Some of these plants have been demonstrated to exhibit antilisterial activity [7]. However, most studies on the antilisterial activity of plant extracts have been conducted in vitro—little information exists regarding the practical use of such antimicrobial extracts in real foods. The objective of this study is to determine the potential use of some edible Thai plant extracts in inhibiting the growth of *L. monocytogenes* on refrigerated cooked pork. The use of extracts rather than purified compounds in this experiment was designed to take advantage of all active compounds present in the extracts.

MATERIALS AND METHODS

Test Organisms and Preparation of Inocula

Cultures of *L. monocytogenes* isolated from food samples, namely raw meat, vegetables and ready-to-eat food (KUS-1, KUS-2 and KUS-3) [8], were used in this study. A reference culture (IFRPD 2068) was kindly supplied by A. D. Hitchins, U.S. Food and Drug Administration, Washington, D.C. Cultures were maintained on tryptic soy agar supplemented with 0.6% yeast extract (TSAYE; Merck) and stored at 4°C until use by subculturing.

L. monocytogenes was activated by culturing in TSAYE broth at 35°C at least twice in 24-hour period prior to use in the experiment. The culture was then centrifuged (c. 2000 g, 10 minutes) and the pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.0). The final inocula were prepared by serial dilution in the buffer such that the final population was about 10^2 or 10^5 cfu g^{-1} of pork.

Plant Extracts and Sample Preparation

Edible Thai plants used in this study are shown in Table 1. They were dried and ground before extracting with 95% ethyl alcohol for 48 hours. The final volume of each extract solution was adjusted to a concentration of 100 mg extract/ml and was then sterilised by a Millipore membrane (0.45 μm).

Fresh pork was purchased from a local retail supermarket and transported under refrigeration (2-7°C) to the laboratory. It was cooked to an internal temperature of 85°C, then cut into 25-g pieces with a sanitised knife, placed in sterile Petri dishes and immediately stored at 4°C before use.

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Table 1. Edible Thai plants tested for antilisterial activity

Common name	Scientific name	Part extracted
Buabok	*Centella asiatica*	Leaf
Buab	*Cucurbitaceae* sp.	Fruit
Chamuang	*Garcinia cowa*	Leaf
Cha-om	*Acacia pennata*	Leaf
Chaphlu	*Piper sarmentosum*	Leaf
Dipli	*Piper retrofractum*	Fruit
Dok Pheka	*Oroxylum indicum*	Flower
Fakthong	*Cucurbita moschata*	Leaf
Hang Plachon	*Emilia sonchifolia*	Leaf
Horapha	*Ocimum basilicum*	Leaf
Hua Plee	*Musa sapientum*	Flower
Kaphrao	*Ocimum tenuiflorum*	Leaf
Khanun	*Artocarpus heterophyllus*	Fruit
Krachai	*Boesenbergia pandurata*	Rhizome
Krachiap Daeng	*Hibiscus sabdariffa*	Leaf
Krathin	*Leucaena leucocephala*	Leaf
Lepkhrut	*Polyscias fruticosa*	Leaf
Makham	*Tamarindus indica*	Leaf
Mamuang	*Mangifera indica*	Leaf
Mamuang Himmapaan	*Anacardium occidentale*	Leaf
Mapring	*Bouea oppositifolia*	Leaf
Maenglak	*Ocimum americanum*	Leaf
Manthet	*Ipomoea batatas*	Leaf
Mara	*Momordica charantia*	Leaf
Mui	*Micromelum minutum*	Leaf
Phak Bung	*Ipomoea aquatica*	Leaf
Phak Chee	*Coriandrum sativum*	Leaf
Phak Chee Farang	*Eryngium foetidum*	Leaf
Phak Chee Lao	*Anethum graveolens*	Leaf
Phak Chiangda	*Gymnema inodorum*	Leaf
Phak Keehuud	*Raphanus sativus*	Fruit
Phak Khana	*Brassica alboglabra*	Leaf
Phak Khom	*Amaranthus lividus*	Leaf
Phak Krachet	*Neptunia oleracea*	Leaf
Phak Kutkhao	*Diplazium esculentum*	Leaf
Phak Mae	*Momordica subangulata*	Leaf
Phak Paem	*Eleutherococcus trifoliatus*	Leaf
Phak Plang	*Basella alba*	Leaf
Phak Sian	*Cleome gynandra*	Leaf

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Table 1. (Continued)

Common name	Scientific name	Part extracted
Phak Tamlueng	*Coccinia grandis*	Leaf
Phak Tang-o	*Chrysanthemum coronarium*	Leaf
Phak Wan	*Melientha suavis*	Leaf
Phak Wan Ban	*Sauropus androgynus*	Leaf
Phlu	*Piper betle*	Leaf
Phrik Khinuu	*Capsicum frutescens*	Leaf
Phrik Thai	*Piper nigrum*	Fruit
Sadao	*Azadirachta indica*	Flower
Takhrai	*Cymbopogon citratus*	Stem
Thammang	*Litsea petiolata*	Leaf
Thua Fakyao	*Vigna unguiculata*	Fruit

Sensitivity Testing and Minimum Inhibitory Concentrations

Sensitivity testing was done by well assay technique using TSA (tryptic soy agar; Merck) incubated at 35°C for 24 hours [9]. The well diameter was 4 mm. Minimum inhibitory concentrations (MIC) were determined according to the method of Richards et al [10].

Treatment of Pork Samples

The plant extract (1.0 ml) was uniformly deposited on the pork sample using a pipette and then spread with a sterile bent glass rod. Controls consisted of pork to which 95% ethanol (1.0 ml) was similarly applied, or pork that was untreated. The treated pork samples were kept in a biological safety cabinet for 15 minutes to allow alcohol to evaporate before being inoculated with the test bacteria. The samples were then inoculated with either low (10^2 cfu g^{-1}) or high (10^5 cfu g^{-1}) population of the reference culture of *L. monocytogenes* (IFRPD 2068). Inoculated pork samples were left to stand undisturbed for 30 minutes to allow residual moisture to be absorbed. They were then separately placed into polyethylene sampling bags and incubated at 4°C. Analysis of *L. monocytogenes* population was conducted after 0, 3, 5 and 7 days of storage.

Microbiological Analysis

On each sampling day, the incubated samples were removed for enumeration of *L. monocytogenes*. Phosphate buffer (225 ml) was added to each sampling bag and the contents were macerated with a Stomacher for 2 minutes. The resulting slurry was serially (1:10) diluted, and 0.1 ml of the diluted slurry was spread-plated in duplicate onto Oxford agar and Palcam agar plates (Merck). They were then incubated at 35°C for 48 hours.

RESULTS AND DISCUSSION

Of the 50 edible plant extracts tested, those of *Micromelum minutum* (Mui), *Artocarpus heterophyllus* (Khanun), *Piper retrofractum* (Dipli) and *Cucurbita moschata* (Fakthong) demonstrated

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the greatest inhibitory effect on the four strains of *L. monocytogenes* and were chosen for further study. As shown in Table 2, *M. minutum* showed potent growth inhibition against *L. monocytogenes* with a clear-zone diameter ranging from 20.89 ± 0.53 mm to 27.29 ± 0.21 mm (mean inhibitory zone of 23.60 mm). Application of *A. heterophyllus* resulted in zones of inhibition ranging from 19.93 ± 0.21 to 24.39 ± 0.83 mm (mean inhibitory zone of 21.89 mm). It is noteworthy that the potent antilisterial activity of these four species (*M. minutum*, *A. heterophyllus*, *P. retrofractum* and *C. moschata*) has not been reported before.

Table 2. Antilisterial activity of four most potent plant extracts

Plant	Diameter of inhibition zone (mm) ± SD[a]			
	L. monocytogenes strain			
	IFRPD 2068	KUS-1	KUS-2	KUS-3
Mui (*Micromelum minutum*)	21.50 ± 0.05	27.29 ± 0.21	24.76 ± 0.75	20.89 ± 0.53
Khanun (*Artocarpus heterophyllus*)	22.48 ± 0.03	24.39 ± 0.83	19.93 ± 0.21	20.75 ± 0.47
Dipli (*Piper retrofractum*)	18.20 ± 0.80	21.25 ± 1.10	19.09 ± 0.71	18.44 ± 0.97
Fakthong (*Cucurbita moschata*)	14.25 ± 0.03	11.33 ± 0.66	14.76 ± 1.14	16.10 ± 0.80

[a] Mean value of four determinations, each from a different plate

MIC assays were also performed to determine the lowest concentrations of the plant extracts that produced an inhibitory effect. *M. minutum* and *A. heterophyllus* were most inhibitory to the four strains of *L. monocytogenes*, with MIC of 625 μg/ml. The extracts of *P. retrofractum* and *C. moschata* were also effective, with MIC of 1250 and 2500 μg/ml (Table 3).

Table 3. MIC against *L. monocytogenes* of four most potent plant extracts

Plant	MIC (μg/ml)			
	L. monocytogenes strain			
	IFRPD 2068	KUS-1	KUS-2	KUS-3
Mui (*Micromelum minutum*)	625	625	625	625
Khanun (*Artocarpus heterophyllus*)	625	625	625	625
Dipli (*Piper retrofractum*)	1250	1250	1250	2500
Fakthong (*Cucurbita moschata*)	2500	1250	2500	1250

M. minutum, which showed the highest activity in the antilisterial screening, is a shrub belonging to the Rutaceae family. It is consumed mainly in the southern part of Thailand as a fresh vegetable served with certain dishes such as rice noodles with hot curry. *M. minutum* has also been used as a folk medicine for fever and giddiness [11]. The medicinal principles of *M. minutum* have not yet been elucidated thoroughly although many bioactive compounds such as coumarins, a flavanone, a quinolone alkaloid and carbazole alkaloids have previously been isolated [11-12].

A. heterophyllus is a tree belonging to the Moraceae family and produces a fruit which is eaten when it is still unripe by boiling with salt. Thais also eat it as a fresh fruit with chili paste, or sometimes

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add it to curry. The methanolic extract of *A. heterophyllus* fruit exhibits a broad-spectrum antibacterial activity [13].

At the storage temperature of 4°C, typically the temperature at which consumers store cooked food, little or no growth of the test strain of listeria was observed in control samples when both low (Table 4a) and high (Table 4b) populations of inocula were applied. However, listeria population on cooked pork with high inoculum which was treated with either *M. minutum* or *A. heterophyllus* extracts was at least 2 to 3 magnetudes of logarithmic scale less than that on control samples. This difference persisted throughout the storage period (7 days) (Table 4b). When samples were inoculated with a low level of inoculum (c. 10^2 cfug^{-1}), *M. minutum* and *A. heterophyllus* extracts were also the most effective in controlling *L. monocytogenes* during storage of pork at 4°C. After 5 days and 7 days of storage, *L. monocytogenes* could not be detected in samples treated with *M. minutum* and *A. heterophyllus* respectively (Table 4a). Samples treated with *P. retrofractum* also suppressed the growth of *L. monocytogenes* although not as effectively as *M. minutum* and *A. heterophyllus* extracts.

Table 4. Population ($\log_{10}$ cfu g^{-1} ± SD) of *L. monocytogenes* (IFRPD 2068 strain) on cooked pork with (a) low inoculum (c. 10^5 cfu g^{-1}) and (b) high inoculum (c. 10^2 cfu g^{-1}) at 4 °C

(a)

Treatment	Time (days)			
	0	3	5	7
None	1.90 ± 0.02	1.85 ± 0.08	2.64 ± 0.11	2.07 ± 0.23
95% ethanol	1.84 ± 0.06	1.90 ± 0.06	2.03 ± 0.08	2.15 ± 0.09
Mui (*Micromelum minutum*)	1.05 ± 0.01	1.21 ± 0.03	ND[a]	ND
Khanun (*Artocarpus heterophyllus*)	1.03 ± 0.01	1.37 ± 0.03	1.23 ± 0.10	ND
Di pli (*Piper retrofractum*)	1.62 ± 0.02	1.71 ± 0.08	2.69 ± 0.08	2.08 ± 0.47
Fak thong (*Cucurbita moschata*)	1.93 ± 0.04	2.05 ± 0.03	2.79 ± 0.10	2.26 ± 0.32

[a] ND = not detected

(b)

Treatment	Time (days)			
	0	3	5	7
None	4.65 ± 0.02	4.91 ± 0.04	4.63 ± 0.06	5.03 ± 0.10
95% ethanol	4.35 ± 0.01	4.75 ± 0.06	4.94 ± 0.11	5.00 ± 0.11
Mui (*Micromelum minutum*)	4.08 ± 0.03	2.03 ± 0.06	2.29 ± 0.08	1.78 ± 0.28
Khanun (*Artocarpus heterophyllus*)	4.78 ± 0.01	2.71 ± 0.03	2.41 ± 0.08	1.80 ± 0.34
Di pli (*Piper retrofractum*)	4.61 ± 0.06	4.12 ± 0.05	3.57 ± 0.14	2.64 ± 0.25
Fak thong (*Cucurbita moschata*)	4.57 ± 0.03	4.51 ± 0.07	4.24 ± 0.19	4.81 ± 0.21

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