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Full Length Research Paper

Contents and antibacterial activity of flavonoids extracted from leaves of *Psidium guajava*

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Four antibacterial flavonoids (morin-3-*O*-lyxoside, morin-3-*O*-arabinoside, quercetin, and quercetin-3-*O*-arabinoside) were isolated from fresh and dried *Psidium guajava* leaves, and their concentrations were determined. Among them, quercetin and morin-3-*O*-arabinoside were the most and the least abundant, respectively. Studies on inhibitory effects of the flavonoids on spoilage and foodborne pathogenic bacteria revealed that they had bacteriostatic mode of action against all tested spoilage and foodborne pathogenic bacteria including *Bacillus stearothermophilus*, *Brochothrix thermosphacta*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Pseudomonas fluorescens*, *Salmonella enterica*, *Staphylococcus aureus* and *Vibrio cholerae*.

Key words: antibacterial activity, flavonoids, guava, *Psidium guajava.*

INTRODUCTION

Foods eaten raw are usually sold in ready-to-eat form, and most of them generally do not contain food preservatives or antimicrobial substances. Many Thai foods fall in this category such as nham (fermented pork sausage), Kem-Buk-Nud (fermented pineapple), Kung Jom (shrimp paste), Puk Dong (fermented vegetable). These foods serve as sources not only for food spoilage bacteria which shorten shelf life of foods but also for pathogenic bacteria which cause food related diseases. Spoilage bacteria are microorganisms that cause foods to deteriorate and develop unpleasant odors, taste and texture. Most of them are species of *Bacillus*, *Clostridium*, *Micrococcus*, *Proteus*, *Pseudomonas*, *Shewanella* and *Xanthomonas* (Lund et al., 2000). Foodborne pathogenic bacteria may cause foodborne diseases with flu-like symptoms such as nausea, vomiting, diarrhea, and/or fever. A variety of bacteria have been known to be pathogens associated with foods including *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Aeromonas hydrophila*, *Staphylococcus aureus* and *Yersinia enterocolitica* (Lund et al., 2000). Food additives with antimicrobial activity can be used to overcome such problems. However, the present uses of chemical based food additives are objected by many consumers. They prefer additives derived from natural products. Many plants can represent a source of natural antimicrobial substances to improve the shelf life and the safety of foods (Cowen, 1999). Among them, *Psidium guajava* or guava is one of the potential plants to be used in foods for such purpose.

P. guajava or guava is a plant in the family Myrtaceae along with clove, allspice and eucalyptus. Native to tropical America, it is now cultivated in many tropical and subtropical countries for its edible fruit (Perez et al., 2008). It has been used as an ingredient in many food recipes and desserts. *P. guajava* has been known to have antimicrobial (Arima and Danno, 2002; Chah et al., 2006; Prabu et al., 2006), anti-inflammatory (Ojewole, 2006), antimalarial (Tona et al., 1998), antitumor (Manosroi et al., 2006; Chen et al., 2007), antiallergic (Seo et al., 2005), antihyperglycemic (Ojewole, 2005; Mukhtar et al., 2006) and antimutagenic (Grover and Bala, 1993) activities. It has been used to treat wounds (Chah et al., 2006), acne lesions (Qadan et al., 2005), cough (Jairaj et al., 1999) and dental diseases (Razak et al., 2006). Flavonoids extracted from guava leaves including morin-3-*O*-lyxoside, morin-3-*O*-arabinoside, quercetin and quercetin-3-*O*-arabinoside were reported to have strong antibacterial action (Arima and Danno, 2002). This study aimed to examine the flavonoids contents of fresh and dried guava leaves and their anti-

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Table 1. Classification and optimal growth parameters for bacteria used in this study.

Bacteria	Classification	Media	Incubation temperature (°C)
E. coli O157:H7 (ATCC 35150)	Pathogenic	Nutrient	37
L. monocytogenes (ATCC 19111)	Pathogenic	Brain heart infusion	37
S. enterica (ATCC 8326)	Pathogenic	Nutrient	37
S. aureus (ATCC 25923)	Pathogenic	Tryptic soy	37
V. cholerae (ATCC 14101)	Pathogenic	Nutrient	37
B. stearothermophilus (ATCC 12980)	Spoilage	Nutrient	55
B. thermosphacta (ATCC 11509)	Spoilage	Nutrient	26
P. fluorescens (ATCC 11150)	Spoilage	Nutrient	25

bacterial activity and mode of action against various strains of spoilage and foodborne pathogenic bacteria.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacteria used in this study were *Bacillus stearothermophilus* (ATCC 12980), *Brochothrix thermosphacta* (ATCC 11509), *E. coli* O157:H7 (ATCC 35150), *Listeria monocytogenes* (ATCC 19111), *Pseudomonas fluorescens* (ATCC 11150), *Salmonella enterica* (ATCC 8326), *S. aureus* (ATCC 25923) and *Vibrio cholerae* (ATCC 14101). The growth conditions of the bacteria are presented in Table 1. Bacterial stock cultures were stored as frozen at -80°C in appropriate broth containing 20% glycerol (v/v).

Plant materials and sample preparation

Fresh leaves of *P. guajava* were purchased from the herb shop "Ban Samunprai" in Pranakorn, Bangkok, Thailand. They were sold with an identification certificate approved by the Royal Forest Department, Bangkok, Thailand. Dried leaves were obtained by baking the fresh leaves in an oven at 70°C for 72 h. Fresh and dried guava leaves were initially extracted by methanol and then subjected to the isolation of flavonoids by column chromatography as previously described by Arima and Danno (2002).

Antimicrobial activity determination

The flavonoids were examined for their antimicrobial activities against the spoilage and foodborne pathogenic bacteria named above using the microdilution method described by Amsterdam (1996) with some modifications. Briefly, each tested compound was added into a microtiter plate containing appropriate broth to obtain the concentration ranging from 10 to 200 µg/ml. The bacteria to be tested were added to the wells containing the compound to obtain a final concentration of 10^4 CFU/ml.

A positive control (without tested compounds) and a negative control (without tested bacteria) were included for each plate. After incubation at optimal temperature, bacterial growth was inspected at 24 h. Results were reported as the minimal inhibitory concentration (MIC) required to cause no growth of the bacteria. Each MIC value was obtained from five experiments. The same protocol was used to determine the MIC of oxytetracycline for the inhibition of all tested pathogenic bacteria.

Examination of mode of action

The flavonoids (at final concentrations equal to the MIC values) were added to 4.9 ml of bacterial cultures (10^3 CFU/ml). After incubation at optimal temperature for 24 h, 100 µl of the mixtures were inoculated into 4.9 ml of fresh culturing broth. As a control, 100 µl of untreated cultures of bacteria at a concentration of 10^3 CFU/ml were transferred to 4.9 ml of fresh culturing broth. The optical density at a wavelength of 600 nm (OD_{600nm}) of the tested and control cultures was determined at the time of inoculation and after incubation for 24 h. The same protocol was used to determine mode of action of oxytetracycline on all tested pathogenic bacteria.

Statistical analysis

Analysis of variance was performed using the General Linear Models procedure of Statistical Analysis System (SAS Institute Inc, Cary, NC, USA). Duncan's new multiple range test was used to obtain pairwise comparisons among sample means. Evaluations were based on a 5% significance level ($p < 0.05$).

RESULTS AND DISCUSSION

When fresh leaves of *P. guajava* were baked in an oven at 70°C for 72 h, the yield of dried leaves was about 10.2% (w/w). The isolation of flavonoids from both types of leaves revealed that the concentrations of all flavonoids extracted from dried leaves were higher than those extracted from fresh leaves (Table 2). However, the difference between the concentrations of each flavonoid extracted from both types of leaves was lower than 10 times: about 2.3, 2.8, 2.7 and 2.8 times for morin-3-*O*-lyxoside, morin-3-*O*-arabinoside, quercetin-3-*O*-arabinoside and quercetin, respectively. These results suggested that the flavonoids were lost during the drying process of guava leaves. However, further investigations are required to identify the actual cause of the loss of flavonoids in dried leaves.

Regardless of types of guava leaves used for isolation, quercetin and morin-3-*O*-arabinoside were the most and the least abundant flavonoids, respectively. The concentrations of quercetin in fresh and dried leaves were about 63.9 and 179.3, respectively while those of morin-3-*O*-arabinoside in fresh and dried leaves were about 28.8

Table 2. Concentrations of flavonoids isolated from guava leaves.

Flavonoids	Concentration (µg/g of guava leaves) [a]		Concentration ratio [b] (times)
	Fresh leaves	Dried leaves	
morin-3-*O*-lyxoside	28.8 ± 3.2	65.4 ± 4.8	2.27
morin-3-*O*-arabinoside	20.5 ± 2.8	57.2 ± 3.6	2.79
quercetin-3-*O*-arabinoside	52.2 ± 6.7	138.6 ± 8.1	2.66
quercetin	63.9 ± 8.5	179.3 ± 9.2	2.81

[a]Results are mean ± S.D. values of five replicates; [b]Concentration ratio = concentration of flavonoid of dried leaves/concentration of flavonoid of fresh leaves.

Table 3. MICs of flavonoids isolated from guava leaves against spoilage and foodborne pathogenic bacteria.

Pathogens	MIC (µg/ml)[a]				
	ML	MA	QA	Q	OT
E. coli O157:H7	72 ± 4.0	70 ± 0.0	70 ± 6.3	74 ± 4.9	70 ± 6.3
L. monocytogenes	62 ± 4.0	58 ± 4.0	60 ± 0.0	60 ± 0.0	58 ± 7.5
S. enterica	114 ± 4.9	110 ± 0.0	110 ± 6.3	112 ± 4.0	112 ± 4.0
S. aureus	80 ± 0.0	82 ± 4.0	82 ± 4.0	80 ± 0.0	76 ± 4.9
V. cholerae	52 ± 4.0	54 ± 4.9	52 ± 4.0	50 ± 6.3	56 ± 4.9
B. stearothermophilus	152 ± 4.0	150 ± 0.0	150 ± 0.0	154 ± 4.9	152 ± 7.5
B. thermosphacta	120 ± 0.0	122 ± 4.0	120 ± 0.0	122 ± 4.0	124 ± 4.9
P. fluorescens	42 ± 4.0	40 ± 6.3	42 ± 4.0	44 ± 4.9	40 ± 0.0

[a]Results are mean ± S.D. values of five replicates; MICs = minimal inhibitory concentrations; ML = morin-3-*O*-lyxoside; MA = morin-3-*O*-arabinoside; QA = quercetin-3-*O*-arabinoside; Q = quercetin; OT = oxytetracycline.

and 65.4, respectively (Table 2). Of all flavonoids studied in this work, quercetin has been the most frequently studied flavonoid. It was reported to be present in numerous vegetables and fruits including onions (284 - 486 mg/kg), kale (110 mg/kg), French beans (32 - 45 mg/kg), broccoli (30 mg/kg), apples (21 - 72 mg/kg), lettuce (14 mg/kg) and tomatoes (8 mg/kg) (Hertog et al., 1992). Compared to these quercetin concentrations, guava leaves can be considered to contain a relatively high content of the compound.

P. guajava leaves have long been recognized for their antibacterial activity. They were shown to inhibit both Gram-positive and Gram-negative bacteria such as *S. aureus*, *Streptococcus mutans*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Bacillus cereus*, *Proteus* spp., *Shigella* spp. and *E. coli* (Cowen, 1999; Perez et al., 2008). Since the flavonoids extracted from guava leaves were believed to be responsible for the activity, it is interesting to examine morin-3-*O*-lyxoside, morin-3-*O*-arabinoside, quercetin-3-*O*-arabinoside and quercetin for their antibacterial activity against several strains of spoilage and foodborne pathogenic bacteria including *B. stearothermophilus*, *B. thermosphacta*, *E. coli* O157:H7, *L. monocytogenes*, *P. fluorescens*, *S. enterica*, *S. aureus*, and *V. cholerae*. The results showed that the flavonoids were able to inhibit all of the bacteria used in this study with different degree of inhibition (Table 3). *P. fluorescens* and *B. stearothermophilus* were the most and the least sensitive strains to the flavonoids, respectively. For each examined bacteria, MIC values of the four flavonoids had no significant difference ($p < 0.05$), indicating the participation of all four flavonoids in antibacterial activity of guava leaves. Besides flavonoids, other compounds were also found in guava leaves such as anthocyans, alkaloids, tannins, and terpenoids (Cowen, 1999). However, these compounds extracted from guava have never been reported to have antibacterial activity. It was also shown in this study that the MICs of the flavonoids for every tested bacterium had no significant difference ($p < 0.05$) from those of oxytetracycline (Table 3). From these results, it is possible that the f lavonoids may be used as natural antimicrobial substances to replace antibiotics for controlling bacterial infections.

In order to determine whether the flavonoids had bactericidal or bacteriostatic mode of action on the sensitive bacteria, the ability of the flavonoid inhibited bacteria to resume their growth in fresh culturing broth was observed. The results showed that the bacteria inhibited by all of the flavonoids could resume their growth in fresh media within 24 h (Table 4). These results suggested that the flavonoids had bacteriostatic mode of action on the

Table 4. Recovery ability of the flavonoids-inhibited bacteria in fresh media.

Pathogens	OD600nm[a]									
	ML		MA		QA		Q		OT	
	0[b]	24[b]	0[a]	24[a]	0[a]	24[a]	0[a]	24[a]	0[a]	24[a]
E. coli O157:H7	0	0.70 ± 0.01	0	0.74 ± 0.03	0	0.78 ± 0.03	0	0.73 ± 0.03	0	0
L. monocytogenes	0	0.87 ± 0.04	0	0.84 ± 0.03	0	0.86 ± 0.02	0	0.80 ± 0.06	0	0
S. enterica	0	0.75 ± 0.03	0	0.78 ± 0.03	0	0.73 ± 0.03	0	0.79 ± 0.05	0	0
S. aureus	0	0.82 ± 0.04	0	0.88 ± 0.05	0	0.80 ± 0.02	0	0.79 ± 0.03	0	0
V. cholerae	0	0.87 ± 0.03	0	0.80 ± 0.03	0	0.82 ± 0.04	0	0.86 ± 0.04	0	0
B. stearothermophilus	0	0.73 ± 0.03	0	0.74 ± 0.01	0	0.80 ± 0.03	0	0.75 ± 0.02	0	0
B. thermosphacta	0	0.71 ± 0.02	0	0.73 ± 0.03	0	0.74 ± 0.02	0	0.66 ± 0.03	0	0
P. fluorescens	0	0.90 ± 0.05	0	0.86 ± 0.02	0	0.83 ± 0.04	0	0.80 ± 0.04	0	0

[a]OD_{600nm} (optical density at a wavelength of 600 nm) values are the mean of five replicates; [b]Time after inoculation of the flavonoids-inhibited bacteria into fresh broth (h); ML = morin-3-*O*-lyxoside; MA = morin-3-*O*-arabinoside; QA = quercetin-3-*O*-arabinoside; Q = quercetin; OT = oxytetracycline.

bacteria. In contrast, oxytetracycline was shown to have bactericidal mode of action on all of the tested bacteria indicated by the lack of ability of the drug inhibited bacterial cells to grow in fresh media within 24 h (Table 4). In previous study, guava leaves' flavonoids were also shown to have bacteriostatic effects on fish pathogenic bacteria including *Aeromonas hydrophila*, *Aeromonas salmonicida* subsp. *salmonicida*, *Flavobacterium columnare*, *Lactococcus garvieae*, *Streptococcus agalactiae* and *Vibrio salmonicida* (Rattanachaikunsopon and Phumkhachorn, 2007). The use of antimicrobial substances with bacteriostatic mode of action may have less side effects than those with bactericidal mode of action. The latter ones tend to kill all of the bacteria in the body including normal flora whereas the former ones just retard the growth of the bacteria which are further killed by the immune responses of the body. By this way, normal flora which is not harmed by the immune responses is only temporary inhibited.

The findings presented in this report showed that the flavonoid isolated from guava leaves (morin-3-*O*-lyxoside, morin-3-*O*-arabinoside, quercetin and quercetin-3-*O*-arabinoside) might be potential biologically active compounds for use as food preservatives to improve the shelf-life and the safety of foods.

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