Inhibitory and Killing Activities of Medicinal Plants against Multiple Antibiotic-resistant *Helicobacter pylori*

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Multiple antibiotic-resistant *Helicobacter pylori* (*H. pylori*), one of the major causes of gastric cancer, is now increasingly reported. The aim of this study was to screen medicinal plants widely used in Thailand as possible sources of medicines that can be used to treat *H. pylori* infection. Twenty-four extracts from 13 kinds of Thai herbs were tested for their antibacterial activity against 20 strains of antibiotic-resistant *H. pylori*. Inhibition of growth was tested by the paper disc agar diffusion method. Most strains of *H. pylori* examined were proved to be susceptible to seven medicinal plants; *i.e.*, *Peltophorum pterocarpum*, *Piper betle*, *Punica granatum* (*P. granatum*), *Quercus infectoria* (*Q. infectoria*), *Tamarindus indica*, *Uncaria gambir*, and *Walsura robusta*. Among these extracts, *P. granatum* and *Q. infectoria* exhibited the greatest inhibitory potencies. Minimal inhibitory concentrations (MIC) were determined by the agar dilution method in Petri dishes with a Millipore filter membrane, and minimal bactericidal concentrations (MBC) were assessed with the extract that gave a significant MIC value against each bacterial strain by placing the Millipore filter membrane onto a fresh Isosensitest agar plate. Ethanolic extracts of *P. granatum* and *Q. infectoria* significantly reduced the growth of all strains of *H. pylori*, with the best MIC values at 0.8 and 3.1 mg/ml, and the best MBC values at 3.1 and 6.2 mg/ml, respectively. Effective fractions partially purified from both plant species yielded MICs and MBCs that were at least 10-fold less compared with the crude extracts. From the data obtained, it is hoped that *P. granatum* and *Q. infectoria* will become useful sources with which to develop new therapeutic agents for *H. pylori* infection.

Key words —— *Helicobacter pylori*, *Punica granatum*, *Quercus infectoria*, antibacterial activity, medicinal plant

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative spirally shaped bacterium that has been implicated to cause not only gastritis and peptic ulcer disease but also gastric carcinoma and lymphoma.[1–3] Unless specifically treated, infection with the gastric pathogen *H. pylori* is lifelong. Infection with this bacterium induces the development of an active chronic gastritis. While chronic inflammation is the major outcome of infection, this disorder often develops into a number of more serious conditions such as peptic ulcer disease (PUD), gastric cancer and B cell mucosa-associated lymphoid tissue (MALT) lymphoma. For example, approximately 20% of infected persons develop PUD during their lifetime.[4, 5]

Currently, antimicrobial therapy represents the sole approach for the eradication of *H. pylori* infection. The eradication of *H. pylori* with antibiotics significantly decreased the recurrent rates of gastric and duodenal ulcers in both adults and children.[6] A wide variety of antimicrobial regimens have been used for the treatment of *H. pylori* infection with varying degrees of success.[7]

It is obvious that the appearance of antibiotic-resistant strains decreases the efficacy of eradication therapy. Alternative approaches on the use of plant extracts to cure *H. pylori* infection have become increasingly reported. Previously, we have reported that aqueous and ethanolic extracts of certain medicinal plants have antibacterial activity against a number of pathogenic bacteria.[8–10] This includes enterohaemorrhagic *Escherichia coli*,[8] methicillin-resistant *Staphylococcus aureus*,[9] and opportunistic pathogens in AIDS patients.[10] The aim of this study was to investigate the effects of some selected plants, the ingredients of which may be useful for treating *H. pylori*. We have attempted to find some bioactive compounds that are simple, affordable, and have minimal side effects.

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MATERIALS AND METHODS

Plant Collection —— Thirteen well-recognized traditional Thai medicinal plants used to cure gastrointestinal diseases were collected in Thailand in September 2003 on the basis of traditional practices by Thai herbalists. They were air-dried, and their botanical identification was kindly made by Associate Professor T. Supavita, Department of Pharmacognosy, Faculty of Pharmacy, Prince of Songkla University, Thailand. A classified reference voucher specimen was deposited at the Herbarium of Prince of Songkla University. The parts of the plants, their uses in traditional medicine,[11] as well as the percentage yield for each extract are summarized in Table 1.

Plant Extraction —— The plant parts were washed with distilled water, dried at 60°C overnight, cut into small pieces, and crushed in a mechanical mortar. Powdered samples (100 g) were soaked either in water or 95% ethanol (500 ml, w/v) at room temperature for 7 days and then filtered through Whatman No. 2 filter paper. The filter was extracted three times, and the combined filtrate was evaporated under reduced pressure at 60°C until they became completely dry. The aqueous extract was dissolved in 250 mg/ml water, and the ethanolic extract was dissolved in dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) before use.

Fractionation of Active Compounds —— The ethanolic exudates from *Punica granatum* (*P. granatum*, 26.5 g) and *Quercus infectoria* (*Q. infectoria*, 41.2 g) were partially purified as follows. The exudate was dissolved in 95% ethanol a concentration of 10% (w/v), and applied onto a silica gel column (Merck 60GF$_{254}$ 70–230 mesh; 500 g; column *i.d.*, 5 cm). In the purification of *P. granatum* exudate, the column was eluted by a linear gradient from hexane-ethyl acetate (100 : 0 to 0 : 100, v/v; total volume, 8.5 l) to 5% methanol-ethyl acetate (100 : 1900, v/v; total volume, 2 l) and 100% methanol (total volume, 4 l). In the case of *Q. infectoria*, the column was developed by a gradient from chloroform to methanol. Each fraction (250 ml) was monitored by thin layer chromatography (TLC) on silica gel 60GF$_{254}$ TLC aluminium sheets (Merck; layer thicknesss 0.2 mm) with chloroform : methanol : H_2O (6 : 3.7 : 0.3, v/v/v) as the mobile phase. After air drying, spots on the plate were visualized under UV light (200–400 nm). Desired fractions were then concentrated to complete dryness. *P. granatum* and *Q. infectoria* yielded 20 and 100 fractions, respectively. Fractions with similar TLC patterns were pooled as depicted in Fig. 1. Some fractions from *P. granatum* exudate were further purified to yield Pg1, 2 and 3 (Fig. 1A). Fraction IV containing a white solid in yellowish oil gave Pg1 by washing with hexane. Fractions VII (white solid) and XV (dark red hexagonal planar solid) were recrystallized from methanol : chloroform (8 : 2, v/v) to Pg2 and Pg3, respectively. Fractions 1–9 (i) of the exudate from *Q. infectoria* contained only trace amounts of extracts. Fractions 10 and 11 (ii: Qi1), frac-

Table 1. List of Medicinal Plants Used in the Antimicrobial Assay

Botanical species	Family	Plant part	Anticancer[a]	Anti-ulcerogenic[a]	Extract yield (%)[b]	
					Aqueous	Ethanolic
Andrographis paniculata (Burm.f) Nees.	*Acanthaceae*	leaf	+	–	ND	11.2
Centella asiatica (L.) Urb.	*Apiaceae*	leaf	+	+	ND	6.8
Curcuma longa L.	*Zingiberaceae*	rhizome	+	+	ND	15.9
Garcinia mangostana L.	*Clusiaceae*	pericarp	+	–	ND	ND
Peltophorum pterocarpum (DC.) Backer ex K. Heyne	*Fabaceae*	bark	–	–	8.6	7.1
Piper betle L.	*Piperaceae*	leaf	–	–	ND	12.4
Psidium guajava L.	*Myrtaceae*	leaf	+	+	2.8	8.0
Punica granatum L.	*Punicaceae*	pericarp	+	–	8.0	13.0
Quercus infectoria Oliv.	*Fagaceae*	fruit	–	–	37.8	32.4
Tamarindus indica L.	*Fabaceae*	leaf	–	–	37.1	4.8
Uncaria gambir Hunter Roxb.	*Rubiaceae*	leaf, stem	–	–	59.8	65.4
Walsura robusta Roxb.	*Meliaceae*	wood	–	–	2.3	4.3
Zingiber cassumanar Roxb.	*Zingiberaceae*	rhizome	+	+	ND	ND

a) See reference 11 for these effects. *b*) Each value represents the percentage (w/w) on the basis of the weight of dried plant. ND = not done.

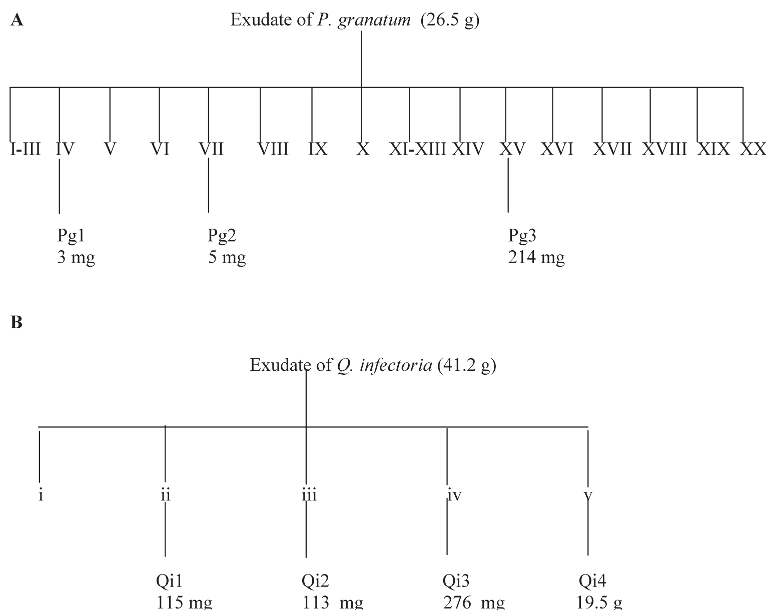

Fig. 1. Fractionation of the Exudate of Medicinal Plants
Schematic representations of the fractionation of exudates from *Punica granatum* pericarp (A) and *Quercus infectoria* nutgall (B) are shown.

tions 12–17 (iii: Qi2), fractions 18–26 (iv: Qi3), and fractions 27–100 (v: Qi4) were pooled, and each combined fraction was designated as indicated (Fig. 1B). When the 1H-nuclear magnetic resonance (NMR) spectrum was measured for structural elucidation, it was taken in CDCl3, using a 500 MHz Varian Unity Inova (Merck, Darmstadt, Germany). Fractions Pg3, Qi2, Qi3, and Qi4 were used for further study. The others were not further investigated since they have no UV absorbance or their yields were too low to assay for antibacterial activities.

Bacterial Strains Tested —— Reference guidelines for culturing and antibiotic sensitivity testing were used.[12] Biopsy specimens sampled from the gastric antrum and body of the stomach were received in Amies or Stuart's transport media and maintained between 4°C and 7°C. Following the Gram stain, the biopsy specimens were cultured without prolonged delay on Campylobacter-selective agar (CSA)[13] to enable the growth and detection of *H. pylori.* The plates were incubated with a lid uppermost at 37°C in 10% CO_2 and 95% relative humidity. Some strains required up to 5–7 days incubation. These plates were examined at 3 day intervals for 12 days. All strains were maintained frozen in Brain Heart Infusion broth (Oxoid, Basingstoke, Hampshire, U.K.) containing 31% (w/v) glycerol in liquid nitrogen. They were thawed just before use and inoculated directly onto CSA. After 48–72 hr incubation, the cultures were checked for purity by phase-contrast microscopy. Biochemical tests including rapid urease, oxidase, and catalase reactions were performed to verify each culture.

Paper Disc Agar Diffusion Method —— A sterile filter paper disc (6 mm) was soaked with 10 µl of plant extract (250 mg/ml extraction solvent) so that each disc was impregnated with 2.5 mg of a substance whose antimicrobial activity was to be examined. Bacterial suspensions were adjusted to a McFarland turbidity of 3.0 (approximately 9.0×10^8 cfu/ml) and cultured on Isosensitest agar plates (Oxoid) enriched with 5% horse blood (Oxoid) by dipping a sterile swab into the suspension and swabbing over the entire plate surface in three directions. Both a wet disc and a dry disc (dried at 37°C overnight) were applied to the surface of the Isosensitest agar (pH adjusted to 4–6) seeded with the test bacteria culture and then the cultures were incubated anaerobically. Antibiotic susceptibility discs (10–30 µg) were used as controls. The antibacterial activity was evaluated by measuring the annular radius of the inhibition zone. The urease test was performed to elicidate a clear zone.[14] The experiments were performed in triplicate and the mean of the diameter of the inhibition zones (annular radii) was calculated.

Determination of Minimal Inhibition Concentration (MIC) and Minimal Bactericidal Concentration (MBC) —— The MIC was determined according to an established method.[15] Briefly, one mi-

croliter of each bacterial strain containing approximately 10^4 colony forming unit (CFU) was seeded on an Isosensitest agar plate supplemented with different fractions of the effective extracts at concentrations starting from the MIC values of crude extracts. The plates were incubated anaerobically at 37°C for 48–72 hr. Observations were performed at least in triplicate and the results expressed as the lowest concentration of a plant extract that produced complete suppression of colony growth (MIC). The MBC was determined with the extract that gave MIC values against each bacterial strain by placing a Millipore filter membrane onto an Isosensitest agar plate.

RESULTS AND DISCUSSION

The plants were initially screened for their antibacterial activity against 20 different clinical strains of *H. pylori* with multiple resistance to amoxicillin, clarithromycin, and metronidazole. Two reference strains, ATCC 43504 and ATCC 43579, were used as controls. Among 26 crude aqueous and ethanolic extracts tested, only 12 extracts (46.15%) of 7 plant species were demonstrated to have antibacterial activity against these strains (Table 2). The extracts from *P. granatum*, *Q. infectoria*, *Uncaria gambir*, and *Walsura robusta* (*W. robusta*) produced inhibition zones against all strains of *H. pylori* tested. The inhibition zones ranged from 4.95 to 16.5 mm. Both aqueous and ethanolic extracts of *P. granatum* and *Q. infectoria*, and the ethanolic extract of *W. robusta*, exhibited high activity against all strains tested. The maximum zone (16.5 mm) of antibacterial effect against *H. pylori* was demonstrated with the ethanolic extracts from *P. granatum*.

The antibacterial effects, expressed as MIC and MBC, of both aqueous and ethanolic extracts of each medicinal plant against each *H. pylori* strain are illustrated in Figs. 2 and 3. The ethanolic extracts of both *P. granatum* and *Q. infectoria* were among the most active, showing very strong activity against all *H. pylori* strains, with the best MIC and MBC values being 0.8, 3.1 and 3.1, 6.2 mg/ml, respectively. Partially purified fractions of both plant species yielded MICs and MBCs that were at least 10-fold less compared with the crude values (Table 3). Thus, we purified or partially purified crude extracts from the above two plants as described in the Experimental Section. Although the components involved in the samples obtained should be further clarified, preliminary experiments suggest the following (data not shown). Of three fractions from *P. granatum*, Pg1 did not contain any ingredient showing UV absorption. Based on data such as the TLC profile and 1H-NMR spectra,

Table 2. Antibacterial Activity of Aqueous and Ethanolic Extracts of Medicinal Plants (2.5 mg/disc) against *H. pylori*

H. pylori strain	Mean values of radii of inhibition zone in wet disc/dry disc (mm)[a]						
	Peltophorum ptercarpum	*Piper betle*	*Punica granatum*	*Quercus infectoria*	*Tamarindus indica*	*Uncarcia gambir*	*Walsura robusta*
Aqueous extract:							
H. pylori (20 strains)	4.5/4.3 (4)	2.6/3.0 (5)	13.7/12.9 (20)	11.7/12.0 (20)	ND	4.9/5.1 (20)	ND
H. pylori ATCC 43504	3/2	3/2	13/15	13/12	ND	5/5	ND
H. pylori ATCC 43579	−/−	−/−	12/12	10/10	ND	6/5	ND
Ethanolic extract:							
H. pylori (20 isolates)	4.4/4.0 (8)	3.8/3.9 (9)	16.1/15.9 (20)	13.7/13.1 (20)	3.6/3.6 (14)	5.5/5.3 (20)	8.7/8.5 (20)
H. pylori ATCC 43504	3/4	1/1	14/12	10/10	5/4	2/4	10/9
H. pylori ATCC 43579	−/−	−/−	15/14	11/11	5/4	7/7	8/8

Andrographis paniculata, *Centella asiatica*, *Curcuma longa*, *Garcinia mangostana*, and *Zingiber cassumanar* produced no inhibition zones for all strains tested. *a*) Each value represents the mean of inhibition zones of susceptible strains isolated from human gastrointestinal tract or the mean of three assays for reference strains (ATCC 43504 and ATCC 43579). Numbers of susceptible strains among 20 strains are shown in parentheses. – = no inhibition zone; ND = not done.

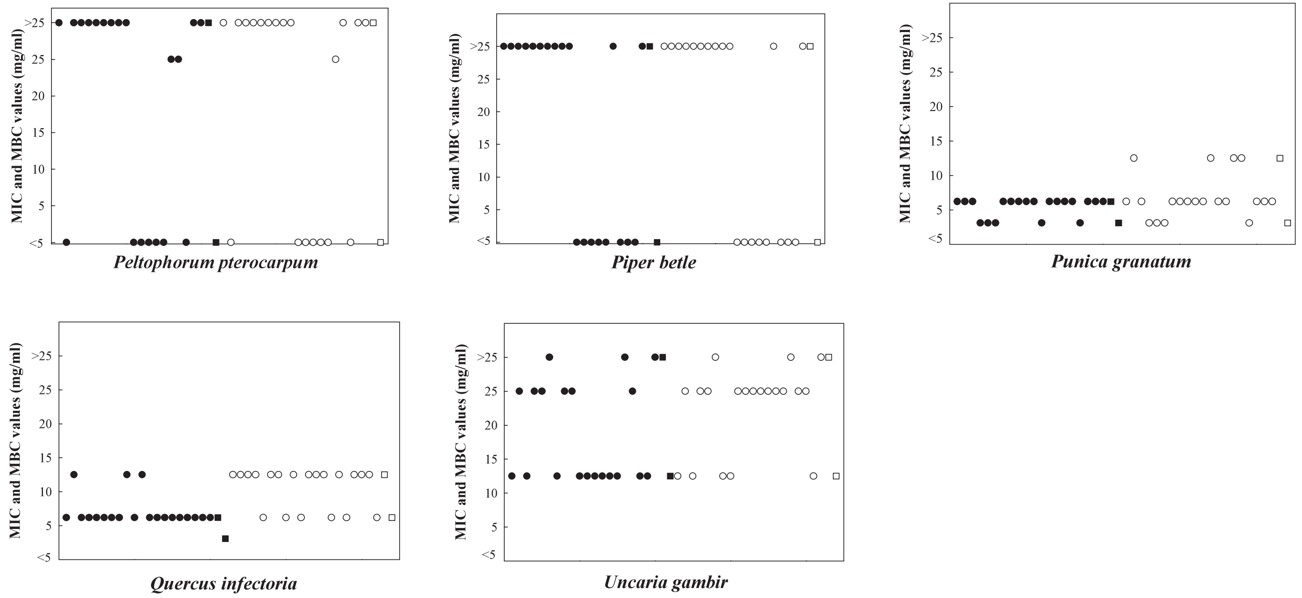

Fig. 2. The MIC and MBC of Aqueous Extracts from Medicinal Plants against *H. pylori*

The closed (●) and open (○) circles indicate the MIC and MBC, respectively, against 20 strains of *H. pylori* isolated from human gastrointestinal tracts. The closed (■) and open (□) squares indicate the MIC and MBC, respectively, against 2 reference strains (ATCC 43504 and ATCC 43579). Each plot represents the value against different strains of *H. pylori*.

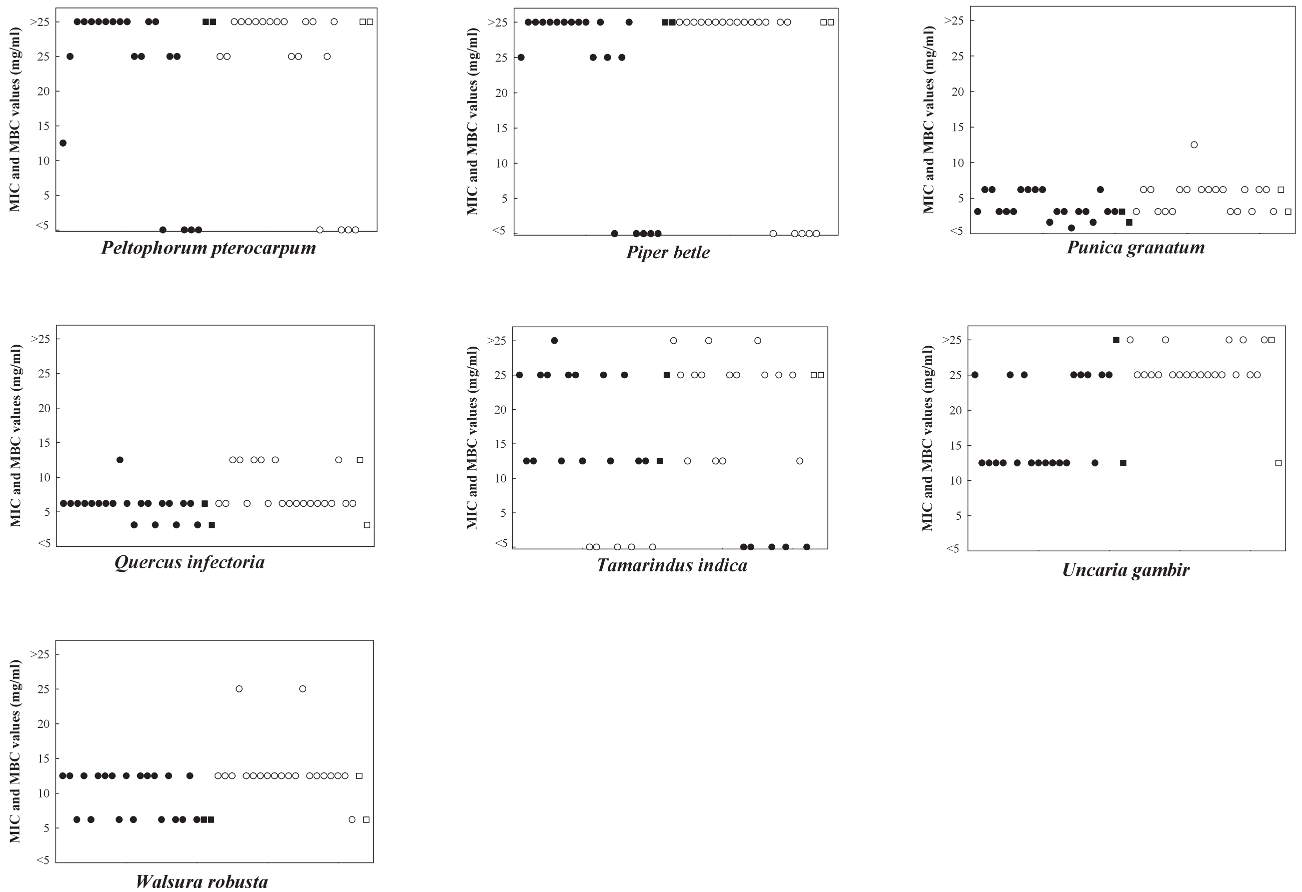

Fig. 3. The MIC and MBC of Ethanolic Extracts from Medicinal Plants against *H. pylori*

The closed (●) and open (○) circles indicate the MIC and MBC, respectively, against 20 strains of *H. pylori* isolated from human gastrointestinal tracts. The closed (■) and open (□) squares indicate the MIC and MBC, respectively, against 2 reference strains (ATCC 43504 and ATCC 43579). Each plot represents the value against different strains of *H. pylori*.

Table 3. MICs and MBCs of Partially-Purified Fractions of *P. granatum* and *Q. infectoria* against *H. pylori*

Effective fraction	MIC/MBC (mg/ml) on *H. pylori* strain				
	PSU HP 4	PSU HP5	UNSW 03-03-21-002	UNSW 03-03-25-001	ATCC 43504
P. granatum Fraction Pg3	0.32/0.32	0.32/0.32	0.16/0.16	0.16/0.16	0.32/0.32
Q. infectoria Fraction Qi2	0.16/0.16	0.16/0.16	0.16/0.16	0.16/0.16	> 2.5 /NA
Q. infectoria Fraction Qi3	0.32/0.32	0.16/0.16	0.32/0.32	0.16/0.16	0.32/0.32
Q. infectoria Fraction Qi4	0.32/0.32	0.32/0.32	0.32/0.32	0.32/0.32	> 2.5 /NA

NA = not applicable.

while Pg2 seemed to be a mixture of stigmasterol and fisitosterol (1 : 1), Pg3 is thought to be a pure compound belonging to the tannin group. Regarding the purified samples from *Q. infectoria*, Qi1 and Qi2 were suggested to contain aliphatic hydrocarbons with long alkyl chains and two phenolic components, respectively. Qi3 appeared to be a phenolic compound. Qi4, which contained active components, was obtained in a large quantity, but its further purification is currently underway. In this study, Pg3, Qi2, Qi3 and Qi4 were further examined for their anti-*H. pylori* activities.

Although the majority of individuals infected by *H. pylori* are asymptomatic, a proportion of them develop peptic ulcers. This organism is the major cause of gastric cancer and has been classified as a Class I carcinogen by the World Health Organization (WHO). It is well-established that an asymptomatic *H. pylori* infection could be a risk factor for gastric cancer. Due to the increasing rate of antibiotic-resistant organisms, many workers have attempted to eradicate the organism with natural products. Plants contain a number of organic components including alkaloids, flavones, phenols, quinines, terpenoids, and tannins, all of which are known to have antibacterial activity.[16] Among the plants that exhibited an antibacterial effect, *P. granatum* and *Q. infectoria* were the most efficient, probably due to the production of novel metabolites capable of inhibiting *H. pylori* growth. *P. granatum* has been extensively studied. This medicinal plant possesses a high amount of tannin (25%). The antimicrobial properties of this substance are well-established.[17] Recently, the antimicrobial properties of polar fractions, which contain ellagitannin and punicalagin, of *P. granatum*, were reported by other workers.[18] In contrast, very limited studies have been done on *Q. infectoria*, and thus this plant species is being brought into focus in this laboratory. Preliminary results from our laboratory indicate the activity is due to hydrolysable tannins.

Plants also contain a number of water-soluble proteins, lectins, and carbohydrates which may bind specifically to sugar residues, polysaccharides, glycoproteins or glycolipids such as adhesins present on the cell surface of *H. pylori*. Lengsfeld *et al.*[19] have demonstrated that adhesion of *H. pylori* to stomach sections is almost completely inhibited by pre-incubating with a fresh juice preparation of the fruit of the *Abelmoschus esculentus* (L.) Moench (okra plant). Many other plant extracts including turmeric, borage, and parsley were also reported to possess similar ability.[20] We have previously described that both *P. granatum* and *Q. infectoria* can increase the cell hydrophobicity of *H. pylori*.[21] Modulation of cell surface hydrophobicity by the plants may synergistically facilitate the elimination of the bacterial cells from the human body.

Our finding tentatively suggests important therapeutic implications for some herbal preparations with antibacterial properties for patients with *H. pylori*-induced PUD or gastric cancer. The high activity of both *P. granatum* and *Q. infectoria* against all strains of *H. pylori* could allow their use in the treatment of an *H. pylori* infection. The partially-purified fractions of *P. granatum* and *Q. infectoria* were the most effective against *H. pylori*, and had the same MIC and MBC values (0.16 mg/ml). In addition, many plants also have anti-ulcerogenic or anti-cancer effects. They may enable a treatment that is simple and relatively inexpensive by incorporation into the normal diet of the patient since the plants are already known to be safe and are commonly employed in traditional Thai medicine with

no toxicity having been reported. Alternatively, they could be used in combination with antibiotics, possibly increasing the success of eradication, as has been demonstrated earlier with cranberry juice.[22] However, many more studies are needed to confirm the *in vivo* effects of plant ingredients. Such information would be more important if administration of the pure forms of these substances to patients is desired. *Q. infectoria* is rather interesting since it is inexpensive and can be recovered at very high extract yields. This medicinal plant should be further analyzed as it might provide a new effective compound against *H. pylori* infections. Further studies are in progress in this laboratory to determine more precisely the effects of different fractions of the plant in order to provide an alternative treatment of *H. pylori* infection.

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