Traditional Thai Medicines inhibit *Helicobacter pylori in vitro* and *in vivo*: Support for ethnomedical use

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Research

Abstract

In Thailand, traditional plant-based medicines have always been used to treat gastrointestinal ailments, including gastritis, peptic ulcer disease (PUD) and diarrhea. Since *Helicobacter pylori* (HP) is an etiological agent of PUD, we have used an ethnomedical approach for screening plant extracts as potential treatments for HP infections, including over 20 species from Thailand. International Memoranda of Agreement were established between UIC and Mahidol University in Thailand. Medicinal plants were collected, identified and extracted. Susceptibility testing was performed with 15 HP strains using the agar dilution procedure guidelines of the Clinical and Laboratory Standards Institute. *In vivo* studies included evaluating bacterial load, as well as acute and chronic inflammation in HP-infected Mongolian gerbils. Extracts of *Curcuma longa* L. and *Boesenbergia rotunda* (L.) Mansf. significantly reduced HP-induced gastric lesions, as assessed both macroscopically and microscopically in Mongolian gerbils. The treatments reduced acute and/or chronic inflammation in a prevention model of HP-induced gastritis.

Introduction

Although the incidence and mortality of gastric cancer has declined in industrialized nations, it still remains the second leading cause of cancer deaths worldwide, particularly in Southeast Asia (Jemal *et al*. 2002, Kneller *et al*. 1992). Globally, as many as 1 million people die each year of gastric cancer (Anon 2006). Gastric cancer evolves through a multi-step mechanism in which the Gram-negative bacterium *Helicobacter pylori* (HP) plays a major role (IARC 1994).

Helicobacter pylori (HP) is a spiral or helical-shaped aerobic bacillus that colonizes the gastric epithelial surface, and can withstand the stomach's environment by microaerophilic growth capability and high urease activity (Forman & Graham 2004). Chronic HP infections are associated with the development numerous gastrointestinal disorders, including dyspepsia, duodenal and gastric ulceration, gastric cancer, and mucosa-associated lymphoid tissue (MALT) lymphoma (Breuer-Katschinski *et al*. 1999, Figueriredo *et al*. 2002, Forman *et al*. 2004). In 1994, the International Agency for Research on Cancer classified HP as a human carcinogen, and a definite cause of gastric cancer (IARC 1994).

According to statistics from the World Health Organization, approximately 50% of the population in industrialized nations, and 80% of the populations in developing countries infected with HP (Perez-Perez *et al*. 1990). In Thailand, the incidence of HP infection is similar to other countries in Southeast Asia, with HP antibodies present in approximately 17.5% of children ages 5-9 years, but increasing to 75% in the 30- to 49-year-old group (Mahachai *et al*. 2000). However, although there is a high preva-

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lence of HP infection in Thailand, the incidence of gastric atrophic changes, intestinal metaplasia, and gastric cancer are significantly lower than other countries (Atisook *et al*. 2003, Matsuhisa *et al*. 2003, Sriamporn *et al*. 2002). The reasons for this paradox are not well understood but appear to be related to diet and life style (Bhamarapravati *et al*. 2003). The diet in Thailand includes a wide range of unique fruits, vegetables and spices, many of which are also used ethnomedically for the treatment of gastrointestinal disorders (Murakami *et al*. 1994).

Ethnobotanical searches and reviews Traditional Thai medical texts, as well as the Napralert database for plants used in Thailand for the treatment of gastrointestinal and HP infections, led to a list of over 50 plant species, of which 20 (as 25 plant parts) were collected in Thailand for testing. The plant parts were extracted and the extracts tested for anti-HP activity *in vitro*, of which 12 extracts were shown to be active *in vitro* (13). Here, we report new *in vitro* and *in vivo* data on two Thai plants from the Zingiberaceae, namely finger-root (*Boesenbergia rotunda* (L.) Mansf.) and turmeric (*Curcuma longa* L.), both of which are used in Thailand for the treatment of gastrointestinal ailments, including peptic ulcer disease (Mahady 2005).

Materials and Methods

Reviews of Traditional Thai medical texts and the Napralert database for plants used in Thailand for the treatment of gastrointestinal and HP infections were performed as described (Bhamarapravati *et al*. 2003). These searches led to a list of over 50 plant species from Thailand, of which the results of 20 were previously reported (Bhamarapravati *et al*. 2003)(Figure 1). Two plant species were selected for further *in vitro* and *in vivo* testing: *Boesenbergia rotunda* (finger-root) and *Cucurma longa* (turmeric) based on their use in Traditional Thai medicine for the treatment of gastrointestinal ailments.

The rhizomes of these plants were collected in Thailand after obtaining a Memorandum of Agreement between the University of Illinois at Chicago and Mahidol University, Bangkok, Thailand. Five hundred grams of finger-root rhizomes and 1 kg of dried turmeric rhizomes were obtained from Jatujak market, Jatujak, Bangkok, Thailand. The collected plants were identified by Ms. Thaya Jenjittikul, Department of Plant Science, Faculty of Science, Mahidol University, Bangkok, Thailand. Herbarium specimens were deposited at Mahidol University, Faculty of Science. The outer surface of the rhizomes were cleaned with soap and water, the plant parts were cut into 1 cm slices and then dried in 55^{0}C hot air oven for 24-48 h. The dried rhizomes were then ground to a fine powder and extracted twice in an Erlenmeyer flask containing 95% ethanol (2 x 500 ml) at room temperature on a rotary shaker for 24 h. Each extract was filtered through Whatman no. 1 filter paper and the resultant filtrate was concentrated to dryness under reduced pressure. The extracts were then defatted with 500 ml of hexane and the resulting defatted extracts were concentrated to dryness under reduced pressure. The concentrated extracts were stored in coded sterile borosilicate glass vials under desiccation at -20ºC. A 10.0 mg sample of each plant extract was used for the *in vitro* susceptibility testing against the various HP strains. For the animal studies, 100 grams of the extracts were prepared.

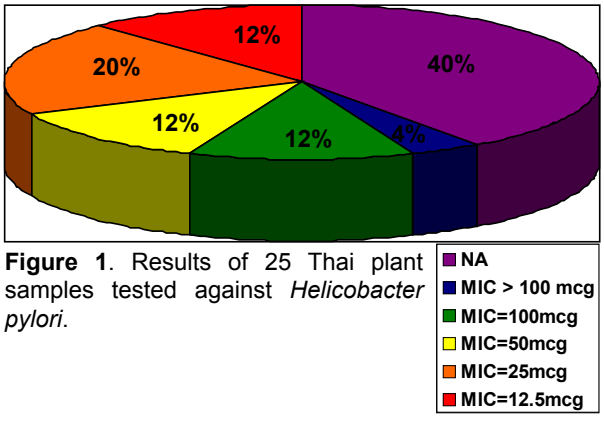

Figure 1. Results of 25 Thai plant samples tested against *Helicobacter pylori*.

In Vitro susceptibility Testing

Susceptibility testing was performed using the agar dilution procedure guidelines of The National Committee for Clinical Laboratory Standards (NCCLS 2005). The plant extract were dissolved using a minimal volume of solvent. The solvents used to dissolve the extracts were dimethyl sulfoxide (DMSO), ethanol, or sterile distilled water. Sterile distilled water was used for further dilutions (serial) of the dissolved plant extracts. Final concentrations of the extracts tested included 100, 50, 25, and 12.5 µg/ml for each sample. The *in vitro* HP susceptibility tests were performed exactly as previously described (Bhamarapravati *et al*. 2003, Mahady *et al*. 2003a,b). A total of five cagA+ strains of HP were tested, rodent adapted strain B128, M23-3, GTD7-13, G1-1, SS1 and the American Type Culture Collection (ATCC) (Rockville, MD) strain (#43504) of HP were used in the susceptibility testing. Clinical isolates were obtained from Dr. Rick Peek, Division of Gastroenterology, Vanderbilt University, Nashville, TN. The identification of each organism has been confirmed by Gram stain appearance and a positive urease test. *Staphylococcus aureus* ATCC 29213 was included in the susceptibility testing of the plant extracts and amoxicillin for validation of results.

An inoculum of each isolate was prepared by suspending the organism in 4.5 ml of sterile tryptic soy broth and adjusting the turbidity to that of a 2.0 McFarland Standard using a spectrophotometer at 625 nm. This density produces a suspension of approximately 1 x 106 CFU/ml of HP. The organisms were inoculated onto agar plates containing the plant extracts via a 32-prong inoculating device. The device delivers 8 µl per spot resulting in a final inoculum of approximately 1 x 104 CFU/spot. After the spots dried, the

plates were incubated at 37^{0}C in 10% CO2 and examined for growth after 5 days. All procedures were performed in duplicate. The minimum inhibitory concentration (MIC) was determined for each plant extract. The MIC is defined as the lowest concentration of an extract or compound at which there is no visible growth (Mahady *et al*. 2003). To ensure that the vehicle did not affect HP growth, minimal to maximal volumes of vehicle solvent were also tested. For quality control and comparative analysis, the antibiotics amoxicillin and metronidazole were also tested with each batch of plant extracts, as a positive control. For the animal studies, the cagA+ HP strain B128 was maintained as previously described (Israel *et al*. 2001).

Mongolian gerbil model

Specific pathogen-free male Mongolian gerbils (Charles River, Wilmington, Massachusetts, USA), 6 weeks old, were housed in an air-conditioned biohazard room with a 12 h light-dark cycle, and were fed a commercial diet (PURINA 5100) and water ad libitum for 24 hours. The animals were handled according to the guidelines of the AAC and the protocols for this study were approved by the Institutional Animal Care Committee, at the University of Illinois at Chicago prior to the initiation of the work.

In the four-armed study, group A (n = 10) negative control group and did not receive the treatment or HP challenge. Group B (n =10) served as a positive control and received the HP-challenge but no treatment. Both groups A and B received a standard diet chow (Purina 5100). Groups C and D were the treatment groups and were fed the same chow supplemented with the plant extracts (100 mg/kg body weight) for 3 weeks prior to challenge with HP, and then 6 more weeks after challenge. At 9-weeks of age, all animals except Group A (10 negative controls) were fasted for 12 h and then inoculated with the rodent-adapted HP cag+ strain B128 by gavage (0.5 ml, 1.7 × 106 CFU/animal). Negative control animals were given sterilized broth alone (n = 10). After inoculation, each animal was fasted for 4 h and then fed a basal diet or basal diet supplemented with turmeric extract or finger root extract (dose calculated at 100 mg/kg body weight/day for six weeks). Body weights, as well as diet and water intake were measured three times a week, respectively, and animals were monitored daily for their general health.

Six weeks post-infection, all animals were sacrificed under CO2 euthanasia and their stomachs were resected, opened along the greater curvature, and washed twice with saline. The macroscopic gastric lesions (edema and hemorrhage) in the glandular stomach were recorded, and the wet weight of the whole stomach was measured. The glandular stomach was divided into halves, and one half was fixed in 10% neutral buffered formalin for histological examination, and the other half was transferred to 1.0 ml of sterile 0.1 M phosphate buffered saline (PBS, pH 7.4), homogenized, and plated on selective Trypticase soy agar/5% sheep blood plates containing vancomycin (20 ug/ml), nalidixic acid (10 µg/ml), bacitracin (30 µg/ml), and amphotericin B (2 µg/ml) (Sigma Chemical Company, St. Louis, MO) and grown for 3 to 5 days, as previously described (19). After 5 days, the colonies were counted to determine the level of HP colonization of each stomach. Colonies were identified as HP based on their characteristic morphology, and by urease and oxidase activities; colony counts were expressed as log CFU per stomach. Colonization density was determined by quantitative culture and acute inflammation and chronic inflammation (mononuclear cell infiltration independent from lymphoid follicles) were each graded from 0-3 in the gastric antrum, as previously described (Israel *et al*. 2001; Table 1).

Statistical analyses

An unpaired t test or a Mann-Whitney U test was applied to determine the differences in quantitative data for gastric lesions and HP infection between two groups. P values of <0.05 were considered to be statistically significant.

Results

Thai medicinal plants for gastrointestinal ailments

Searches of the ethnomedical Thai literature and the Napralert database resulted in a list of over 50 plant species used in Thailand for the treatment of gastrointestinal ailments. Prioritization of the 50 plant species for collection and testing purposes was performed by associating each of the plant species with previously published experimental reports of antibacterial, anti-ulcer, anti-inflammatory and anti-oxidant activities. This resulting prioritization led to a list of 25 plants that were top priority for test-

Table 1. Grading criteria for chronic inflammation and activity of gastritis

Grade	Chronic inflammation	Activity
0	No increase in number of inflammatory cells	Same
1	Uniform infiltration of lamina propria by lymphocytes, plasma cells, and some eosinophils	Scattered neutrophils in the lamina propria with no leukopedesis in the region of the gastric pits
2	Moderately dense infiltration of the lamina propria by lymphocytes and plasma cells	Moderate number of neutrophils in the lamina propria with microabscess in the region of gastric pits
3	Very dense lymphoplasma cell infiltration in the lamina propria	Extensive neutrophils in the lamina propria with obvious cryptitis.

Table 2. Top priority plant samples that were tested.

Plant taxa sampled	Thai name	Parts sampled (source)
Artemisia vulgaris L. (Asteraceae)	โกฐจุฬาลำ	leaf[1]
Barringtonia acutangula (L.) Gaertn. (Lecythidaceae)	จิกนา	leaf[1]
Boesenbergia rotunda (L.) Mansf. (Zingiberaceae)	กระชาย	rhizome
Bridelia ovata Decne. (Euphorbiaceae)	มะกา	leaf[1]
Cassia grandis L.f. (Fabaceae)	กาฬพฤกษ์	leaf[1]
Cleome viscosa L. (Capparaceae)	ผักเสี้ยนผี	leaf[4] & stem[4]
Curcuma longa L. (Zingiberaceae)	ขมิ้นชัน	rhizome
Cycas siamensis Miq. (Cycadaceae)	ตาลปัตรฤาษี	spongy integument[2] & sarcotesta[2]
Dioscorea hispida Dennst. var. *hispida* (Dioscoreaceae)	มันกลอย	aerial tuberous stem[1]
Kaempferia galanga L. (Zingiberaceae)	หอมเปราะ	rhizome[2]
Litsea elliptica Blume (Lauraceae)	ทำมัง	leaf[1]
Melaleuca quinquenervia (Cav.) S.T. Blake (Myrtaceae)	เสม็ด	leaf[6]
Moringa oleifera Lam. (Moringaceae)	มะรุม	bark[3]
Myristica fragrans Houtt. (Myristicaceae)	จันทน์เทศ	aril[1]
Myristica sp. (Myristicaceae)	จันทน์ป่า	aril[1]
Persicaria chinensis (L.) H. Gross. (Polygonaceae)	เอื้องเพ็ดม้า	leaf[5] & aril[5]
Piper retrofractum Vahl (Piperaceae)	ดีปลี	leaf[1]
Pouzolzia pentandra (Roxb.) Benn. (Urticaceae)	ขอบชะนางแดง	leaf[4]
Syzygium aromaticum (L.) Merr. & L.M. Perry (Myrtaceae)	กานพลู	leaf[1]
Zingiber officinale Roscoe (Zingiberaceae)	ขิง	rhizome

[1]One Hundred Year Herbal Garden, Ministry of Public Health, Makham, Chantaburi, Thailand, February 2001.
[2]Jatujak market, Jatujak, Bangkok, Thailand, February, 2001.
[3]Author's garden, Bangkhen, Bangkok, Thailand, February 2001.
[4]Siri Rukachard Herbal Garden, Mahidol University, Salaya, Nakornpathom, Thailand, February 2001.
[5]Salaya, Nakornpathom, Thailand, February 2001.
[6]Baan Phe, Rayong, Thailand, February 2001.

ing (Table 2). Twenty-one of these plants were previously tested *in vitro* (Bhamarapravati *et al*. 2003). Here, we report that two additional species on the priority list, namely *Boesenbergia rotunda* and *Curcurma longa*, were also found to be active both *in vitro* and *in vivo*, bringing the hit rate to 56% thereby validating this ethnomedical screening approach (Figure 1).

In vitro susceptibility

The ethanol extracts of Thai turmeric and finger-root rhizomes inhibited the growth of all 6 CagA+ strains of HP *in vitro*. The MIC ranges for both finger-root and turmeric were 3.125 – 6.25 µg/ml (Table 3). Thus, these two plants were very active *in vitro*, and ranked in the top 10% of all Thai plants tested (Figure 1).

Effects in Mongolian gerbils

In this work, we investigated whether the treatment of HP-infected Mongolian gerbils with finger-root or turmeric extracts reduced bacterial load, as well as acute and chronic inflammatory responses to HP infection. Administration of the finger-root or turmeric extracts in the chow for 9

Table 3. Minimum inhibitory concentrations of 95% ethanol extracts of *Boesenbergia rotunda* and *Curcuma longa* rhizomes in 6 CagA+ strains of *Helicobacter pylori*. Data are the result of three experiments in triplicate.

Thai plant extracts	Minimum Inhibitory Concentration (MIC µg/ml) CagA+ HP strains					
	M23-3	GTD7-13	G1-1	SS1	ATCC 43504	B128
Boesenbergia rotunda	3.125	3.125	3.125	3.125	3.125	3.125
Curcuma longa	6.25	12.5	12.5	6.25	6.25	6.25

Table 4. Reduction in acute and chronic inflammatory parameters, erosion and cryptitis induced by *Helicobacter pyrlori* infection in Mongolian gerbils.

Treatment Group (n)	Epithelial Cell Degeneration	Erosion	Cryptitis	Chronic Inflammation		Acute Inflammation	
				Mucosal	Submucosal	Mucosal	Submucosal
A (10)	0	0	0	0	0	0	0
B (10)	2.0	2.0	1.1	2.5	1.8	2.2	1.5
C (10)	0.3**	0.2**	0.25**	1.0*	0.3**	0.7*	0.1*
D (10)	1.5	1.0*	0.5*	1.2*	0.8*	2.0	0.9
Group A: no treatment and no *Helicobacter pylori* challenge. Group B: *Helicobacter pylori* challenge but no treatment. Group C: *Helicobacter pylori* challenge treated with finger-root extract. Group D: *Helicobacter pylori* challenge treated with turmeric extract.						* P < 0.05	** P < 0.01

weeks, at a dose of 100 mg/kg bw, did not adversely affect food intake or body weights of the gerbils. The results of this experiment are shown in Table 4. In Group A, no HP was be detected, nor was any inflammation observed. In Group B, 80% of animals inoculated with HP were infected and developed severe gastritis with edema and hemorrhage in the antrum. Microscopic erosion with infiltration was observed with marked infiltration of inflammatory cells in the lamina propria and submucosa. This infiltration was predominantly polymorphonuclear leukocytes and lymphocytes, although some macrophages and neutrophils were also observed. Gastric changes were also severe in the pyloric region, but moderate in the fundic region. The average microscopic score for gastritis of the HP-inoculated control animals (Group B) was 2.5, while in the negative control animals (Group A), the average microscopic score was 0 (Table 4). The average stomach weight of control gerbils inoculated with HP was approximately 1.5-fold of that for animals without inoculation.

Treatment with the finger-root extract for 9 weeks significantly reduced HP load in challenged Mongolian gerbils as compared with Group B ($p < 0.05$; Figure 2). The parameters of HP-induced acute and chronic inflammation were also significantly ($p < 0.05$) decreased in Group C as compared with the HP-challenged untreated Group B. (Table 4). Both chronic (mean: 2.5 vs. 1.0; control vs. extract, respectively) and acute mucosal (mean: 2.2 vs. 0.7; control vs. extract, respectively, $p < 0.05$) inflammation scores, as well as acute and chronic submucosal inflammatory parameters (mean: 1.8 vs. 0.3 and 1.5 vs. 0.1 control vs. extract respectively, $p < 0.01$) were decreased in gerbils treated with the finger-root extract. These changes were paralleled by significant reductions in the severity of epithelial cell degeneration (2.0 control vs. 0.3 extract, $p < 0.05$), cryptitis (1.1 control vs. 0.25 extract, $p < 0.05$) and erosions (2.0 control vs. 0.2 extract, $p < 0.01$). Thus, treatment significantly reduced both acute and chronic muscosal and submucosal inflammation, cryptitis, as well as epithelial cell degeneration and erosion induced by HP infection (Table 4). Importantly, the extract did not increase morbidity or mortality of the animals.

Treatment with the turmeric extract (Group D) did not significantly reduce bacterial load or acute inflammatory parameters as compared with group B, but did significantly ($p < 0.05$) reduce HP-induced chronic mucosal and submucosal inflammation, cryptits, and erosion. Chronic mucosal (mean: 2.5 vs. 1.2; control vs. extract, respectively, $p < 0.05$) and submucosal inflammatory parameters (mean: 1.8 vs. 0.8, control vs. extract respectively, $p < 0.05$) were decreased in gerbils treated with turmeric (Table 4).

Discussion

Thailand has long incorporated the use of food and spice plants into everyday medical practice. This custom was introduced to Thailand with Buddhism 700-1000 years ago (Bunyapraphatsara 1990). While the practice was interrupted during the wars in Thailand in the mid-eighteenth century, King Rama III later revived it again, in 1821 (Bu-

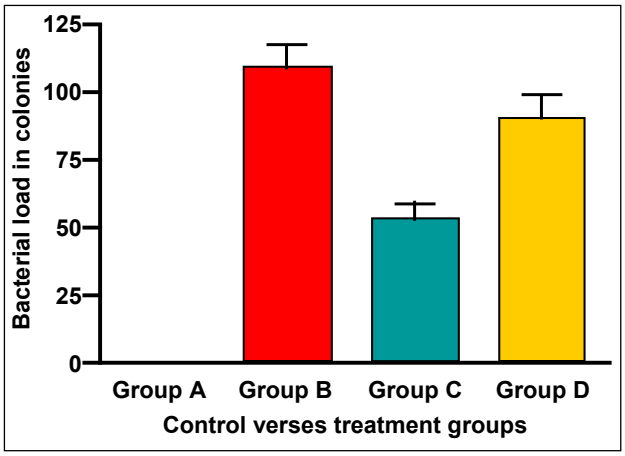

Figure 2. Reduction in bacterial load per treatment group. Group A, Monogolian gerbils were not challenged with HP; Group B, Mongolian gerbils challenged with HP but received no treatment; Group C, Mongolian gerbils challenged with HP but received treatment of 100 mg/kg b.w. of finger-root extract ($p < 0.05$); Group D, Mongolian gerbils challenged with HP and received treatment of turmeric extract (100 mg/kg b.w.)

nyapraphatsara 1990). Hundreds of stone plaques inscribed with knowledge of all kinds including medical texts, were established at Wat Photharaam, an old temple built during the Ayuthaya period. Royal medical texts were complied in 1871 by a committee of medical doctors appointed by King Rama V were revised and published, and are the basis for Thai traditional medicine practice today (Bunyapraphatsara 1990). This ethnomedical approach to healthcare is currently promoted by the Thai government, through the Ministry of Health. Thus, unlike the West, ethnomedicine in Thailand is not an alternative, but is actually integrated into standard medical care, as for many people in Thailand become the mainstay of pharmacotherapy.

Curcuma longa (turmeric) has been used in Thailand for hundreds of years for the treatment of peptic ulcer disease (Mahady *et al*. 2002). Curcumin (Figure 3), one of the polyphenolic chemical constituents derived from turmeric rhizomes has been shown to prevent gastric and colon cancers in rodents. Many mechanisms have been proposed for the chemopreventative effects, however here we demonstrate that turmeric inhibits the chronic inflammatory responses to HP infection, as well as erosion and cryptitis *in vivo*. Interestingly, the chemical structure of the curcumin is similar to that of the gingerols from Zingiber officinale (ginger), that are also very active against HP infections (Figure 2; Mahady *et al*. 2003a).

The second plant species tested in this work, *Boesenbergia rotunda* (L.) Mansf. (Zingiberaceae, finger-root) was also very active both *in vitro* and *in vivo*. Finger-root is used frequently in Thai cooking as a culinary spice, as well as ethnomedically to treat gastrointestinal ailments and oral diseases. In this investigation, finger-root extracts reduced HP load and the acute and chronic inflammatory response to HP challenge in the Mongolian gerbil model. The HP strain used for this investigation was B128, a cag+ strain of HP. CagA is the strain-specific HP gene that is linked to the development of premalignant and malignant histological lesions, and thus infections caused by cagA+ strains significantly increase the risk for developing severe gastric inflammation, atrophic gastritis and noncardia gastric adenocarcinoma (Censini *et al*. 1996). Loss or inactivation of the entire cag locus profoundly attenuates the severity of gastritis and development of atrophy in Mongolian gerbils infected with *H. pylori* (Magari *et al*. 2005). Thus, cag+ strains induce more inflammation than cag- strains and chronic inflammation is thought to play a central role in the accumulation of genetic events leading to transformation and cancer by increasing the number of target cells or by promoting the proliferation of initiated cells. (Magari *et al*. 2005).

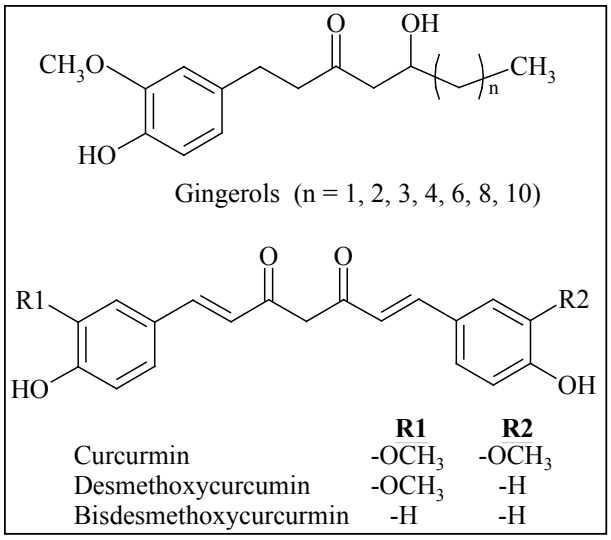

Figure 3. Structures of the gingerols, the anti-HP constituents of ginger (*Zingiber officinale*) and the curcumins, the active constituents of turmeric (*Curcuma longa*).

Conclusions

Our investigations have demonstrated that the ethnomedical use of specific Thai plants for the treatment of gastrointestinal disorders have a plausible mechanism of action in that they inhibit the growth of HP *in vitro* and *in vivo*. In fact, using an ethnomedical approach to screen for inhibitors of HP, led to a 56% hit rate, of which 78% of the plant extracts were very active with a an MIC of < 50 µg/ml *in vitro*. More importantly, in experiments with Mongolian gerbils, finger-root significantly reduced bacterial load, and both finger-root and turmeric treatments significantly reduced the acute and chronic inflammatory response to HP infections. These data suggest that not only can these Thai plants be used for the prevention and treatment of HP infections and they may also be chemopreventative against gastric cancer, which may explain the lower incidence of gastric cancer in Thailand.

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