

Antibacterial activity of *Boesenbergia rotunda* (L.) Mansf. and *Myristica fragrans* Houtt. against *Helicobacter pylori*

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Abstract

Bhamarapravati, S., Juthapruth, S., Mahachai, W. and Mahady, G.
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Songklanakarin J. Sci. Technol., 2006, 28(Suppl. 1) : 157-163

Helicobacter pylori, a gram-negative bacterium, is recognized as the primary etiological agent for the development of gastritis, dyspepsia, peptic ulcer as well as gastric and colon cancer. In developing countries the incidence of *H. pylori* infection ranges from 50-100%. Two Thai plants, namely *Boesenbergia rotunda* (L.) Mansf. and *Myristica fragrans* Houtt., have been used to treat dyspepsia and peptic ulcer in Thai Traditional Medicine. Their crude extracts were previously reported to possess anti-*H. pylori* activity. This investigation proposed to test previously isolated bioactive compounds from *B. rotunda* and *M. fragrans* if they possessed anti-*H. pylori* activity. Primary cultures of *H. pylori* from local hospital patients in Thailand were used in the investigation. *In vitro* anti-*H. pylori* testing had been performed with pinostrobin and red oil from roots of *B. rotunda*, and dihydroguaiaretic acid from arils of *M. fragrans*. Clarithromycin (MIC 120 µg/mL) was used as a positive control. All three compounds showed positive clear zone in agar diffusion test at p<0.05 in all

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Received, 1 September 2004 Accepted, 21 December 2005

10 clinical cultures. Pinostrobin, red oil and dihydroguaiaretic acid autoclaved in blood agar medium had MIC of 125, 150, 100 µg/mL and MBC of 150, 175, 125 µg/mL, respectively. All three compounds have their activities against *H. pylori* in the same range of that of drug currently used in the treatment of peptic ulcer. Thus, all three compounds from *B. rotunda* and *M. fragrans* show good potential for further drug development. This investigation demonstrates that food and spice plants used in Thai Traditional Medicine for treatment of dyspepsia and peptic ulcer contain compounds which inhibit the growth of *H. pylori in vitro*. The result suggests that ingredients of some Thai food in regular diet may contribute to the low incidence of gastric cancer in the Thai population by affecting the growth of *H. pylori*.

Key words : antibacterial activity, *Boesenbergia rotunda* (L.) Mansf., *Myristica fragrans* Houtt.

Helicobacter pylori (HP) was first isolated in 1982 by Marshall and Warren in Western Australia from gastric mucus of patients with chronic gastritis and duodenal ulcers (Marshall, 1983), later named as a new genus *Helicobacter*.

In laboratory conditions, these bacterial strains typically grow under strictly microaerobic condition at 37°C. *Helicobacter* will grow at 37°C on a variety of rich agar bases supplemented with 5% whole blood or serum.

HP is well recognized as being the primary etiological agent responsible for the development of gastritis, dyspepsia, peptic ulcer disease and gastric cancer. In developing countries, a high prevalence of HP infection is associated with an increased incidence of gastric cancer (Frenck and Clemens, 2003). Thailand, however, while having a high prevalence of HP infections, has a lower than expected gastric cancer rate than other developing countries. It has been suggested that diet contents and life style of the Thais may explain this discrepancy (Bhamarapavati et al., 2002).

The diet in Thailand includes a wide range of vegetables and spices indigenous to the region, e.g. chili pepper, galangal, black pepper, finger root and mace. Root and rhizome of finger root plant, *B. rotunda* (L.) Mansf., and mace (aril of *M. fragrans* Houtt.) are used both as food and in Traditional Thai Medicines to treat stomach discomfort and peptic ulcer. Crude extract of these two plants were reported to have chemopreventive and anti-*H. pylori* activities (Bhamarapavati et al., 2003a,b).

Bhamarapavati et al. (2000) showed methanolic/dichloromethane extract of *B. rotunda*

partitioned in chloroform possessed antidermatophytic activity against *Epidermophyton floccosum*, *Microsporum gypseum* and *Trichophyton mentagophyte*. The most active compound from hexane/ethylacetate column chromatography, yellowish crystal, was identified as a flavonoid compound named pinostrobin (Figure 1). Sakaengarm et al. (2003) also reported from our laboratory the isolation of dihydroguaiaretic acid (Figure 1) from crude mace extract. It possessed antifungal activity against *Collectotrichum gloeosporioides* from mango host.

This investigation determined the susceptibility of 10 Thai clinical strains of *H. pylori* against bioactive compounds from root of finger root *B. rotunda* and aril of *M. fragrans*, namely pinostrobin (P) and dihydroguaiaretic acid (DGA). Red oil (RO), a mixture of a few minor compounds in the adjacent fraction from pinostrobin in *B. rotunda* root extract was also tested.

Materials and Methods

Compounds

B. rotunda rhizomes and roots were purchased from Nakornpathom Province, Thailand, in June 2002. P and RO were extracted and fractionated from fascicled roots of *B. rotunda* (Bhamarapavati et al., 2000, Jianpermpoolpol et al., 2003).

Dried aril of *M. fragrans* was purchased from Chaokom-pure spice shop, Bangkok, Thailand, in March 2002. DGA was obtained from dried aril of *M. fragrans* using chromatographic methods on silica gel column (Sakaengarm et al., 2003).

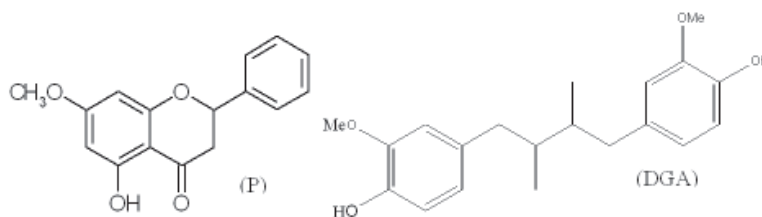


Figure 1. The structure of the pinostrobin (P) (Bhamarapavati *et al.*, 2000) and 4,4'-dihydroxy-3,3'-dimethoxylygnan (dihydroguaiaretic acid or DGA) (Sakaengarm, 2003)

Bacterial Isolates

The specimens were obtained from patients at Internal Medicine Department, Chulalongkorn University Hospital, Bangkok, Thailand, during the period between May 2001 and November 2002. Ten patients with informed consent having upper gastrointestinal symptoms, namely gastritis, dyspepsia and peptic ulcer were enrolled in the study of antibacterial activity of compounds *B. rotunda* and *M. fragrans* against *H. pylori*. All project participants had not been subjected to eradication therapy in the previous two months.

All patients underwent upper gastrointestinal endoscopy as a part of normal investigation. Two biopsy specimens taken as a part of normal investigation were taken from the antrum and the gastric body of each patient. Each biopsy specimen was placed in 0.1 mL transport medium and sent to the laboratory.

Culture and Identification Method

Gastric biopsy specimens were inoculated on selective medium brain heart infusion agar plus 7% sheep blood with antibiotics (vancomycin and amphotericin B) and on one without antibiotic. Plates were incubated at 37°C for 48-72 h in an anaerobic jar (BBL Microbiology Systems, Cockeysville, MD) under microaerophilic conditions. The plates were visually inspected for small, translucent and glistening colonies with a convex elevation and entire edge.

Determination of Urease Activity Assay

Bacterial suspension (50-100 μ L) was added to a cuvette containing 0.25-1.0 mL of a 3 mmol

phosphate buffer solution (PBS) pH 6.8, 0.1 mL of phenol red (7 μ g/mL), and 0.4 mL of urease (330 μ g/mL). Color intensity was measured at different time intervals using a spectrophotometer (Shumatzu, Japan) at 560 nm wavelength.

Evaluation of Compounds

Anti-*H. pylori* Activity

Each *H. pylori* was grown on blood agar plates for 3 d under microaerophilic conditions. Suspensions of the cultures were prepared in sterile saline phosphate buffer. Fresh blood agar plates were seeded with 0.1 mL of bacterial suspensions (total of $1 \times 10^7 - 10^8$ cells per plate).

The method used was modified from that of Malekzadeh *et al.* (2001) and Fukai *et al.* (2002). Each compound (200 μ g/mL in DMSO) was saturated on 6 mm diameter paper discs (BBL, Becton Dickinson, Cockeysville, MD). Each disc was air dried, then placed on a lawned plate. Discs saturated with distilled water and clarithromycin (CLAR), respectively, were served as negative and positive controls. All plates were incubated under microaerophilic conditions at 37°C for 72 h. Then, diameter of inhibition zone on each plate was recorded. The same procedure was used to test all 10 clinical isolates in triplicate.

Minimum Inhibitory Concentration (MIC) and Minimum Bacteriocidal Concentration (MBC)

The test compounds were initially dissolved in DMSO and then further diluted with DMSO. Paper discs were saturated with 25, 40, 75, 100, 150, 175 and 200 μ g/mL of each compound and let dried. Each disc was placed on blood agar with

5% defibrinated sheep blood containing 1×10^7 - 10^8 cells and incubated under microaerophilic conditions for 72 h. MIC of each compound on each bacterial strain was determined as the minimum concentration inhibiting visible *H. pylori* growth at 72 h. MBCs were established by the minimum concentration showing lack of growth upon re-inoculation of *H. pylori* from compound-treated plates to blood agar plates after 72 h.

Results and Discussion

Patients participating in this study came to the hospital showing upper gastrointestinal symptoms, namely gastritis, dyspepsia and peptic ulcer. They were all of Thai nationality. The median age of the consenting patients was 45 years; ranging from 16 to 89 years. Gender information was not included for this study. Age range of the patients in this study was in accordance with the report by Frenck and Clemens (2003). Frenck and Clemens stated that while prevalence of *H. pylori* infection had dropped significantly in many parts of North America, Western Europe and Korea, *H. pylori* infection in developing countries occurred earlier in life, and gender did not seem to be related to this infection.

Anti-*H. pylori* Activity

Results from anti-*H. pylori* activity of the compounds from the two Thai plants are shown in Table 1.

Results from Table 1 show that DGA from aril of *M. fragrans* was the most active compound among all compounds tested, including clarithromycin. The results were consistent in all *H. pylori* clinical strains tested. P and RO both showed significant clear zone. When treatment from all clinical isolates were accounted for, the results were all significantly different at $p \leq 0.05$ using ANOVA (Table 2).

DGA and P both inhibited growth of *H. pylori* after 3 h exposure. Visible colony of *H. pylori* could not be detected after incubation for 10 d on blood agar plates in both treatments (data not shown). No growth of any microbes was detected visually in DGA and P tested plates at day 10.

RO inhibited growth of *H. pylori* after 3 h exposure. Visible colonies of *H. pylori* could not be detected after incubation for 10 d on blood agar plates (data not shown). However, RO tested plate at day 10 contained colonies of other bacteria. Judging by the colony morphology all bacterial colonies seemed to be normal flora of the gastric area, i.e., *Escherichia coli*. Urease test from the

Table 1. Anti-*H. pylori* activity of pinostrobin, red oil, and dihydroguaiaretic acid against ten clinical isolates of *H. pylori* from Thailand.

| Isolate Number | Diameter of Inhibition Zone (mm) ^a | | | | |
|----------------|---|-------------------|----------------|-----------------|------------------|
| | Water | CLAR ^b | P ^b | RO ^b | DGA ^b |
| 1 | 0 | 28 | 22 | 15 | 32 |
| 2 | 0 | 25 | 23 | 16 | 35 |
| 3 | 0 | 27 | 21 | 12 | 33 |
| 4 | 0 | 26 | 22 | 12 | 34 |
| 5 | 0 | 28 | 23 | 11 | 35 |
| 7 | 0 | 27 | 25 | 13 | 35 |
| 8 | 0 | 28 | 25 | 14 | 34 |
| 9 | 0 | 25 | 26 | 16 | 32 |
| 10 | 0 | 26 | 22 | 12 | 33 |

^a Rounded up mean from triplicate samples.

^b Concentration 200 µg/mL.

Table 2. Anti-*H. pylori* activity effect seen as average inhibition zone.

| Compound 200 µg/ml | Diameter of Inhibition Zone* (mm) |
|-----------------------|--------------------------------------|
| Water | 0 ^a |
| CLAR | 26.7±1.22 ^d |
| P | 23.2±1.72 ^c |
| RO | 13.5±1.88 ^b |
| DGA | 33.7±1.22 ^e |

* Mean ± Standard Deviation

Means with the same letters in the same column are not significantly different at $p \leq 0.05$ in one-way ANOVA.

content of RO plates at day 10 showed 80% less enzyme activity compared to that of water treated plates.

All *H. pylori* isolates used in this experiment were obtained from patient biopsies. All tissues came with other normal flora of the gastric area. It was possible that antibacterial activity of DGA and P was broad spectrum since no microbe was observed in DGA treated plate by day 10. There was no significant difference between isolates in sensitivity to each of the compounds.

Since other microbes were observed in RO-treated plate on day 10, it was possible that bioactivity of RO was *H. pylori* specific. As a result RO seemed to be a very good candidate for treating *H. pylori in vivo* because RO did not kill other normal flora of the gastric area.

Minimum Inhibitory Concentration (MIC) and Minimum Bacteriocidal Concentration (MBC)

Four *H. pylori* clinical strains were used in the test. Results are shown in Tables 3 and 4. All the clinical strains tested showed similar response

in MIC to the tested compounds. However, strain 10 seemed to be the most sensitive clinical strain to all the compounds tested.

P, RO and DGA Blood had MIC of 125, 150 of 100 µg/mL and MBC of 150, 175, 125 µg/mL, respectively. The control drug, clarithromycin, had the MIC of 120 µg/mL in this assay (result not shown). DGA has a lower MIC than an existing antibacterial drug, clarithromycin, in this investigation.

Many flavonoids have been recently reported to possess anti-*H. pylori* activity (i.e., Fukai *et al.*, 2002, Konstantinopolou *et al.*, 2003). Malekzadeh *et al.* (2001) reported that ethanolic extract of Terminalia chebula Retz fruit had antibacterial activity against *H. pylori*. Bioactive compound from *T. chebula* was not isolated in the above work. However, other work found ethyl gallate and gallic acid from *T. chebula* active against *Staphylococcus aureus* (Sato *et al.*, 1997). The work implied that these two lignin-type compounds might be responsible for the anti-*H. pylori* activity of *T. chebula* fruit extract. Our discovery that pinostrobin and

Table 3. Anti-*H. pylori* activities (MIC, µg/mL) of P, RO and DGA.

| | Strain 1 | Strain 5 | Strain 9 | Strain 10 |
|-----|----------|----------|----------|-----------|
| P | 150 | 150 | 125 | 125 |
| RO | 175 | 175 | 175 | 150 |
| DGA | 125 | 125 | 100 | 100 |

Table 4. Anti-*H. pylori* activities (MIC and MBC µg/mL) of P, RO and DGA.

| | MIC | MBC |
|-----|-----|-----|
| P | 125 | 150 |
| RO | 150 | 175 |
| DGA | 100 | 125 |

dihydroguaiaretic acid possess anti-*H. pylori* activity adds to a growing list of bioactivities these two groups of compounds possess.

B. rotunda has long been used as herb in Thai cooking. Rhizome and root of Krachai were used in Thai traditional medicine to alleviate stomach discomfort and to treat oral diseases, such as aphthous ulcer and dry mouth. It has also been used as diuretic, antidysenteric, and antidermatophytic remedies (Saralamp *et al.*, 1996). Many parts of *M. fragrans* are used as herbs and spices. Fresh fruit is consumed in Indonesia and Southern Thailand as preserves and pickles (personal unpublished data). Mace is the most popular spice in European food because it helps preserve food for long-term storage (Bremness, 1994). Incorporation of these two plants in daily diet might help keep the population of *H. pylori* at a low level.

Although there is a direct correlation between *H. pylori* infection and gastric cancer in the world population, the incidence of gastric cancer in Thailand is quite small (Mahachai *et al.*, 2000). The result of this investigation shows that vegetable and spice used in Thai cooking and in Thai Traditional Medicine for treatment of gastrointestinal disorders have compounds which inhibit the growth of *H. pylori in vitro*. The unique plant food consumed by the Thai may control the infection by *H. pylori* (gastric cancer causative agent), and explain the low incidence of gastric cancer in the Thai population. The result also verifies the efficacy of the Thai Traditional Medicine remedy using *Boesenbergia rotunda* (L.) Mansf. and *Myristica fragrans* Houtt. in treating gastric ulcer.

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