Inhibition of urease and growth of *Helicobacter pylori* by herb extracts

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Abstract

Helicobacter pylori (HP) is associated with human gastroduodenal diseases such as gastritis, peptic ulcer and gastric carcinoma. HP produces strong urease, which is considered to play a critical role in pathogenesis of gastroduodentis. On the other hand, herb extracts have been used for stomach diseases as oriental traditional medicines for thousands of years. In the present study, ethanol extracts of 44 herbs and water extracts of twelve hozais were investigated on their inhibitory effects against urease activity and growth of HP *in vitro*. Urease activity and growth of HP were not influenced by 12 hozai extracts and most of these herb ethanol extracts. Among them, 1.25 mg/ml extract of Malloti Cortex and Myricae Cortex potently inhibited the urease activity of HP. From further fractionation, water extracts of these two herbs dose-dependently and strongly inhibited the urease activity, but did not inhibit the growth of HP. Five mg/ml of ethanol extracts of the following nine herbs, Rhei Rhizoma, Zingiberis Rhizoma, Saussureae Radix, Sophorae Radix, Acori Graminei Rhizoma, Gambir, Aloe Pulverata, Kaempferiae Rhizoma and Paeoniae Radix, showed inhibitory effects on the growth of HP, but showed no inhibitory effects on the urease activity of HP. An ether fraction from ethanol extract of Kaempferiae Rhizoma potently inhibited the growth of HP.

Key words *Helicobacter pylori*, urease, growth, Myricae Cortex, Kaempferiae Rhizoma.
Abbreviation HP, *Helicobacter pylori*.

Introduction

Helicobacter pylori (HP) was isolated from the gastric antrum of chronic gastritis patients by Warren and Marshall in 1983.[1] HP is a gram-negative, spiral bacterium and produces strong urease. Ammonia generated by hydrolysis of the urea protects this acid-sensitive bacterium from gastric acid,[2] and damages directly the gastric mucosal cells.[3,4] HP also produces a vacuolating toxin and its toxicity may be potentiated by urease-mediated ammonia production.[5] Therefore, HP and HP urease are considered to play critical roles in the pathogenesis of gastritis and peptic ulcer,[6] and is associated with stomach cancer.[7] From these results, eradication of the bacteria and inhibition of the urease are important for the treatment of patients with human gastroduodenal diseases.

Several trials have shown that HP is eradicated by mixed therapeutic agents such as antibiotics, bismuth subsalicylate, proton pump inhibitors and H_2-blockers in the U.S.A. and western Europe.[8-10] However, all these drugs are administered for a long period for eradication so that adverse side-effects often occurred in patients.[11] In Japan, long-term and combined treatments of those drugs have not been permitted.[12]

On the other hand, herb extracts have been used as oriental traditional medicines for thousands of years in Japan, China and other Asian countries. These traditional medicines are administered orally for various diseases in human. Also, many oriental

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medicines including herbs have been used for gastritis in folk cure. Therefore, it is worthwhile to screen these traditional medicines (Hozai) and herbs that may directly act on HP growth and urease in human stomach. In the present study, ethanol-extracts of 44 herbs and water-extracts of 12 hozais were investigated on their inhibitory effects against urease activity and growth of HP *in vitro*.

Materials and Methods

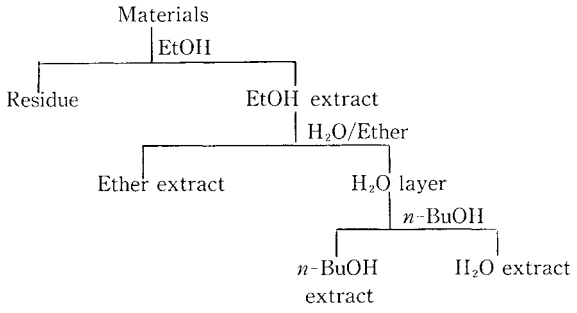
Cell : HP ATCC 43504 was kindly provided by the Research Institute for Microbial Diseases, Osaka University. It was inoculated on to brucella HK agar plates (Kyokuto Seiyaku Kogyo, Japan) and cultured for 4 days at 37°C in an anaerobic jar with Anaero Pack Campylo (Mitsubishi Gas Chemical Co., Japan).

Preparation of ethanol extract of herbs : All herbs were purchased from Tochimoto Tenkaido, Japan. Materials were extracted with ethanol at room temperature, and lyophilized. The weight (g) of materials and lyophilized extracts were shown in Table I. Gambir and Aloe Pulverata were used without ethanol extraction. Some effective herb extracts were fractionated further with ether, *n*-butanol and water as described in Fig. 1. Twelve hozais were kindly donated by Tsumura Co., Japan.

Preparation of HP urease : HP was inoculated from an agar plate into 10 ml of brucella broth (BBL, USA) supplemented with 10 % fetal calf serum in a 50 ml flask, which was placed in an anaerobic jar, and cultured micro-aerobically for 4 days at 37°C. The harvested cells were washed with 5 ml of 20 mM phosphate buffer (pH 7.0) containing 1 mM β-mercaptoethanol and 1 mM EDTA·2Na, then resuspended in the same buffer and used for the assay of urease activity.

Measurement of urease activity : Reaction mixtures composed of 50 μl of each enzyme solution and 1 or 0.1 %w/v test extract ethanol solution (final concentrations of test compounds were 1.25 and 0.125 mg/ml, respectively) were preincubated at 37°C for 15 min, and 300 μl of 100 mM phosphate buffer (pH 7.0) containing 400 mM urea were incubated at 37°C for 30 min, after which 100 μl of 1N H_2SO_4 was added. For ammonia determination using the indophenol method,[13] 2.5 ml each of phenol reagent (1 % phenol and 0.005 % sodium nitroprusside) and alkali reagent (5.5 % $Na_2HPO_4 \cdot 12H_2O$, 0.5 % NaOH and 0.1 % NaOCl) were added to each reaction mixture. After incubation at 65°C for 20 min, the absorbance at 630 nm was measured. In order to determine the 50 % inhibition (I_{50}) values, mixtures of 50 μl each of enzyme and test extract solution at various concentrations were preincubated at 37°C for 15 min. All experiments were done in triplicate. The activity was calculated as a percentage of that of the control experiment, in which 50 μl solvent was added in place of the test extract solution. The I_{50} value was determined using the program provided by Dr. H. Ono (Tokyo University).

Growth inhibition assay of HP : The ethanol extracts or the hozais were added to 18.6 ml brucella broth (BBL, USA) with 2 % Bacto agar (Difco, USA) and autoclaved at 120°C for 20 min. Then, the agar plates containing herb extracts of various concentration were supplemented with 1.4 ml horse serum. For



(g)	Malloti Cortex	Myricae Cortex	Kaempferiae Rhizoma	Paeoniae Radix
Materials	400	400	400	400
Extracts				
EtOH	12.0	35.9	19.6	26
Ether	3.5	2.1	14.5	3
H_2O	1.2	21.5	2.2	8.5
n-BuOH	6.8	12.3	2.4	14.4

Fig. 1 Fractionation procedure of ethanol extract. The weights (g) of materials and of recovered extracts were represented in the table.

Table I List of 44 herbs

Source		Materials (g)	Extracts (g)
Magnoliae Cortex	Kouboku	100	12.56
Pogostemonis Herba	Koukakko	100	6.49
Curcumae Rhizoma	Ukon	100	8.24
Capsici Fructus	Tougarashi	100	10.03
Rhei Rhizoma	Daio	100	5.98
Swertiae Herba	Senburi	100	17.67
Zingiberis Rhizoma	Shokyo	100	4.72
Anisi Stellati Fructus	Daiuikyo	100	12.36
Cinnamomi Cortex	Keihi	100	4.51
Scutellariae Radix	Ougon	100	3.76
Piperis Longi Fructus	Hihatsu	100	6.15
Anisi Fructus	Anisu	100	3.99
Perillae Semem	Shisoshi	100	4.94
Foericuli Fructus	Uikyo	100	4.82
Citri Leiocarpae Exocarpium	Seihi	100	4.44
Zingberis Siccatum Rhizoma	Kankyo	100	5.26
Kaempferiae Rhizoma	Sanna	100	3.72
Saussureae Radix	Mokko	100	6.59
Zedoariae Rhizoma	Gajutsu	100	3.86
Paeoniae Rubra Radix	Sekishaku	100	10.80
Gentianae Scabrae Radix	Ryutan	100	8.69
Sophorae Radix	Kujin	100	3.52
Myristicae Semen	Nikuzuku	100	8.09
Atractylodis Rhizoma	Byakujutsu	100	5.22
Zanthoxyli Fructus	Sansho	100	14.62
Paeoniae Radix	Shakuyaku	100	8.54
Acori Graminei Rhizoma	Sekishoukon	100	2.72
Magnoliae Officinalis Cortex	Karakouboku	100	15.40
Atractylodis Lanceae Rhizoma	Soujutsu	100	6.43
Alpiniae Officinarum Rhizoma	Ryoukyo	100	5.45
Mume Fructus	Ubai	100	28.87
Evodiae Fructus	Goshuyu	100	6.10
Coptidis Rhizoma	Ouren	100	2.00
Isodonis Herba	Enmeisou	100	3.42
Myricae Cortex	Youbaihi	100	16.61
Crataegi Fructus	Sanzashi	200	3.12
Calumbae Radix Pulverata	Koronbomatsu	100	1.99
Malloti Cortex	Akamegashiwa	100	2.24
Picrasmae Lignum	Nigaki	300	2.32
Ginseng Radix	Ninjin	300	2.02
Corydalis Tuber	Engosaku	509.4	1.88
Panacis Japonici Rhizoma	Chikusetsuninjin	200	3.23
Gambir	Asennyaku	3.00*	
Aloe Pulverata	Aroematsu	3.01*	

*These were used without ethanol extraction.

screening experiments, each plate contained 100 mg of extract. HP was inoculated into the agar plates which was placed in an anaerobic jar, and cultured microaerobically for 4 days at 37°C. All experiments were performed in duplicate and the control was measured at the same time in each experiment. The growth of HP was judged with five grades as well (+++), worse (++), slight (+), little (±) and complete no (−) growth of HP. Amoxicillin and ampicillin were purchased from Sigma Co. (USA).

Protein determination : The amounts of protein were measured using the Folin-Lowry method with bovine serum albumin (Fraction V, Sigma Co., USA) as a standard.[14]

Results

Effects of ethanol extracts on HP urease

Table II shows the effects of 44 herb ethanol extracts on HP urease activity. Most of these herbs were not inhibitory on the enzyme activity. Among them, fourteen extracts inhibited the HP urease activity and especially, extracts of Rhei Rhizoma, Malloti Cortex, Myricae Cortex and Mume Fructus potently inhibited the urease activity of HP at 1.25 mg/ml. Furthermore, extracts of Malloti Cortex and Myricae Cortex inhibited the activity at 0.125 mg/ml, but the extract of Mume Fructus was not effective at the concentration for which reasons are not yet clarified. Therefore, we fractionated further the ethanol extracts of Malloti Cortex and Myricae Cortex.

The effects of fractions from the ethanol extracts of Malloti Cortex and Myricae Cortex on the enzyme activity are shown in Fig. 2. Each fraction of both extracts dose-dependently inhibited the urease activity. The water extracts of both herbs strongly inhibited the urease activity and ether extracts did so weakly. I_{50} values of Malloti Cortex and Myricae Cortex of crude ethanol extracts were 388 and 34.6 μg/ml. Those of H_2O extracts were 269 and 37.5 μg/ml

Table II Effects of ethanol extracts on urease activity of *H. pyloi.*

Source	Inhibition (%) Sample (mg/ml) 1.25	0.125	Source	Inhibition (%) Sample (mg/ml) 1.25	0.125
Magnoliae Cortex	0	0	Coptidis Rhizoma	0	0
Pogostemonis Herba	0	0	Crataegi Fructus	0	0
Curcumae Rhizoma	0	0	Calumbae Radix Pulverata	0	0
Capsici Fructus	0	0	Picrasmae Lignum	0	0
Swertiae Herba	0	0	Ginseng Radix	0	0
Zingiberis Rhizoma	0	0	Corydalis Tuber	0	0
Piperis Longi Fructus	0	0	Panacis Japonici Rhizoma	0	0
Anisi Fructus	0	0	Gambir	0	0
Perillae Semem	0	0	Scutellariae Radix	11.6	10.1
Foericuli Fructus	0	0	Aloe Pulverata	16.4	18.2
Citri Leiocarpae Exocarpium	0	0	Paeoniae Radix	22.2	3.9
Zingberis Siccatum Rhizoma	0	0	Sophorae Radix	26.8	10.0
Kaempferiae Rhizoma	0	0	Cinnamomi Cortex	30.3	17.4
Saussureae Radix	0	0	Anisi Stellati Fructus	30.4	0
Zedoariae Rhizoma	0	0	Myristicae Semen	33.3	15.1
Gentianae Scabrae Radix	0	0	Alpiniae Officinarum Rhizoma	52.2	17.0
Atractylodis Rhizoma	0	0	Paeoniae Rubra Radix	64.1	11.3
Zanthoxyli Fructus	0	0	Isodonis Herba	72.3	22.3
Acori Graminei Rhizoma	0	0	Rhei Rhizoma	85.3	46.2
Magnoliae Officinalis Cortex	0	0	Malloti Cortex	88.0	72.0
Atractylodis Lanceae Rhizoma	0	0	Myricae Cortex	95.2	76.6
Evodiae Fructus	0	0	Mume Fructus	98.1	0

Specific activity of urease was 1.49 IU/mg protein.

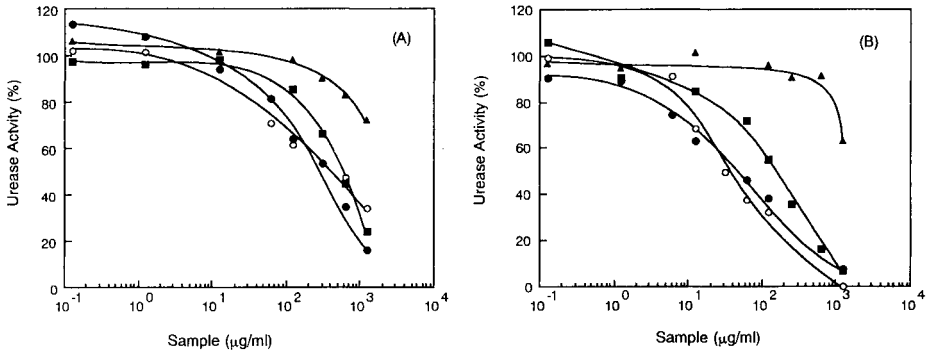


Fig. 2 Effects of fractions from Malloti Cortex (A) and Myricae Cortex (B) ethanol extracts on HP urease activity. ○, ethanol ; ●, H_2O ; ■, *n* BuOH ; ▲, ether.

and of *n*-BuOH extracts were 512 and 85.8 μg/ml, respectively, and those of ether extracts were more than 1 mg/ml at final concentrations.

Effects of herb and hozai extracts on HP growth

Table III shows the effects of 44 herb ethanol extracts on the growth of HP. Nine ethanol extracts exhibited inhibitory effects on the growth of HP as shown in grades + and −, of their final concentrations at 5.0 mg/ml media. Especially, Kaempferiae Rhizoma and Paenoniae Radix completely inhibited the growth. As standards, amoxicillin and ampicillin, which are used for eradication of HP, were tested under the same procedure and conditions. Both antibiotics exhibited inhibitory effects in grade +, at 1.0 μg/ml

Table III Effects ethanol extracts on growth of *H. pylori*.

Source	Growth	Source	Growth
Curcumae Rhizoma	+	Scutellariae Radix	+
Anisi Stellati Fructus	+	Perillae Semem	+
Cinnamomi Cortex	+	Foericuli Fructus	−
Piperis Longi Fructus	+	Citri Leiocarpae Exocarpium	−
Anisi Fructus	−	Zingberis Siccatum Rhizoma	−
Zedoariae Rhizoma	−	Gentianae Scabrae Radix	−
Paeoniae Rubra Radix	−	Atractylodis Rhizoma	−
Myristicae Semen	+	Zanthoxyli Fructus	−
Atractylodis Lanceae Rhizoma	+	Magnoliae Officinalis Cortex	−
Alpiniae Officinarum Rhizoma	+	Mume Fructus	+
Coptidis Rhizoma	−	Evodiae Fructus	+
Isodonis Herba	−	Calumbae Radix Pulvrata	−
Myricae Cortex	−	Picrasmae Lignum	−
Crataegi Fructus	−	Rhei Rhizoma	+
Malloti Cortex	−	Zingiberis Rhizoma	+
Ginseng Radix	−	Saussureae Radix	−
Corydalis Tuber	+	Sophorae Radix	−
Panacis Japonici Rhizoma	+	Acori Graminei Rhizoma	−
Magnoliae Cortex	−	Gambir	−
Pogostemonis Herba	−	Aloe Pulverata	−
Capsici Fructus	−	Kaempferiae Rhizoma	−
Swertiae Herba	−	Paeoniae Radix	−

Each plate (20 ml medium) contained 100 mg of extract.

media. Twelve hozai extracts did not inhibit the growth of HP as shown at 5.0 mg/ml media in Table IV. These hozai extracts had no effect on HP urease activity at the same concentration (data not shown).

In order to identify which components of Kaempferiae Rhizoma or Paeoniae Radix have the inhibitory effects on HP growth, the ethanol extracts were fractionated as Fig. 1 to assay for the growth inhibition. As shown in Table V, the most effective fraction was an ether extract of Kaempferiae Rhizoma, and the inhibition of the growth was dose-dependent. Other extracts did not significantly inhibit the growth of HP at final concentrations of 2.5 mg/ml media. Isolation of the inhibitor(s) in the ether extract is now in progress.

Table VI shows the effects of kaempferol and paeoniflorin, the known component of Kaempferiae Rhizoma and Paeoniae Radix, respectively, and some other components of traditional medicine or herbs on the growth of HP. Caffeine and quercetin considerably inhibited the growth at 2.5 mg/media ml, however, caffeine inhibited the growth a little at one-tenth dose, 0.25 mg/ml.

Table IV Effects of Kampo hozai extracts on growth of *H. pylori*.

	Growth
Anchu-san	++
Sho-saiko-to	−+−
Saiko-keishi-to	+++
Hange-shashin-to	+++
Oren-gedoku-to	+++
Hange-koboku-to	−+−
Ninjin-to	++
Shigyaku-san	+++
Rikkunshi-to	++−
Heii-san	++−
Oren-to	+−
Sai-shaku-rikkunshi-to	+++

Each plate (20 ml medium) contained 100 mg of extract.

Table V Effects of Kaempferiae Rhizoma and Paeoniae Radix extracts on growth of *H. pylori*.

	Extract	mg/ml	Growth
Kaempferiae Rhizoma	H_2O	2.5	+−+
	n-BuOH	2.5	+−
	Ether	0.25	+−+
		1.0	+−
		2.5	+
Paeoniae Radix	H_2O	2.5	+−+
	n-BuOH	2.5	+−
	Ether	2.5	+−+

Table VI Effects of some compounds on growth of *H. pylori*.

	mg/ml	Growth
Kaempferol	0.5	++
Paeoniflorin	0.5	+++
Ellagic acid	0.25	+++
	2.5	++
Caffeine	0.25	++
	2.5	+
Quercetin	0.25	+++
	2.5	++
Gallic acid	2.5	+++
	5.0	++

Discussion

Traditional medicines have been used for many centuries, and some of them are known to be very effective for the stomach disease, however, a study has not been made on the gastroduodenal ulcer which is caused by HP. Recently, plaunotol from Plau-noi (*Croton Sublyratus Kurz*) was reported to have an antibacterial activity on HP.[15] Also, ecabet sodium from Pini Resina, binds to urease of HP to inhibit its activity and also affects the viability of HP in the acidic condition supplemented with urea.[16, 17] In the present study, we investigated the effects of 44 herb ethanol extracts and 12 hozai water extracts on the urease activity and growth of HP.

In the urease inhibition assay, Malloti Cortex and Myricae Cortex strongly inhibited the urease activity. The water extract of Myricae Cortex inhibited the urease activity stronger than the other fractions. Myricae Cortex contains tannins for about 15 %,[18] therefore it is assumed that tannins in the water extract inhibited HP urease activity. Further studies are required to isolate the inhibitory compound(s) from the water extract of Myricae Cortex. The inhibi-

tory effect of ethanol extract of Malloti Cortex was different between Table II and Fig. 2. From these data, components of ethanol extracts of Malloti cortex may be altered during the storage and further fractionation of inhibitory compounds is considered to be difficult.

Traditional medicines have been usually prepared not only as an extract of one kind of herb but also a mixed extract of many kinds of different herbs, which are called hozai. We tested the effects of 12 hozai water extracts, which have been commonly and commercially used for gastroenteropathy, on urease activity and growth of HP. The urease activity and growth of HP were not affected after incubation with 100 mg of the water extracts, showing that these hozais were not effective for HP urease activity and growth. The dose of these extracts for humans are about 7-10 g a day, which corresponds to the amounts of the extract used here. These hozai extracts used were not expected to have strong inhibitory effects on HP growth *in vivo*.

The eradication of HP has been known to cure gastritis and prevents the relapse of duodenal ulcer.[19] Some traditional medicines have antibacterial activities, such as berberine from Phellodendri Cortex and Coptidis Rhizoma. In the present study, nine ethanol extracts showed inhibitory effects on the growth of HP at 100 mg/plate ; 5 mg/ml. Especially, Kaempferiae Rhizoma and Paeoniae Radix completely inhibited the growth. From the further fractionation, the ether layer of Kaempferiae Rhizoma dose - dependently inhibited the growth of HP. Antibiotics showed inhibitory effects on growth at three order lower concentration. Therefore, further purification and modification of active components in Kaempferiae Rhizoma extract are necessary for clinical use.

In this study we found that ethanol extracts of Malloti Cortex and Myricae Cortex were potent inhibitors of the urease of HP. Ethanol extracts of Kaempferiae Rhizoma and Paeoniae Radix were potent inhibitors of the growth of HP. It is known that urease activity is essential for HP to protect from gastric acid and enable HP to infect human gastric mucosa.[20] However, acetohydroxamic acid, a strong urease inhibitor,[21] had an inhibitory effect on the urease activity of HP but no effect on growth *in vitro*.[22] An HP mutant, which lacked the ability to complex and form the active urease enzyme, was viable at the neutral pH, with or without urea.[23] Several herb ethanol extracts described above in the present study, inhibited urease activity or growth of HP, but no herb ethanol extracts inhibited both of them. These observations suggest that the urease activity is irrespective to the growth of HP, at least, *in vitro*.

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和文抄録

Helicobacter pylori (HP) は，高いウレターゼ活性を持つ胃内感染菌で，現在，胃炎・胃潰瘍の発症因子として注目されている。一方，生薬には，伝統医学の中で何千年にもわたり胃の薬として使用されたきたものがある。今回44種の生薬エタノール抽出エキスと，12種の方剤水抽出エキスのHPのウレアーゼ活性と増殖に対する阻害効果を検討した。消化管疾患に用いられている12種の方剤エキスは，HPのウレアーゼ活性と生育に影響しなかった。HPウレアーゼは，44種の生薬の中で，0.125 mg/mlの赤芽柏と楊梅皮で強く阻害された。さらに分画したところ，この2つのエキスの水抽出画分に濃度依存的な強いウレアーゼ阻害活性があることが明らかになったが，HP増殖抑制効果は認められなかった。HP増殖では，5 mg/mlで9種の生薬エキス，大黄，生姜，木香，苦参，石菖根，阿仙薬，アロエ末，山奈，芍薬に抑制効果が認められた。しかし，これらはウレアーゼ活性には影響しなかった。山奈エキスのエーテル画分は強力に生育を阻害した。

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