Antibacterial activity of traditional medicines and an active constituent lupulone from *Humulus lupulus* against *Helicobacter pylori*

Mizue OHSUGI,[a] Purusotam BASNET,[a] Shigetoshi KADOTA,*[a] Eiji ISHII,[b] Toshihide TAMURA,[c] Yasushi OKUMURA[d] and Tsuneo NAMBA[a]

[a] *Research Institute for Wakan-Yaku (Traditional Sino-Japanese Medicines), Toyama Medical and Pharmaceutical University,* [b] *Department of Health and Epidemology, Osaka City Institute of Public Health and Environmental Sciences,* [c] *Hyogo College of Medicines,* [d] *Bioscience Research Laboratory, Asahi Breweries Ltd.*

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Abstract

Twenty-seven natural medicines, which have been traditionally used in China, Indonesia, Vietnam or Japan, were examined for *in vitro* antibacterial activity against *Helicobacter pylori* by disc method. Among these, Hop (female inflorescence of *Humulus lupulus* L.), MAGNOLIAE OFFICINALIS CORTEX (barks of *Magnolia officinalis* REHD. et WILS.) and Bangkawang (seeds of *Pachyrrhizus erosus* URBAN) significantly inhibited the growth of *Helicobacter pylori*. Hop was subjected for further study as it is largely used to flavor beer and its evaluation for antibacterial activity seemed to be very pertinent. The activity guided chemical analysis led to the isolation of an active compound, lupulone (**1**), from the hexane soluble fraction of the methanol extract of Hop, which showed significant antibacterial activity against three clinically isolated strains including a macrolide resistant strain and two standard strains of *Helicobacter pylori*.

Key words *Helicobacter pylori, Moraceae, Humulus lupulus,* β-acids, lupulone, disc-MIC.

Abbreviations BSA, bovine serum albumine ; cfu, colony forming units ; CSF, chloroform soluble fraction ; DMSO, dimethylsulfoxide ; HP, *Helicobacter pylori* ; HPLC, high performance liquid chromatography ; HSF, hexane soluble fraction ; MIC, minimum inhibitory concentration ; PPI, proton pump inhibitor.

Introduction

One half to one third of the world's population harbors *Helicobacter pylori* (HP). Numerous gastroduodenal ulcers or gastric cancers are imputed to this bacteria.[1] The administration of proton pump inhibitor (PPI) causes an ascending of gastric pH, but it can not make complete eradication.[2] Most antibiotics are sensitive to HP, however the affect of gastric acidity plays a role which ultimately weakens their antibacterial activity. PPI in combination with antibiotics is commonly used to eliminate HP. As stated above, PPI increases gastric pH and improves activity of the antibiotics.[3] There is another report using antiprotozoan drug, a known antibacterial agent against HP in combination with PPI and antibiotics which is an example of triple therapy method.[4] Unfortunately, these antibiotics and/or metronidazole resistant strains of HP soon appeared.[5] Under this circumstance, search for a more efficient and less expensive drug from natural resources such as traditional medicine is being considered. As one of a series of studies,[6] we have reported that trichorabdal A which was isolated from *Rabdosia trichocarpa,* showed antibacterial activity against HP. In this paper, we studied the antibacterial activity of twenty-seven natural medicines used in various regions of Asia in

*〒930-01 富山市杉谷2630
富山医科薬科大学和漢薬研究所　門田重利
2630 Sugitani, Toyama 930-01, Japan

order to find antibacterial compounds against HP, even those resistant strains. From Hop, the female inflorescence of *Humulus lupulus* L., lupulone (**1**) was isolated and found to be very effective.

Materials and Methods

Strains used and culture condition : Three clinical isolates (CLO1, CLO2 and CLO36), including a erythromycin resistant strain (CLO36), and two standard strains (NCTC11637 and 11916) of HP. *Helicobacter* strains were grown on Brucella agar (Becton Dickinson Microbiol. System, USA) supplemented with horse defibrinated blood at 5 % (blood agar). They were cultured at 37°C in a glove box under a humidified microaerobic atmosphere consisting of 80 % nitrogen, 15 % carbon dioxide and 5 % oxygen for three days. They were harvested, suspended in Brucella broth including 0.5 mg/ml bovine serum albumin fraction V (BSA, Sigma Chemical Co., USA, A-4503) sterilized with 0.2 μm filter and modulated at approximately 10^7 colony forming units (cfu)/ml for an inoculation.

Drug samples : CORYDALIS TUBER, GENTIANAE SCABRAE RADIX, MAGNOLIAE OFFICINALIS CORTEX, MALLOTI CORTEX, PICRASMAE LIGNUM, SOPHORAE SUBPROSTRATAE RADIX and SWERTIAE HERBA were purchased from Uchida Pharmaceutical Co. Ltd., Japan. Hop was supplied by Asahi Breweries Ltd.. Pule, Jungrahab, Kuwalot, Kananga, Tapak lemam, Manggis, Daun Jati Belanda, Duku Langsat, Merica Bolong, Kudu, Bangkawang, Apuket, Great frangipanni, Burahol, Mahoni and Suren were collected in Indonesia, and Trâm bâu collected in Vietnam. Propolis collected at Atibaia sp in Brazil was provided by Nihon Propolis Co. Ltd., Japan. VESPAE NIDUS from the market of Hong Kong was purchased from Uchida Pharmaceutical Co. Ltd., Japan (Table I).

Table I Crude drug samples used for antibacterial activity against *Helicobacter pylori*.

Crude drug name	Scientific name	Family name	Parts used
1. CORYDALIS TUBER	*Corydalis yanhusuo* W.T.WANG	Papaveraceae	tubers
2. GENTIANAE SCABRAE RADIX	*Gentiana scabra* BUNGE	Gentianaceae	roots and rhizomes
3. MAGNOLIAE OFFICINALIS CORTEX	*Magnolia officinalis* REHD. et WILS.	Magnoliaceae	bark
4. MALLOTI CORTEX	*Mallotus japonicus* MÜELL.-ARG.	Euphorbiaceae	bark
5. PICRASMAE LIGNUM	*Picrasma quassioides* BENN.	Simaroubaceae	woods
6. SOPHORAE SUBPROSTRATAE RADIX	*Sophora subprostrata* CHUN et CHEN	Leguminosae	roots
7. SWERTIAE HERBA	*Swertia japonica* MAKINO	Gentianaceae	whole plant
8. Hop	*Humulus lupulus* L.	Moraceae	female inflorescence
9. Pule	*Alstonia scholaris* R.BR.	Apocynaceae	bark
10. Jungrahab	*Baeckea frutescens* L.	Myrtaceae	leaves
11. Kuwalot	*Brucea javanica* MERRILL	Simaroubaceae	leaves, fruits
12. Kananga	*Cananga odorata* HOOK. f. et THOMS.	Annonaceae	bark
13. Tapak lemam	*Elephantopus scaber* L.	Compositae	whole plant
14. Manggis	*Garcinia mangostana* L.	Guttiferae	fruit peels
15. Daun Jati Belanda	*Guazuma ulmifolia* LAM.	Sterculiaceae	leaves
16. Duku Langsat	*Lansium domesticum* CORR.	Meliaceae	seeds, fruit peels
17. Merica Bolong	*Melaleuca leucadendron* L.	Myrtaceae	fruits
18. Kudu	*Morinda citrifolia* L.	Rubiaceae	fruits
19. Bangkawang	*Pachyrrhizus erosus* URBAN	Leguminosae	seeds
20. Apuket	*Persea americana* MILL.	Lauraceae	bark
21. Great frangipanni	*Plumeria alba* L.	Apocynaceae	bark
22. Burahol	*Stelechocarpus burakol* HOOK.f.et THOMS.	Annonaceae	bark, seeds
23. Mahoni	*Swietenia mahagoni* JACQ.	Meliaceae	seeds
24. Suren	*Toona sureni* MERRILL	Meliaceae	leaves
25. Trâm bâu	*Combretum quadrangulare* KURZ.	Combretaceae	leaves
26. Propolis	*Apis mellifera* L.	Apidae	honeybee glue
27. VESPAE NIDUS	*Vespula flaviceps* SMITH etc.	Vespidae etc.	hives

Extraction of samples : Each dried sample of CORYDALIS TUBER, GENTIANAE SCABRAE RADIX, MAGNOLIAE OFFICINALIS CORTEX, MALLOTI CORTEX, PICRASMAE LIGNUM, SOPHORAE SUBPROSTRATAE RADIX and Hop were divided into three parts and separately extracted with H_2O, MeOH and MeOH : H_2O (1 : 1) ; SWERTIAE HERBA was extracted with EtOH : H_2O (7 : 3) ; Pule, Jungrahab, Kuwalot, Kananga, Tapak lemam, Manggis, Daun Jati Belanda, Duku Langsat, Merica Bolong, Kudu, Bangkawang, Apuket, Great frangipanni, Burahol, Mahoni and Suren were extracted with MeOH ; Trâm bâu and Propolis were extracted with H_2O and MeOH, separately ; VESPAE NIDUS was extracted with H_2O. All the extractions were performed under refluxed condition. The supernatant was filtrated and evaporated under reduced pressure to get their corresponding extracts.

Extraction and purification of antibacterial compounds from Hop : Hop (100 g) was extracted three times with methanol (300 ml×3) under reflux for 3 h each time. The supernatant was filtrated and a portion of the filtrate was evaporated under reduced pressure to obtain the methanol extract (26.69 g). A portion (22.08 g) of the methanol extract was suspended in methanol and partitioned with hexane (300 ml×2) stirring for 2 h each at room temperature. The hexane phase was evaporated under reduced pressure to give hexane soluble fraction (HSF, 8.72 g). The methanol phase was extracted with chloroform (300 ml×2) stirring for 1 h each at room temperature to obtain chloroform soluble fraction (CSF, 6.06 g) and the residue (5.06 g). HSF was subjected to HPLC analysis using Shim-pack PREP-ODS column (5.5 mm×25 cm). Mobile phase was CH_3CN : H_2O (18 : 7). The HPLC profile of HSF is shown in Fig. 1. α-and β-acids were the mixtures comprising 4 and 3 compounds, respectively. Lupulone (**1**) and humulone (**2**) were purified by the preparative HPLC.

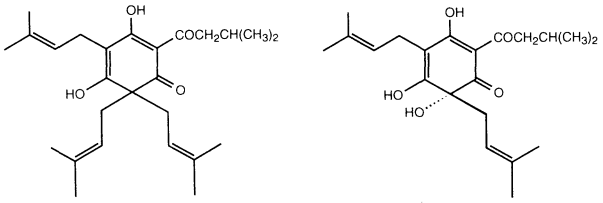

Chart 1 Structures of lupulone (**1**) and humulone (**2**).

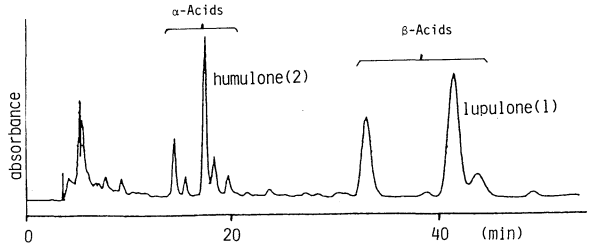

Fig. 1 HPLC chromatogram of the hexane soluble fraction of the methanol extract of Hop. HPLC condition : column, Shim-pack PREP-ODS ; column size, 5.5 (I. d.) ×250 mm ; eluent, acetonitrile-water (18 : 7) ; flow rate, 1.0 ml/min ; detector setting, UV 302 nm.

Sample preparation : It should be noted that the samples targeted for antibacterial tests were insoluble in water and therefore, they were dissolved in dimethylsulfoxide (DMSO), a low volatile solvent, and diluted with DMSO to 10, 1 and 0.1 mg/ml. The methanol extract of Hop, its fractions and the isolated compounds were also dissolved in DMSO and diluted with DMSO to 5.0, 1.0, 0.5, 0.25, 0.13, 0.063, 0.031, 0.016 and 0.008 mg/ml. These solutions were used for antibacterial tests by disc method.

Antibacterial activity by disc method : Water insoluble property of these compounds made it impossible to examine the antibacterial activity by *in vitro* agar dilution method. It is mainly because of the fact that the water insoluble substances do not spread well and the minimum inhibitory concentration (MIC) values of the substances cannot be evaluated accurately.[7] So their antibacterial activity has been studied by using disc method.[8,9] Albumin agar (Brucella agar supplemented with BSA fraction V sterilized with 0.2 μm filter at 0.5 mg/ml) was found to be the most sensitive for the evaluation of antibacterial activity in this method.[9] Therefore, about 10^6 cfu of HP was inoculated on albumin agar (20 ml) in 90 mm Petri dishes. DMSO solution of drug extracts, Hop fractions or purified compounds were charged (20 μl) in 8 mm thin paper disc (Toyo Roshi Kaisha, Ltd., Japan). The charged paper was put on the inoculated agar plate and incubated at 37°C under the microaerobic condition for three days. The inhibitory zone from the edge of the disk was measured. In every strain, no inhibi-

tory zone was observed around the disc charged with DMSO.[7] On the other hand, use of DMSO in combination with test samples clearly displayed the inhibitory zones which were suspected even below 1 mm. However, the zones below 1 mm were difficult to measure. The minimum concentration of the substance required to produce more than 1 mm of inhibitory zone around the disc was termed as disc-MIC.

Results and Discussion

Forty-seven extracts of twenty-seven traditional medicines used traditionally in China, Indonesia, Vietnam or Japan, were examined for their inhibitory effects against the growth of HP by disc method. Sixteen extracts exhibited disc-MIC values less than 10 mg/ml against three strains, CLO1, NCTC11916 and NCTC11637 of HP (Table II, those exhibiting disc-MIC higher than 10 mg/ml are not shown). Among these, the methanol extract of Hop, MAGNOLIAE OFFICINALIS CORTEX and Bangkawang exhibited the strongest antibacterial activity, whose disc-MIC values were less than 1 mg/ml.

Recently, MAGNOLIAE OFFICINALIS CORTEX was reported to be very sensitive to HP.[10] Bangkawang is traditionally used for dermatosis in Indonesia.[11] The female inflorescence of *H. lupulus* is commonly known as Hop and widely used to flavor beer. Most Hop-derived compounds were reported to have antibacterial activity against gram positive as well as gram negative bacteria. However, their affects towards gram negative were alleged to be weaker than gram positive bacteria.[12] Hop, as mentioned earlier, is one of the ingredients of beer and there is no report available so far about its antibacterial activity against HP, a gram negative bacteria. We therefore, for the first time investigated its antibacterial activity using the disc method. As shown in Fig. 2, disc-MIC values of the methanol extract of Hop against NCTC11637 and NCTC11916 strains were 0.13 and 0.063 mg/ml, respectively, while 0.13 mg/ml against CLO1, CLO2 and CLO36 strains : the activities were nearly identical against five strains. Disc-MIC values of HSF were almost equivalent to those of the methanol extract, while those of CSF and the residue were higher than the methanol extract against each strain. An HPLC fraction of HSF containing α- and β- acids showed a little stronger antibacterial activity : the

Table II Antibacterial activity of crude drug extracts against *Helicobacter pylori*.

		inhibitory zone (+ or −*)								
		CLO1			NCTC11916			NCTC11637		
sample	solvent	10**	1	0.1	10	1	0.1	10	1	0.1
1. CORYDALIS TUBER	MeOH	+	−	−	+	−	−	+	−	−
3. MAGNOLIAE OFFICINALIS CORTEX	MeOH	+	+	−	+	+	−	+	+	−
	MeOH-H_2O(1 : 1)	+	+	−	+	−	−	+	+	−
4. MALLOTI CORTEX	MeOH	+	−	−	+	−	−	+	−	−
	MeOH-H_2O(1 : 1)	+	−	−	+	−	−	+	−	−
	H_2O	+	−	−	+	−	−	+	−	−
6. SOPHORAE SUBPROSTRATAE RADIX	MeOH	+	−	−	+	−	−	+	−	−
7. SWERTIAE HERBA	EtOH-H_2O(7 : 3)	+	−	−	+	−	−	+	−	−
8. Hop	MeOH	+	+	−	+	+	−	+	+	−
	MeOH-H_2O(1 : 1)	+	−	−	+	−	−	+	−	−
10. Jungrahab	MeOH	+	−	−	+	−	−	+	−	−
19. Bangkawang	MeOH	+	+	−	+	+	−	+	+	−
24. Suren	MeOH	+	−	−	+	−	−	+	−	−
25. Trâm bâu	MeOH	+	−	−	+	−	−	+	−	−
	H_2O	+	−	−	+	−	−	+	−	−
26. Propolis	MeOH	+	−	−	+	−	−	+	−	−

Helicobacter pylori's strain : CLO1, NCTC11916, NCTC11637 ; *+ : ≧ 1 mm, −: 0mm ; **tested concentration (mg/ml) of extracts by disc method.

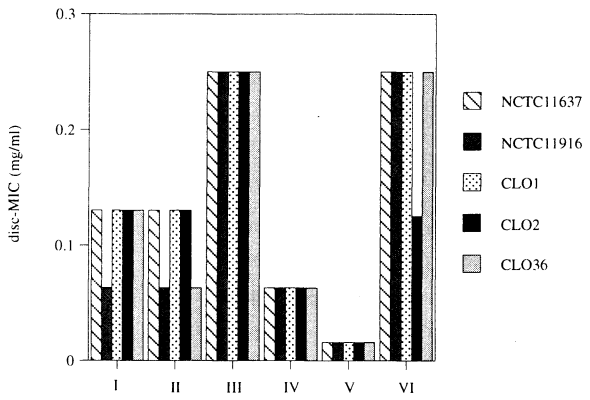

Fig. 2 DIsc-MIC of the methanol extract, fractions and compounds of Hop. I, methanol extract ; II, hexane soluble fraction ; III, chloroform soluble fraction ; IV, α- and β-acids ; V, lupulone ; VI, humulone ; Disc-MIC of residue was 8 mg/ml against five strains.

disc-MIC value was 0.063 mg/ml against five strains. From this fraction, lupulone (**1**) and humulone (**2**), which belong to α- or β- acids, respectively, were isolated and purified. The disc-MIC value of humulone (**2**) against five strains, was quite higher than the methanol extract, while that of lupulone (**1**) was 0.016 mg/ml, the lowest among the tested samples. It is therefore to be concluded that lupulone (**1**) is the active constituent of Hop against HP, as lupulone (**1**) showed very strong antibacterial activity against three clinically isolated strains including an erythromycin resistant strain, as well as two standard strains in this study.

It has been reported that the MIC values of Penicillin G against HP NCTC11916 and 11637 strains by disc method were 1.6 and 3.1μg/ml, respectively, while the values against both strains by agar dilution method were 0.025 μg/ml.[6] A ratio of MIC of Penicillin G by disc method to it by agar dilution method is 64-124. From this ratio, when MIC by agar dilution method is assumed to be 1/10 to 1/100 fold of disc-MIC, MIC of lupulone (**1**) by agar dilution method is presumed to be 0.63-13 μg/ml. The MIC of omeprazole was reported to be 12.5-50 μg/ml.[13] Then the antibacterial activity of lupulone (**1**) seems to be comparable to PPI or still stronger than PPI. Its high efficacy is futher supported by the fact that erythromycin was reported to be ineffective against CLO36 strain even at a concentration of 100 μg/ml, whereas lupulone (**1**) was effective below 100 μg/ml (Fig. 2). This evidence infers that lupulone (**1**) was more effective than erythromycin against the macrolide resistant strain. Thus, lupulone (**1**) was concluded to be a potent antibacterial agent that could be used for clinical trial.

和文抄録

Helicobacter pylori は胃・十二指腸疾患患者の胃粘膜から高頻度に検出されることなどから，これらの疾患の原因の一つであると考えられるようになった。そのため，これらの疾患の治癒促進や再発防止のためには，*H. pylori* の除菌が有効であると考えられる。これまで，除菌法として PPI (proton pump inhibitor) と抗菌剤，さらに抗原虫剤などを加えた併用療法が用いられ，高い除菌効果も示されている。しかし，副作用の発現率が高く，耐性株が出現するなどの問題があり，より簡便で副作用が少ない除菌法が望まれるようになった。そこで，こうした薬物を天然物中に検索するために，27 種の天然物について *in vitro* における *H. pylori* に対する抗菌活性スクリーニングを行ったところ，ホップ (*Humulus lupulus* L. の雌花序) 及びその含有成分である lupulone (**1**) に強い活性が認められた。

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