Original Article

The Isolation of the Anti-Helicobacter Pylori Compounds in Seeds of *Arctium lappa* Linn.

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ABSTRACT

Three lignan compounds namely 3'-demethyl arctigenin (compound 1), arctigenin (compound 2) and arctigenin glucoside (compound 3) were isolated from dried seeds of *Arctium lappa* L. (Compositae) by bioassay-guided fractionation. From 500 g of dried seeds, the yields of compounds 1, 2 and 3 were 26.2, 38.4 and 22.1 mg, respectively. The structures of these compounds were determined by extensive NMR studies. Crude extracts and isolated compounds showed a strong antibacterial activity against a clarithromycin-resistant *H. pylori* strain. Specifically, at a concentration of 50 μg/mL, compounds 1 and 2 each exerted a 100% inhibition against *H. pylori* compared to a standard amoxicillin (5 μg/mL) and clarithromycin (1 μg/mL), while compound 3 and crude extract showed a 95% and 86% inhibition, respectively. In summary, these lignans may be useful as lead compounds in the development of a new class of anti-H. pylori agents.

Keywords: *Helicobacter pylori, Arctium lappa,* lignan

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Introduction

Fructus Arctii (Goboshi in Japanese), the dried fruits of *Arctium lappa* L. (Compositae), is one of the most popular traditional Chinese medicines and is officially listed in the Chinese Pharmacopoeia. It has been widely used for dispelling pathogenic wind-heat, promoting eruption, relieving sore throat, removing toxic substances and subduing swelling. A variety of lignans, including arctigenin and its glucoside arctiin have been isolated. Ichihara et al[1-3] reported the novel lignans including Lappaol A, B, C, D, E, F, and H. Wang and Yang[4] reported a new lignan namely neoarctin B and the other

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five compounds identified as daucosterol, arctigenin, arctiin, matairesinol, and lappaol F. Han et al[5] reported a novel lignan, diarctigenin, that was elucidated as bis-5',5'-arctigenin, together with the known butyrolactone derivatives. Eventhough *A. lappa* is a good source of lignans, the isolation of lignans is typically achieved through several complex procedures of partition, chromatography or precipitation[6]. More interestingly, arctiin, a major lignan compound in Fructus Arctii, has been found to exert protective effects on 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP)-induced carcinogenesis particularly in the mammary gland in the promotion period as tested in Sprague–Dawley rats and a remarkable anti-tumor-promoting effect on carcinogenesis test on mouse skin tumors induced by 7,12-dimethylbenzanthracene. Recently, Huang et al[7] reported that arctiin could significantly induce cell detachment and decrease cell numbers via the up-regulation of MUC-1 mRNA and protein in PC-3 cells.

Helicobacter pylori is a pathogenic microorganism colonizing in the stomach of half of people worldwide. It plays a crucial role in the pathogenesis of gastritis and peptic ulcer disease and increased risk of gastric adenocarcinoma[8]. Current antibiotics used for the treatment of *H. pylori* infections include clarithromycin (CLA), metronidazole (MTZ), amoxicillin, and tetracycline. The resistance to CLA and MTZ can appear due to point mutations. The search of new drugs for the development of alternative therapies is very important since therapies could be ineffective and undesirable side effects may occur. Several natural products used for the treatment of gastric disorders have an antimicrobial activity against *H. pylori*[9-16]. The aim of this study was to determine the antimicrobial activity of *A. lappa* against clarithromycin-resistant strain of *H. pylori.*

Materials and Methods

The NMR system with 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR, and TMS as internal standard was used in this study. The spectra were measured on a Varian Unity 400 spectrometer. Column chromatography system consisted of silica gel 60 N (Cica-Reagent) and silica gel (Wakogel C-200). The HPLC system used silica gel (Capcell pak C-18, 250 x 4.6 mm) and silica gel (Pegasil ODS C-18, 250 x 20 mm).

Plant Materials

Dried seeds of *Arctium lappa* Linn. (Compositae) were purchased from a commercial source (Tochimoto Co., Ltd., Japan, Lot no.1805049) in October 2005. A voucher specimen (Code no. KT127) has been deposited in laboratory of biochemical and pharmacological for phytomedicine at Kitasato Institute for Life Sciences, Kitasato University, Tokyo, Japan.

Extraction and Isolation

The dried seeds of *A. lappa* (0.5 kg) were macerated with methanol (2 L) at room temperature for 3 days. The extract was filtrated and concentrated in a rotary vacuum evaporator (Eyela) with vacuum controller (Buchi) to give the yellowish syrupy extract (14.27 g). The methanolic extract (7 g) was then fractionated by open column chromatography on silica gel (210 g) in glass column (6x38 cm) using gradual elution with $CHCl_3$ and MeOH in varied ratios (10:0, 50:1, 10:1, 5:1, 1:1, 1:5, and 0:10) with a volume of 840 mL for each ratio to give

14 fractions (420 mL/fraction).

Fractions 2, 3, 4, 5, 6, 7 and 9 were further tested for antibacterial activity against *H. pylori*. Fractions 2, 3, 4 (total 1.42 g) were combined and then fractionated by open column chromatography on silica gel (45 g) in glass column (4.5x30 cm) using gradual elution with $CHCl_3$ and MeOH in 4 different ratios (10:0, 50:1, 10:1, and 5:1) with 180 mL for each ratio to give 8 subfractions (90 mL/fraction). The anti *H. pylori* subfraction 2.5 (0.55 g) was further re-chromatographed on silica gel (15 g) in glass column (2x30 cm) with C_6H_{14}:$CHCl_3$ in 6 different ratios (10:1, 5:1, 1:1, 1:5, 1:10, and 0:10) with 60 mL for each ratio to give 12 subfractions (30 mL/fraction). The active subfractions, including 2.5.0 (0.48 g), 2.5.10 (0.19 g) and 2.5.11 (0.23 g) were then purified by preparative TLC ($CHCl_3$:MeOH 10:1) to give 3 subfractions of each original one. The highest potency (100%), subfraction 2.5.0 was then purified by HPLC. The HPLC system consisted of Shimadzu separation module and UV detector, and was equipped with a Pegasil ODS column (250 x 4 mm i.d., 5 μm particle size). The linear solvent gradient of CH_3CN (from 40% to 100%) containing 0.05%TFA was applied over 50 min at a flow rate of 8 mL/min, and UV absorption at 205 nm was measured to obtain compound 1 (26.2 mg) and 2 (38.4 mg). Their structures were identified as 3'-demethyl arctigenin (1) and arctigenin (2), respectively.

Fraction 7 (4.27 g), the main fraction of the methanolic extract, was fractionated by open column chromatography on silica gel (135 g) in glass column (4.5x30 cm) using gradual elution with $CHCl_3$ and MeOH in 3 different ratios (10:1, 1:1, and 1:10) with 540 mL for each ratio to give 6 subfractions (270 mL/fraction). The highest potency (96%), subfraction 7.4 was then purified by HPLC system, equipped with a Pegasil ODS column (250 x 4 mm i.d., 5 mm particle size), using a linear solvent gradient of CH_3CN (from 30% to 50%) containing 0.05% TFA over 30 min at a flow rate of 8 mL/min and UV absorption at 205 nm to obtain four subfractions. The HPLC subfractions (1 and 2) were then eluted with isocratic solvent of 35% CH_3CN containing 0.05% TFA at a flow rate of 8 mL/min over 30 min to obtain compound 3 (22.1 mg). The structure of compound 3 was identified as arctiin (arctigenin glucoside).

Bacterial Strains and Cultures

Clarithromycin-resistant *H. pylori* strain (RC-1) was isolated and established from a human gastric biopsy specimen obtained from Kitasato Institute Hospital, Tokyo, Japan. Until processed, the strain was grown on brain-heart infusion (BHI; Difco Laboratories, Detroit, MI) agar plates supplemented with 1) 5% defibrinated horse blood, 2) 1 mg/mL glucose, 3) 250 mg/mL bovine serum albumin (BSA; Iwai Chemicals Co.) and 4) 1 vial of skirrow supplement (Oxoid, Basingstoke, UK), and were incubated for 4 days in a microaerobic gas environment (15% CO_2, 5% O_2 and 80% N_2). The colonies were harvested from the plates, suspended in 20 mL BHI broth containing 10% fetal bovine serum (FBS; Sigma-Aldrich Co.) and 1 mg/mL glucose, and incubated at 37 °C overnight with agitation on a rotary shaker at 100 rpm in microaerobic gas condition.

Anti Helicobacter pylori activity

The anti-H. pylori assay was carried out according to the *in vitro* infection model developed in the laboratory by Takahashi et al.[17]. Briefly, sample solutions (1 mg/mL) were prepared by dissolving the extracts in 10%MeOH.

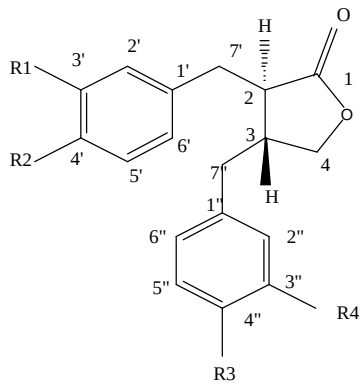
Amoxycilin (Sigma®, A8523) (0.5 mg/mL) and clarithromycin (Sigma®) (0.01 mg/mL) were used as positive control and negative control, respectively. 5 μL of the plant extracts and 1 μL of the control were added in a 96-well plate and dried up in the desiccator. All experiments were performed in triplicate.

H. pylori RC-1 was cultured at 37 °C for 48 h in the broth under microaerobic gas conditions with plate mixer. The culture was checked by phase contrast microscopy to ensure its purity. The preliminary experiments by Takahashi *et al* (2004)[17] showed that a suspension of cells yielding an optical density at 600 nm (OD_{600}) of 0.25 corresponding to $1x10^8$ colony-forming units (CFU)/mL) of living bacteria. The medium consisting of 100 μL *H. pylori* (OD_{600} = 0.0018) were then added in each well to provide a cell concentration of $0.72x10^6$ CFU/mL. After incubation at 37 ◦C under microaerobic gas conditions with plate mixer for 23 h, 100 μL of 20% Alamar Blue (Biosource), a oxidation-reduction sensitive dye, was added in the medium to determine cell growth. After incubation with plate mixer for 1 h, the absorbance was measured at 600 nm using microplate reader (Labsysterms Fluoroskan II, Dainippon Sumitomo Pharma). The activity was calculated by the following equation:

$$\text{Activity} = 100 \times (OD_{control} - OD_{sample}) / OD_{control}$$

Results

The three compounds were isolated and elucidated by spectroscopic method using ^{1}H-NMR, ^{13}C-NMR, distortionless enhancement by polarization transfer (DEPT), and correlation spectroscopy (COSY) compared to the previous report of the chemical constituents as shown in tables 1 and 2, and identified as 3'-demethyl arctigenin (compound 1), arctigenin (compound 2) and arctiin (arctigenin glucoside) (compound 3), respectively. From 500 g of the dried seeds, the yields of compounds 1, 2 and 3 were 26.2, 38.4 and 22.1 mg, respectively. The chemical structures of these compounds are indicated in figure 1.



Compound	R_1	R_2	R_3	R_4
1	OH	OH	OMe	OMe
2	OMe	OH	OMe	OMe
3	OMe	OGlu	OMe	OMe

Figure 1 Chemical structures of compounds 1, 2 and 3

Table 1 ^{1}H-NMR spectral data of compounds 1 - 3 (CD_3OD)

H	1	2	3
2	2.5 (1H, m)	2.5 (1H, m)	2.5 (1H, m)
3	2.6 (1H, m)	2.6 (1H, m)	2.6 (1H, m)
4a	3.9(1H, dd)	3.9(1H, dd)	3.9(1H, dd)
4b	4.2 (1H, dd)	4.2 (1H, dd)	4.2 (1H, dd)
2'	6.5 (1H, d)	6.5 (1H, d)	6.5 (1H, d)
5'	6.8 (1H, d)	6.8 (1H, d)	6.8 (1H, d)
6'	6.6 (1H, dd)	6.6 (1H, dd)	6.6 (1H, dd)
7'	2.9 (2H, m)	2.9 (2H, m)	2.9 (2H, m)
2"	6.4 (1H, d)	6.4 (1H, d)	6.4 (1H, d)
5"	6.7 (1H, d)	6.7 (1H, d)	6.7 (1H, d)
6"	6.5 (1H, dd)	6.5 (1H, dd)	6.5 (1H, dd)
7"	2.8 (2H, m	2.8 (2H, m	2.8 (2H, m
OH	6 x 2	6	-
O-Me	3.8 (3H, s) x 2	3.8 (3H, s) x 3	3.8 (3H, s) x 3
Glu-1	-	-	4.8 (1H, d)

Table 2 ^{13}C-NMR spectral data of compounds 1 - 3 (CD_3OD)

C	1	2	3
1	181.5	181.5	181.4
2	47.7	47.7	47.6
3	42.4	42.4	42.5
4	72.9	72.9	72.9
1'	130.7	130.7	132.7
2'	113.8	113.8	114.8
3'	149	149	149.2
4'	146.4	146.4	146.9
5'	116	116	117.9
6'	123	123	122.9
7'	35.4	35.4	35.4
1"	132.8	132.8	134.2
2"	113.6	113.6	113.6
3"	150.5	150.5	150.7
4"	149.1	149.1	150.5
5"	113	113	113
6"	122	122	122.1
7"	38.9	38.9	38.9
Methyl	-	56.4	56.7
Methyl	56.3	56.3	56.5
Methyl	56.3	56.3	56.4
Glu-1	-	-	102.9
Glu-2	-	-	74.9
Glu-3	-	-	77.8
Glu-4	-	-	71.3
Glu-5	-	-	78.2
Glu-6	-	-	62.5

Table 3 Anti-Helicobacter pylori activities of compounds isolated from *Arctium lappa* compared to standard amoxicillin and clarithromycin

Compounds	Concentration (μg/mL)	% Inhibition (mean ± S.D.)
1	50	99.5 ± 0.7
2	50	100.5 ± 0.7
3	50	95.5 ± 0.7
AMOX[a]	5	99.7 ± 0.5
CLAR[b]	1	25.0 ± 4.8
Crude extract	50	85.5 ± 3.5

[a] amoxycillin was used as positive control.
[b] clarithromycin was used as negative control.

The anti-Helicobacter pylori activities of isolated compounds (1-3) based on the *in vitro* infection model is shown in Table 3. At a concentration of 50 μg/mL, compounds 1 and 2 each exerted a 100% inhibition against *H. pylori* equal to a standard amoxicillin (5 μg/mL) while compound 3, crude methanolic extract and standard clarithromycin showed 95%, 86% and 25% inhibition, respectively.

Discussions

The pathogenic role of *H. pylori* infection in the development of gastroduodenal diseases[18] and the impact of resistance on the clinical outcome[19] stimulated the search for newer treatments and the use of natural agents as alternative therapies[11-12, 14-15, 20-23].

We evaluated methanolic extract of *A. lappa* L. against clarithromycin- resistant *H. pylori* strain. The findings suggested that this methanolic extract could contain most active substances, lignan compounds, possibly due to the fact that cold treatment (maceration) is a very mild condition while hot treatment (decoction) implies longer and stronger heating. Compared with the antimicrobial activity of amoxycillin (5 μg/mL) and clarithromycin (1 μg/mL), we reported the inhibitory effect of *A. lappa* extracts at low concentration (50 μg/mL). Similar results were obtained from garlic extracts (*Allium sativum*) and bismuth salts with minimum inhibition concentration (MIC) at 0.025 - 0.05 μg /mL[10] and 0.25 μg/mL[9] against *H. pylori,* respectively. Aqueous extracts of *Pteleopsis suberosa* showed inhibitory activity at 62.5 – 500 μg/mL for the decoction and for the methanolic extract at 31.25 – 250 μg /mL[12]. The cold extract, infusion, decoction and simulated digestion of

Larrea divaricata exhibited a marked inhibitory activity at 0.04 – 0.1 μg/mL against clarithromycin and metronidazole-susceptible and resistant *H. pylori* strains[22]. Taken into account that minimal levels of lignans present in methanolic extracts of *A. lappa*, arctigenin (26.2 mg), arctigenin (38.4 mg) and arctigenin glucoside (22.1 mg), could be involved in the anti-H. pylori activity observed with clarithromycin-resistant strain that exerted 100%, 100% and 96% inhibition, respectively. Considering the *A. lappa* therapeutic potential as chemopreventive agents for peptic ulcer or gastric cancer, structure-activity relationship (SAR) studies will help characterize pharmacologic effect of this abundant plant.

Conclusion

In treatment of chronic gastritis and peptic ulcers caused by *Helicobacter pylori*, Chinese and Japanese ethnomedical uses of *A. lappa* may be justified because of a high anti-Helicobacter pylori activity of three lignans: 3'-demethyl arctigenin (1), arctigenin (2), and arctigenin glucoside (3). These compounds may be useful as lead compounds in the development of a new class of anti- H. pylori agents.

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