

Antibacterial activities of *Galla rhois* extracts against *Helicobacter pylori* infection

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Summary

In this study, a medicinal plant, *Galla rhois* (GR), was examined and screened for its anti-*Helicobacter pylori* activity. *Galla rhois* was extracted with 70% ethanol. Inhibitory zone tests, as *in vitro* assays, and an *in vivo* study using a Mongolian gerbil (*Meriones unguiculatus*) model were performed. The safety of the GR extract was then evaluated in an animal study. The *Galla rhois* extract demonstrated a strong anti-*H. pylori* activity as well as strong therapeutic effects against *H. pylori* infection in the *in vivo* animal experiments based on the histological criteria and the rapid urease test. Results of the safety study revealed that the animals had no detectable gross or histological changes after a 28 day treatment with the GR extract. These results demonstrate that the GR extract successfully cured *H. pylori* infections and protected against *H. pylori*-induced pathology. This herb could be a promising treatment for patients with gastric complaints including gastric ulcers caused by *H. pylori*.

Key words: *Galla rhois*, Anti-*Helicobacter pylori*, Antibiotic, Herb

Introduction

Helicobacter pylori is the most important etiological agent of chronic gastritis and peptic ulcers and increases the risk for gastric cancer (Maruta *et al.*, 2001). The Mongolian gerbil is an animal model used for the investigation of human gastric disorders; as *H. pylori* easily infects the stomach of Mongolian gerbils, it induces human-mimicking gastritis, peptic ulcers and intestinal metaplasia in the stomach. In addition, this animal model develops gastric adenocarcinoma following the inoculation of *H. pylori* (Watanabe *et al.*, 1998).

Accumulating evidence has demonstrated that eradicating *H. pylori* in the stomach by oral administration of antimicrobial agents results in the resolution of *H. pylori*-associated gastroduodenal diseases (Salih *et al.*, 2005). In addition, eradication of *H. pylori* also decreases the risk of gastric carcinogenesis (Maruta *et al.*, 2005). Triple combination therapy, using two antibacterial antibiotics and a proton pump inhibitor, can help achieve a high eradication rate (Misiewicz *et al.*, 1997). Nevertheless, such combination therapy does not always successfully eradicate *H. pylori*. In recent years, the increased occurrence of clarithromycin- and/or metronidazole-resistant strains of *H. pylori* have become a problem (Midolo *et al.*, 1996). Furthermore, antibiotics cannot be used for some patients due to drug allergies, and antibiotic therapy is also occasionally associated with adverse events. Moreover, the administration of

long-term antimicrobial agents is considered inappropriate for the prevention of *H. pylori* infections.

Antibiotic chemotherapy occasionally produces side effects and fails to eliminate bacterial infections (Buenz *et al.*, 2007). The occurrence of antibiotic resistant strains is expected to increase; thus, the search for non synthetic antimicrobial substances to cure these infections gains importance (Lee *et al.*, 2012). One hope as an alternative to antimicrobial agents is herbal material that can control these infections. Since natural herbs tend to be a source of greater structural and chemical diversity than combination chemistry libraries, natural products offer a potentially rewarding alternative for the identification of novel antimicrobial agents (Koehn and Carter, 2005). It is therefore important to investigate the use of highly effective and safe herbal therapies for *H. pylori* infection (Lee *et al.*, 2012).

One GR extract has been reported to have strong *in vitro* antiviral and antibiotic effects (Hong *et al.*, 2011). *Galla rhois* is the gall derived from the nutgall sumac tree, *Rhus javanica*, caused by the Chinese sumac aphid (Kubo *et al.*, 2003), *Schlechtendalia chinensis*. *Galla rhois* has been used to treat various diseases, including skin diseases, diarrhea, dysentery, hemorrhage, and leucorrhea (Zhu, 1998). In addition, GR and its components perform various biological activities, including antibacterial (Ahn *et al.*, 1998), and antimetastatic/anti-invasion activities (Ata *et al.*, 1996) and have protective effects on liver cells due to apoptosis and necrosis (Park *et al.*, 2008). Recent studies have

shown that GR inhibits the production of inflammatory cytokines in mast cells (Kim *et al.*, 2005) and that gallates inhibit cytokine-induced nuclear translocation of nuclear factor-kappa B and the expression of leukocyte adhesion molecules in vascular endothelial cells (Murase *et al.*, 1999). Constituents of GR include methyl gallate, 3-galloyl-gallic acid, 4-galloyl-gallic acid isomers, 1,2,3,4,6-penta-Ogalloyl- β -D-glucose, and two inactive phenolic compounds, gallic acid methyl ester and gallic acid (An *et al.*, 2005). Methyl gallate also exhibits strong antimicrobial activity and performs growth-inhibiting activities against *E. coli*, without adversely affecting the growth of lactic acid-producing bacteria (Choi *et al.*, 2009).

In this study, the anti-*Helicobacter* effects of a GR extract were investigated using an *in vitro* inhibitory zone test and an *in vivo* study with a Mongolian gerbil (*Meriones unguiculatus*) model.

Materials and Methods

Preparation of GR extract

Galla rhois extract powder, commercially available as FlavosK™, was obtained from Samkwang Biotech (Jeonbuk, South Korea), isolated from plant material, and analyzed as described previously (An *et al.*, 2005). The crude powder was analysed by silica-gel chromatography (Merck 70-230 mesh, Darmstadt, Germany) and fractionated by a preparative high performance liquid chromatography (Waters Delta Prep 4000, Milford, MA, USA).

Inhibitory zone test

Helicobacter pylori (ATCC 43504; American Type Culture Collection, Manassas, VA, USA) was cultured in 2×10^9 colony-forming units (CFU)/ml in a brain-heart infusion broth containing 10% fetal bovine serum and spread onto a *Brucella* agar plate containing 10% (v/v) calf serum. Filter paper disks (0.7-mm diameter) were prepared. The GR extract was dissolved in DMSO to prepare a 300 mg/ml concentration, and a volume of 5 μ l (1.5 mg) was applied on each filter disk. DMSO was used as the control. The plates were incubated in a micro-aerophilic jar system, featuring a gas composition of 10% CO₂ in the air at 37°C for 72 h. The clear zone around each filter disk was observed and the diameter was recorded.

Animal experiments

Specific pathogen-free (SPF) 3-month-old male and female Mongolian gerbils were obtained from the SPF Animal Facility of the Korean Food and Drug Administration (Seoul, South Korea). All animals were kept at the inspecting facility of Wonkwang University (Iksan, South Korea) for 1 week to allow acclimation before experimentation. Thereafter, they were kept in an isolated SPF barrier room with regulated temperature ($23 \pm 1^\circ\text{C}$), humidity ($50 \pm 5\%$) and light/dark cycle (12/12 h). Animals were fed a sterilized (prepared by 2 M rad radiation) pellet diet (Purina, Seoul, Korea) and sterilized

water *ad libitum*. All experiments were performed in accordance with the Guide for Animal Experimentation of Wonkwang University and approved by the Institutional Animal Care and Use Committee of Wonkwang University. All efforts were made to minimize pain or discomfort to the animals.

Helicobacter pylori (ATCC 43504) was incubated in brain-heart infusion broth (BHIB) containing 10% fetal bovine serum at 37°C under a micro-aerophilic atmosphere for 3 days and allowed to grow to a density of $\sim 2.0 \times 10^9$ CFU/ml. Animals were inoculated twice at 3-day intervals by oral administration of 1.0×10^9 CFU *H. pylori* suspended in 0.5 ml BHIB. The challenged animals were confirmed to be *H. pylori*-positive by polymerase chain reaction (PCR) analysis of their faecal samples as described previously (Lee and Kim, 2006).

The antibacterial effect of the GR extract on *H. pylori* infection was investigated using a Mongolian gerbil model. Gerbils were divided into four groups: a negative control (group I, n = 10); *H. pylori*-uninfected, GR-treated animals (group II, n = 10); *H. pylori*-infected, GR-untreated animals (group III, n = 10); and *H. pylori*-infected, GR-treated animals (group IV, n = 10). The GR extract was administered daily via oral route at the rate of 400 mg/kg/day during a 4 week treatment period. The dose of the GR extract was based on previous studies (Hong *et al.*, 2008; Lee *et al.*, 2008a, b). Body weights were measured once per week and the animals were monitored daily for general health. Four weeks after *H. pylori* inoculation, all animals were put to sleep with ether and their stomachs were opened along the greater curvature and washed with saline. Macroscopic gastric lesions were then observed and evaluated. Half of the glandular mucosa was scraped to detect colonizing *H. pylori*, and the rest was formalin-fixed and embedded in paraffin for histopathological observations. *Helicobacter pylori* colonization was confirmed by both PCR and rapid urease tests as described previously (Lee *et al.*, 2010a). Mucosal damage was evaluated grossly and histopathologically according to previously described criteria (Lee *et al.*, 2010b).

Safety evaluation

For the safety study, the GR extract was orally administered daily at a dosing rate of 400 mg/kg/day for 4 weeks. After 4 weeks, animals were put to sleep and evaluated grossly and histologically according to previously described criteria (Lee *et al.*, 2010b).

Statistical analysis

Values for all parameters under study were recorded for each experimental unit, and statistical analyses were performed using a general linear model. Values are reported as average \pm SD, when appropriate. Student's t-tests were used for pair-wise comparisons. Incidence percentages (95% confidence intervals [CIs]) were calculated with the MiniTab statistical software program (MiniTab, State College, PA, USA). A p-value < 0.05 was considered significant.

Results

From the chromatography analysis for GR composition, tannins, methyl gallate and gallic acid were determined to account for 52.7, 16.4 and 4.3% of the total composition of GR, respectively.

With respect to the *in vivo* experiment, all animals survived to the end of the experimental period. No significant differences were observed in the general appearance of the gerbils in each group. At the end of the experiment, average body weights of *H. pylori*-inoculated and GR-untreated gerbils (group III) were significantly lower than those inoculated with *H. pylori* and treated with GR (group IV, Table 1).

The GR extract exerted a strong anti-*H. pylori* effect in the inhibitory zone test. Anti-*H. pylori* activity was revealed in the following order: GR (14 mm), enrofloxacin (12 mm), kanamycin (7 mm), and gentamicin (6 mm).

No visible gross changes were seen in the gastric mucosa of gerbils in group I (PBS) or II (PBS + GR). Pyloric mucosa was expanded and thickened in the *H. pylori*-inoculated gerbils (group III), accompanied by erosive lesions with bleeding. However, no significant macroscopic difference in the gastric mucosa was observed between group I (PBS) and group IV (*H. pylori* + GR) (Table 2).

Pathological changes in the gastric mucosa were negligible in animals of the non-*H. pylori* inoculated groups (I and II). In contrast, gastric atrophy and ulceration had developed in the gastric mucosa of group III gerbils (*H. pylori* inoculation only), indicating marked mucosal destruction (Fig. 1A). In addition, superficial erosion in the antrum and irregularity of the pyloric glands were frequently observed in group III. However, histopathological changes in group IV (*H. pylori* inoculation followed by GR treatment) had significantly reduced as compared to those of group III (Fig. 1B).

Repeated *H. pylori* inoculation (1.0×10^9 CFU, twice) resulted in a positive reaction (red color) on the rapid urease test 28 days after the final inoculation,

indicating a bacterial infection in the stomach (Table 3). None of the animals treated with the GR extract for 28 days after *H. pylori* infection displayed a positive

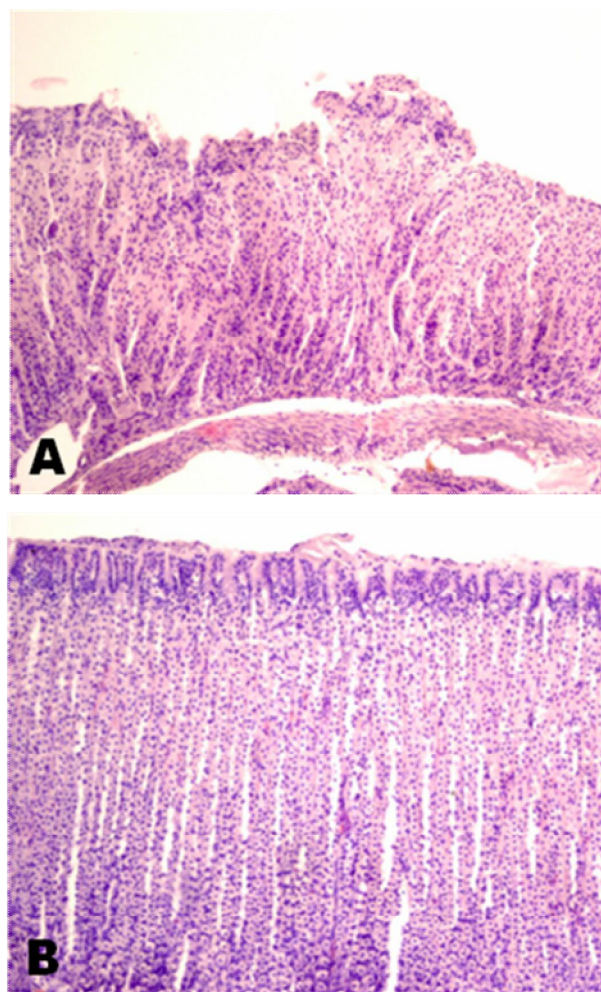


Fig. 1: Histopathological findings of the stomach following *H. pylori* infection and treatment with or without a *Galla rhois* (GR) extract. A) *Helicobacter pylori*-inoculated and GR untreated gerbils, and B) *Helicobacter pylori* inoculation followed by GR treatment, (H&E, $\times 100$)

Table 1: Body weights (g) measurement in different studied groups

Group	Treatment	0 w.p.i. ^a	1 w.p.i.	2 w.p.i.	3 w.p.i.	4 w.p.i.
I	PBS (+PBS)	136.4 \pm 4.3	139.4 \pm 3.2	144.0 \pm 3.2*	148.4 \pm 2.3*	152.6 \pm 2.1*
II	PBS (+GR)	137.0 \pm 6.2	141.0 \pm 4.9	145.4 \pm 4.5*	149.4 \pm 2.9*	153.4 \pm 2.4*
III	<i>H. pylori</i> (+PBS)	136.2 \pm 4.5	135.8 \pm 3.1	136.0 \pm 2.7	136.8 \pm 2.6	137.0 \pm 1.6
IV	<i>H. pylori</i> (+GR ^b)	135.6 \pm 3.7	138.2 \pm 2.9	141.6 \pm 2.3*	144.4 \pm 3.7*	146.4 \pm 2.0*

^a Weeks post-inoculation of *H. pylori*. ^b *Galla rhois* treatment was continuously administered at a 400 mg/kg dose during 4 weeks after infection with *H. pylori*. * Significantly different from the positive control group III ($P < 0.05$). Use one significant figure

Table 2: Gross and histopathological lesion scores in different groups

Groups	Treatment	n	Gross lesion scores ^a	Histopathological lesion scores ^b
I	PBS (+PBS)	10	0 \pm 0*	0 \pm 0*
II	PBS (+GR)	10	0 \pm 0*	0 \pm 0*
III	<i>H. pylori</i> (+PBS)	10	95.6 \pm 10.1	7.4 \pm 1.4
IV	<i>H. pylori</i> (+GR ^b)	10	6.8 \pm 5.4*	1.2 \pm 1.0*

^a Gross lesion scores were calculated with criteria described previously (Lee *et al.*, 2010b). ^b Histopathological lesion scores were calculated with criteria described previously (Lee *et al.*, 2010b). * Significantly different from the positive control group III ($P < 0.05$)

Table 3: Results of the rapid urease test of gastric mucosal tissues in different studied groups

Groups	Inoculation ^a		n.	Positive reaction (positive percent) ^b	Cured animals (therapeutic percent)
	<i>H. pylori</i>	Treatment			
I	No	PBS	10	0* (0%, CI 0-25.9)	-
II	No	GR	10	0* (0%, CI 0-25.9)	-
III	Yes	PBS	10	10 (100%, CI 74.1-100)	0 (0%, CI 0-25.9)
IV	Yes	GR	10	0* (0%, CI 0-25.9)	10* (100%, CI 74.1-100)

^a Treatment was conducted daily during 4 weeks after inoculation with *H. pylori*. ^b The positive percent revealed *H. pylori* colonization, which was observed as a red color change in a yellow-colored medium. * Significantly different from the positive control group III according to CI (P<0.05)

Table 4: Results of the polymerase chain reaction analysis of gastric mucosa tissues in different groups

Groups	Inoculation ^a		n.	Positive reaction (positive percent) ^b	Cured animals (therapeutic percent)
	<i>H. pylori</i>	Treatment			
I	No	PBS	10	0* (0%, CI 0-25.9)	-
II	No	GR	10	0* (0%, CI 0-25.9)	-
III	Yes	PBS	10	10 (100%, CI 74.1-100)	0 (0%, CI 0-25.9)
IV	Yes	GR	10	0* (0%, CI 0-25.9)	10* (100%, CI 74.1-100)

^a Treatment was conducted daily during 4 weeks after inoculation with *H. pylori*. ^b The positive percent revealed *H. pylori* colonization, which was observed as a DNA amplification product. * Significantly different from the positive control group III according to CI (P<0.05)

reaction (95% CI, 0-25.9), in comparison with the 100% (95% CI, 74.1-100) positivity in group III (*H. pylori* inoculation only). The PCR results are shown in Table 4. PCR products were not detected in animals of the non-*H. pylori* inoculated groups (I and II). In contrast, positive PCR reactions were identified in the gastric mucosa of all gerbils in group III (*H. pylori* inoculation only). Nevertheless, PCR products in group IV (*H. pylori* inoculation followed by GR treatment) were not detected (Table 4).

The results of the safety study revealed no significant histopathological changes in the cerebrum, cerebellum, pons, testicles, heart, liver, lungs, kidneys, muscles, spleen, prostate, pancreas, thymus, adrenal glands, small intestine, large intestine, bone marrow, thyroid gland, or seminal vesicles.

Discussion

Galla rhois is found in sumac galls and has been applied in traditional Korean as well as other Oriental medicine techniques to treat diarrhea, persistent coughs, and spontaneous perspiration resulting from the effects of astringents, antidiarrheals, hemostatic drugs, and counterpoison (Ahn *et al.*, 1994). *Galla rhois* is a natural, non-toxic material that contains a number of tannin-derived components, collectively termed as tannic acid, methyl gallate, and gallic acid. Notably, gallotannins, which seem to have antibacterial, antifungal, and antiviral properties, are a class of hydrolysable tannin polymers formed from gallic acid (Ahn *et al.*, 1994; Chen *et al.*, 2006). Tannic acid, a common form of hydrolysable tannin, has an inhibitory effect on the growth of some intestinal pathogens such as *Clostridium* spp., *Escherichia coli*, and *Salmonella typhimurium* (Chung *et al.*, 1998). Methyl gallate inhibits the growth of *E. coli* without negative influences on the growth of lactic acid-producing bacteria (Bae *et al.*, 1998).

In the present study, a strong antibacterial activity of the GR extract against *H. pylori* was shown using the inhibitory zone test and its therapeutic effect on *H. pylori* infections in a Mongolian gerbil model. Both rapid urease and PCR analyses of the stomach demonstrated a clear reduction in *H. pylori* colonization. In addition to the therapeutic effect against *H. pylori* infections, GR extract reduced mucosal inflammation and epithelial proliferation in the stomach of *H. pylori*-infected gerbils. *Helicobacter pylori* eradication might have caused a decrease in the degree of inflammation in the stomach; however, there is a possibility that GR itself has had an anti-inflammatory effect on gastric mucosa.

Although triple therapy (using two antibacterial antibiotics and a proton pump inhibitor) is effective and helps maintain patient compliance due to its short duration, a considerable number of patients experience undesirable side effects such as diarrhea, epigastric pain, nausea, and bloating (Sakamoto *et al.*, 2001). In contrast, GR has relatively few side effects as revealed in our study. Such safety characteristics of GR may, therefore, be appropriate for its use in the prevention and therapy of *H. pylori* infections. As chronic inflammation and increased cell proliferation are features common to the pathogenesis of many human cancers and play a central role in initiating and promoting carcinogenesis, the suppression of inflammation and cell proliferation by GR may contribute to the prevention of *H. pylori*-induced carcinogenesis of the stomach.

In conclusion, due to its strong antimicrobial effects against *H. pylori* infection, GR could be a promising native herb for the treatment of patients with gastric complaints including gastric ulcers caused by *H. pylori*.

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