In vitro and *In vivo* Anti-*Helicobacter pylori* Activities of *Centella asiatica* Leaf Extract

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ABSTRACT: *Helicobacter pylori* infection is associated with an increased risk of developing upper gastrointestinal tract diseases. However, treatment failure is a major cause of concern mainly due to possible recurrence of infection, the side effects, and resistance to antibiotics. The aim of this study was to investigate the activities of *Centella asiatica* leaf extract (CAE) against *H. pylori* both *in vitro* and *in vivo*. The minimum inhibitory concentrations (MICs) against 55 clinically isolated strains of *H. pylori* were tested using an agar dilution method. The MICs of CAE ranged from 0.125 mg/mL to 8 mg/mL, effectiveness in inhibiting *H. pylori* growth was 2 mg/mL. The anti-*H. pylori* effects of CAE *in vivo* were also examined in *H. pylori*-infected C57BL/6 mice. CAE was orally administrated once daily for 3 weeks at doses of 50 mg/kg and 250 mg/kg. CAE at the 50 mg/kg dose significantly reduced *H. pylori* colonization in mice gastric mucosa. Our study provides novel insights into the therapeutic effects of CAE against *H. pylori* infection, and it suggests that CAE may be useful as an alternative therapy.

Keywords: *Centella asiatica* leaf extract, *Helicobacter pylori*, gastric mucosa, colonization

INTRODUCTION

Helicobacter pylori is a common bacterium and a major worldwide cause of gastrointestinal infection in approximately one-half of the world human population (1,2). *H. pylori* infection is associated with the development of upper gastrointestinal tract diseases namely chronic gastritis, as well as gastric and duodenal ulcers (3,4) and gastric carcinoma (5). In fact, *H. pylori* was classified as a Class I carcinogen by the World Health Organization for gastric carcinoma (6). The recommended first line treatments in subjects infected with *H. pylori* is standard triple therapy [a proton pump inhibitor (PPI)], two antibiotics (clarithromycin, amoxicillin, or metronizazole), or Bismuth based quadruple therapy (7,8). However, these therapies are not always successful due to a rapidly-increasing tolerance of the bacteria and recent results are not very encouraging. These treatments also raise concerns mainly infection recurrence, high costs, side effects, and poor compliance to the therapies (9-12). Despite numerous studies, identifying an optimal therapy for eradication of *H. pylori* still remains a challenging clinical problem. Based on the systematic reviews and meta-analysis reports, therapeutic failure mainly resulted from *H. pylori* resistance to the antibiotics (13,14). Therefore, in line with the alternative approaches of naturally occurring medicinal plants, herbal preparations have become more prominent and have been extensively studied worldwide in order to develop new treatment strategies for *H. pylori* infection.

Centella asiatica (L.) Urban is a perennial herbaceous creeper which belongs to the family Apiaceae, and it is commonly named Gotu Kola or Indian Pennywort and has been used as a traditional herbal medicine in Asiatic countries for hundreds of years (15,16). In several studies, *C. asiatica* has been reported to have anti-inflammatory and antioxidant activities (17-19), and *C. asiatica* has been cited in papers regarding anti-gastric ulcers induced by ethanol, aspirin, cold-restraint stress, pylorus ligation, and acetic acid (20-22). In a previous study, we demon-

strated that *C. asiatica* leaf extract (CAE) confers gastro-protective properties against indomethacin-induced gastric injury in rats (23).

To our knowledge, no studies have investigated the effects of CAE on *H. pylori in vivo* on its ability to colonize gastric mucosa. Therefore, the aim of this study is to assess the effects of CAE on *H. pylori* using both *in vitro* and *in vivo* models. The *in vivo* effectiveness of CAE in inhibiting *H. pylori* gastric mucosal colonization was examined in a C57BL/6 mouse model infected with the Sydney strain (SS1) of *H. pylori*.

MATERIALS AND METHODS

Preparation of CAE
A 20 g sample of *C. asiatica* leaf was extracted using the dipping method in 320 mL of 75% ethanol at 30°C for 22 h and filtered using a fabric filter. The filtrate was evaporated using an evaporator (EYELA, Tokyo, Japan) at 60°C (yield 45%, 54°Brix). All other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

H. pylori strains and culture
Fifty-five archived strains of *H. pylori*, which were isolated from biopsy specimens of patients in Korea, and *H. pylori* SS1 (HpKTCC B0890) for animal experiments were obtained from the *H. pylori* Korean Type Culture Collection (HpKTCC, Korea National Microorganisms Research Resource Center, Suwon, Gyeonggi, Korea), and cultured on 10% bovine serum Muller-Hinton agar plates supplemented with 6 μg/mL vancomycin, 8 μg/mL amphotericin B, 10 μg/mL nalidixic acid in an anaerobic chamber with 10% CO_2, 5% O_2, and 85% N_2 at 37°C with ample humidity. The strain was identified on the basis of colony appearance, Gram staining, and positive reactions in biochemical tests (urease and catalase).

Minimal inhibitory concentration (MIC) determination
H. pylori strains were grown on Mueller-Hinton agar plates. The MICs of CAE for *H. pylori* were determined by an agar dilution method following the National Committee for Clinical Laboratory Standards Institute guidelines. Briefly, exponentially growing *H. pylori* cells were suspended in sterile phosphate-buffered saline (PBS) and adjusted to 5×10^8 colony-forming units (CFU) and were then inoculated directly onto various concentrations of CAE-containing agar dilution plates. Final concentrations of CAE ranged from 0.125 to 8 mg/mL. All plates were incubated as previously described for 3 days. The MIC was defined as the lowest concentration of CAE preventing visible growth.

Animals
Specific pathogen-free 6-week-old male C57BL/6 mice were obtained from Central Lab Animal Inc. (Seoul, Korea). Animal care and all experimental procedures were conducted in accordance with the approval and guidelines of the INHA Institutional Animal Care and Use Committee (INHA 121205-171-1) at the medical school in Inha University. The animals were fed standard rat chow and tap water *ad libitum* and maintained under a 12-h dark/light cycle at 21°C. For biosafety, the investigators were fully protected with sterilized clothes, masks, and gloves based on Standard Operation Procedures.

H. pylori infection in C57BL/6 mice and CAE treatment
The mice were randomly divided into 3 experimental groups of 10 mice each (Fig. 1). The treatment groups were treated with CAE (50 or 250 mg/kg) by oral administration once daily for 3 weeks. The control group was treated with equivalent volumes of distilled water. Following a pre-treatment period of 1 week, infection was induced in concomitance with CAE administration as follows: the mice were fasted 4 h before inoculation. Specific pathogen-free C57BL/6 male mice were inoculated 3 times by oral gavage with *H. pylori* SS1 (2×10^9 CFU/mouse) suspended in 500 μL PBS over 5 days. This followed the well-established mouse model of *H. pylori* infection (24). Mice inoculated with *H. pylori* SS1 were kept separately with free access to water and food.

Detection of *H. pylori* colonization
One day after the final administration, the mice were killed and their gastric mucosa was biopsied for the detection of *H. pylori*. The presence of *H. pylori* in gastric mucosa was confirmed by direct bacterial culture, as previously described (25). For culturing, tissue was rubbed across the surface of a plate containing a selective medium of 10% bovine serum, 6 μg/mL vancomycin, 5 μg/mL trimethroprim, 10 μg/mL polymyxin B, 200 μg/mL baci-
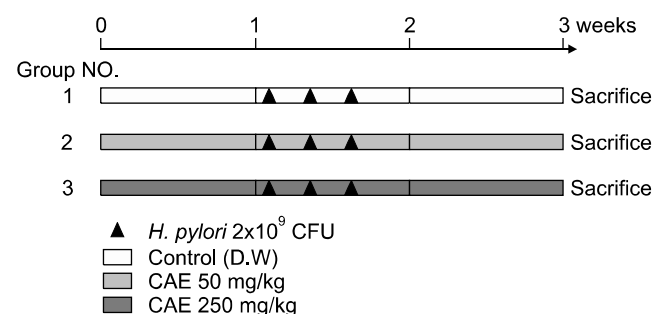

Fig. 1. Experimental protocol for *Helicobacter pylori* infection model. Mice in group 1 were orally administrated distilled water, group 2 and 3 were orally treated *Centella asiatica* leaf extract (CAE) (50 and 250 mg/kg) once daily for 3 weeks. After 1 week CAE treatment, mice in group 1, 2, and 3 were orally administered *H. pylori* [2×10^9 colony-forming units (CFU)] 3 times over 5 days.

tracin, 8 μg/mL amphotericin B, and 10 μg/mL nalidixic acid. Plates were incubated at 37°C under microaerophilic conditions for 5~7 days. Quantitative assessment of *H. pylori* colonization was performed as previously described (26). Briefly, one piece of weighed stomach tissue was homogenized in 200 μL of Brucella broth using a hand homogenizer (Pellet Pestle® Cordless Motor, Kimble Kontes, Rockwood, TN, USA), and the homogenate was diluted 10- and 100-fold in Brucella broth. Fifty micro-liters of each dilution was plated on selective medium containing the antibiotics mentioned above. Plates were incubated at 37°C under microaerophilic conditions for 5~7 days. *H. pylori* strains were identified by Gram staining, positive urease, and catalase tests. *H. pylori* colonies were then counted to determine the number of CFU of the whole stomach tissue.

Statistical analysis

Data were expressed as mean±standard error of the mean (SEM) values. Statistical comparisons between groups were determined by the non-parametric Kruskal-Wallis and Mann-Whitney U tests. Differences were considered statistically significant at a value of $P<0.05$. Statistical calculations were performed using SPSS software on a MS Windows operating system (version 19, SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Several studies have previously reported that extracts from several plants, such as garlic extracts (27,28), cinnamon extracts (29), and tea catechins (30) have anti-*H. pylori* activities. Previous reports that screened 54 Chinese herbs for anti-*H. pylori* activity revealed that *Rheum palmatum*, *Rhus javanica* L., *Coptis japonica*, and *Eugenia caryophyllata* exhibit strong anti-*H. pylori* activities (31).

In addition, Wang and Huang (32) studied herbs derived from Taiwanese folk medicinal plants and highlighted that *C. asiatica* L. Urban had moderate activity against *H. pylori*.

In the present study, we examined anti-*H. pylori* activity of CAE *in vitro* and *in vivo* with the established models of *H. pylori* SS1 infection in C57BL/6 mice.

In order to assess the inhibitory activity of CAE against 55 clinically isolated *H. pylori* strains, the MICs were determined by the agar dilution method. As shown in Fig. 2, the MIC of CAE ranged from 0.125 mg/mL to 8 mg/mL, and the majority of the strains (92%) showed a MIC of 2 mg/mL. CAE at 8 mg/mL concentrations completely inhibited the growth of *H. pylori*.

In order to assess the effectiveness of CAE *in vivo*, we investigated the anti-*H. pylori* activity of CAE on *H. pylori* colonization in C57BL/6 mice. The animals were killed 3 weeks after treatment with CAE. Stomachs were removed and analyzed by direct and quantitative cultures of *H. pylori*. The colonies grown from the selective medium were identified as *H. pylori* by Gram staining, urease, and catalase activity. In the infected group, all animals were successfully infected with *H. pylori*. As shown in Fig. 3, the ability of *H. pylori* SS1 to colonize the gastric mucosa was significantly decreased in 50 mg/kg CAE treated mice compared to mice from the control (G1) group (5.666± 0.062 log CFU *vs*. 5.913±0.041 log CFU, $P<0.05$). There were no toxic effects observed determined by body weight due to the CAE treatment. Our results confirm that oral treatment with CAE for 3 weeks could reduce *H. pylori* SS1 gastric colonization in a significant proportion of mice ($P<0.05$). These data suggest that CAE supplementation as a safe prophylactic/preventive agent for *H. pylori* infection eradication is worth further consideration.

In conclusion, the present study highlights the potential antibacterial activity of CAE against *H. pylori in vitro*, and showed that CAE may possess significant anti-*H. py-*
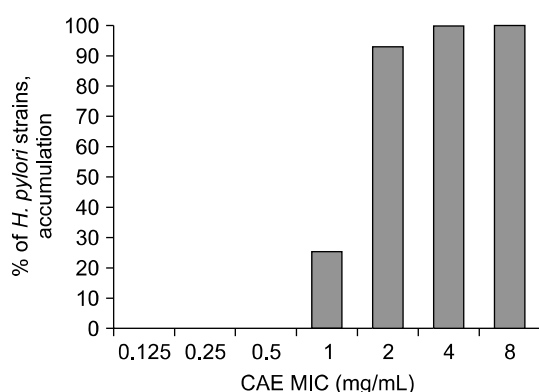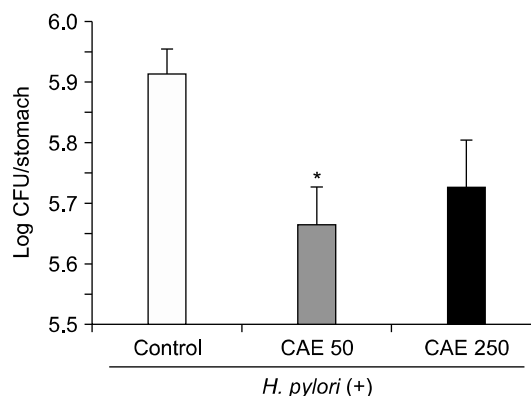

Fig. 2. Distribution of *Centella asiatica* leaf extract (CAE) minimum inhibitory concentrations (MICs) among the *Helicobacter pylori* strains isolated from clinical patients. MICs were determined by the agar dilution method. The antimicrobial activity was examined against 55 different clinical isolates of *H. pylori*.

Fig. 3. Effects of *Centella asiatica* leaf extract (CAE) against *Helicobacter pylori* colonization in C57BL/6 mice. CAE was orally administrated at 50 and 250 mg/kg, once daily for 3 weeks. The results are expressed in mean±SEM (n=10). *P<0.05 compared with control.

lori properties against *H. pylori* SS1 in C57BL/6 mice. This is the first known report to confirm the effects of CAE against *H. pylori* in an animal model. Our study suggests that CAE could be a good candidate for overcoming tolerance of antibiotics for the treatment of *H. pylori*-mediated gastric disease.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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