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***Basic Study***

**Anti-*Helicobacter pylori* activities *of Chenopodium ambrosioides* L. *in vitro* and *in* *vivo***

Ye H *et al.* *C. ambrosioides* against *H. pylori*

Hui Ye, Yu Liu, Ning Li, Jing Yu, Hong Cheng, Jiang Li, Xue-Zhi Zhang

**Hui Ye, Ning Li, Jing Yu,** **Xue-Zhi Zhang,** Department of Integrated Traditional Chinese Medicine, Peking University First Hospital, Beijing 100034, China

**Yu Liu,** Dongzhimen Hospital Affiliated to Beijing University of Chinese Medicine, Beijing 100700, China

**Hong Cheng, Jiang Li,** Department of Gastroenterology, Peking University First Hospital, Beijing 100034, China

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**Data sharing:** Technical appendix, statistical code, and dataset available from the corresponding author at zhang.xuezhi@263.net. Participants gave informed consent for data sharing.

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**Correspondence to:** **Xue-Zhi Zhang,** **Professor**, Department of Integrated Traditional Chinese Medicine, Peking University First Hospital, Xishiku Street, 8th Xicheng District, Beijing 100034, China. zhang.xuezhi@263.net

**Telephone:** +86-10-83572634

**Fax:** +86-10-83572931

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**Abstract**

**AIM:** To investigate the bactericidal effects of *Chenopodium ambrosioides* L. (CAL) against *Helicobacter pylori* (*H. pylori*) both *in vitro* and *in* *vivo*.

**METHODS:** For *in vitro* experiments, the inhibitory activity of CAL was tested using an agar dilution method; *H. pylori* strain NCTC11637 was incubated on Columbia blood agar plates containing serial concentrations of CAL. The minimal inhibitory concentration (MIC) was determined by the absence of *H. pylori* colonies on the agar plate. Time-kill curves were used to evaluate bactericidal activity; the average number of colonies was calculated at 0, 2, 8 and 24 h after liquid incubation with concentrations of CAL at 0.5, 1, and 2 × MIC. For *in vivo* experiments, *H. pylori*-infected mice were randomly divided into CAL, triple therapy (lansoprazole, metronidazole, and clarithromycin), blank control, or *H. pylori* control groups. The eradication ratios were determined by positive findings from rapid urease tests (RUTs) and by histopathology.

**RESULTS:** *In vitro*, the MIC of CAL against *H. pylori* was 16 mg/L. The time-kill curves showed a stable and persistent decreasing tendency with increasing CAL concentration, and the intensity of the bactericidal effect was proportional to dose; the 1 and 2 × MICcompletely inhibited the growth of *H. pylori* at 24 h. *In vivo*, the eradication ratios in the CAL group were 60% (6/10) by RUT and 50% (5/10) by histopathology. Ratios in the triple therapy group were both 70% (7/10), and there was no difference between the CAL and triple therapy groups. Histopathologic evaluation revealed massive bacterial colonizationon the surface of gastric mucosa and slight infiltration of mononuclear cells after inoculation with *H. pylori*, but no obvious inflammation or other pathologic changes in gastric mucosa of mice from CAL and triple therapy groups.

**CONCLUSION:** CAL demonstrates effective bactericidal activity against *H. pylori* both *in vitro* and *in vivo*.

**Key words:** *Helicobacter pylori*; *Chenopodium ambrosioides* L.; Bactericidal activity; Phytotherapy

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**Core tip:** The *Helicobacter pylori* (*H. pylori*) eradication rate of triple therapy has been markedly decreased due to increasing bacterial antibiotic resistance. Natural Chinese medicines, such as *Chenopodium ambrosioides* L. (a Chinese herb derived from Jinhua Weikang Capsule utilized for gastritis and peptic ulcers), represent complementary and collaborative therapies. This report demonstrates that *C. ambrosioides* has *in vitro* and *in vivo* bactericidal effects against *H. pylori*, and may be a good candidate for the treatment of *H. pylori* infection.

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**INTRODUCTION**

*Helicobacter pylori* (*H. pylori*) is the etiologic agent associated with many gastrointestinal diseases, such as chronic gastritis, peptic ulcers, gastric carcinoma, and mucosa-associated lymphoid tissue lymphoma. The eradication of *H. pylori* has thus been utilized as the primary treatment strategy of these diseases for three decades. In the past, standard triple therapy consisting of a proton pump inhibitor and two broad-spectrum antibiotics (usually amoxicillin, clarithromycin or metronidazole) was able to achieve eradication rates > 90%[1]. However, recent studies have demonstrated that the eradication rates of triple therapy have declined below 80%[2–5]. Among the factors causing this decline, antibiotic resistance (particularly clarithromycin and metronidazole resistance) is the primary cause. In China, the eradication rate decreased from 88.9% in 1996[6] to 73.5% in 2012[7], while the prevalence of antibiotic resistance to clarithromycin and metronidazole increased to 65.4 and 78.8%[8], 21.5 and 95.4%[9], and 20.7 and 42.2%[10], respectively, in various regions. Hence, alternative strategies, including sequential, concomitant, and bismuth-based quadruple therapies, have been proposed to counteract increasing antibiotic resistance rates. The updated Chinese guidance for *H. pylori* infection has recommended 10–14 d of bismuth-based quadruple therapy as the initial treatment[11]. However, the additional drugs and prolonged duration result in poorer compliance, severer adverse reactions, and higher costs for sufferers.

The Chinese guidance also describes the application of natural medicine as a complementary and collaborative therapy. The anti-*H. pylori* effects of dozens of herbs and formulas have been investigated, but the findings are limited by varying drug quality and sources, as well as the numerous and complicated components of formulas. Thus, a drug utilized clinically with simple ingredients, reliable and stable quality, and traceable sources of raw materials is an ideal choice. Jinghua Weikang Capsule (JWC) is a popular Chinese patent drug that consists of two ingredients, *Chenopodium ambrosioides* L. (CAL) and *Adina pilulifera* (AP). Our previous works demonstrate the inhibitory, bactericidal, and synergistic effects (with clarithromycin and metronidazole) of CAL against *H. pylori* strain 26695 and antibiotic-resistant strains isolated from patients suffering from *H. pylori*-associated gastric ulcers[12–14]. In addition, multicenter, randomized, controlled clinical trials demonstrated that *H. pylori* eradication of JWC-containing therapy was superior to standard triple therapy and equal to bismuth-based quadruple therapy[15,16]. These results indicate the synergistic effect of the drug, of which CAL is the major effective ingredient, with antibiotics. However, it is not known if CAL alone is capable of eradicating *H. pylori in vivo*. The intragastric environment differs dramatically from agar plates, thus the drug effect might be conflicting. This study investigated the bactericidal activities of CAL against *H. pylori* *in vivo* and *in vitro* to confirm the consistency effects in different environments.

**MATERIALS AND METHODS**

***H. pylori strains and experimental animals***

*H. pylori* strains (NCTC11637 and Sydney strain 1) were kindly provided by the Department of Gastroenterology of Peking University First Hospital and preserved in brain heart infusion broth (No. 783396; OXOID of Thermo Fisher Scientific, Waltham, MA, United States) at -80 °C.

Forty-four specific-pathogen-free (SPF) male Kunming mice (18–22 g) were purchased from Viral River Laboratories (Beijing, China; Certification No. SCXK 2012-0001). All the animals were housed in an SPF environment and had free access to sterile neutral water and standard mouse feed. The experimental procedures in this study were approved by the Experimental Animal Ethics Committee of Peking University First Hospital (Certification No. J201150).

***Drugs and reagents***

The volatile oil of CAL (No. 20110311; Tianjin Tasly Pharmaceutical Co., Ltd., Tianjin, China) was diluted in DMSO (Sinopharm Chemical Regent Co., Ltd., Shanghai, China). Lansoprazole (No. 20130205), metronidazole (No. 20120228), and clarithromycin (No. 20120704; Dalian Meilun Biology Technology Co., Ltd., Dalian, China) were used as the triple therapy control in animal experiments. Columbia blood agar plates with 5% sheep’s blood (No. 20130205; bioMérieux Industry Ltd., France), Columbia agar (No. 848706), Brucella broth (No. 8170491), fetal calf serum (No. NTMO133) and brain heart infusion broth (OXOID) were prepared for *H. pylori* liquid culture and collection.

***Culture and collection of H. pylori***

Frozen stocks of *H. pylori* were recovered at room temperature, inoculated on the Columbia blood agar base, and incubated under a microaerobic environment (15% CO2, 5% O2, 80% N2) at 37°C with over 90% humidity for 48–72 h. The bacterial colonies were collected and prepared in Brucella broth with fetal calf serum for *in vitro* bactericidal tests, or in brain heart infusion broth for oral gavage of animals.

***Inhibitory and*** ***bactericidal activities test***

The inhibitory activity of CAL against the growth of *H. pylori* was assessed using an agar dilution method. Briefly, serial dilutions (1:2) of CAL were added to the Columbia blood agar for final concentrations starting from 512 mg/L down to 1 mg/L. The non-drug agar and DMSO agar served as negative controls. Agar plates were inoculated with *H. pylori* [NCTC11637; 3 × 108 colony forming units (CFU)/mL] and cultured for 72 h. The minimal inhibitory concentration (MIC) was defined as the minimal concentration of CAL required for complete inhibition of *H. pylori* growth.

Bactericidal activity was evaluated using time-kill curves with 0.5, 1.0 and 2.0 × MIC CAL, and blank and DMSO controls. *H. pylori* (NCTC11637; 0.1 mL at 1 × 106 CFU/mL) was added to 90 mm plates with the calculated concentrations of CAL or DMSO and Brucella broth containing fetal calf serum (final volume 10 mL) and cultured with gentle shaking at 37 °C in a microaerobic environment. At 0, 4, 8 and 24 h, 0.5 mL of liquid was removed, serially diluted, and plated on Columbia blood agar plates (*n* = 2 per group). The colonies were counted and averaged after a 72 h incubation.

***In vivo inoculation***

Mice (*n* = 44) were randomly divided into four groups: blank control, *H. pylori* control, CAL, and triple therapy group. Except for the blank controls, all animals received a single intraperitoneal injection of cyclophosphamide (200 mg/kg), followed by a total of five gastric intubations (every other day) with 0.4 mL of *H. pylori* (Sydney strain 1; 12 × 108 CFU/mL). Animals were fasted 24 h before and 2 h after each inoculation. Two weeks after the last inoculation, one mouse was randomly selected from each group and sacrificed to test the bacterial colonization by rapid urease test (RUT) and hematoxylin and eosin (HE) staining of gastric tissue. Giemsa staining was performed to detect *H. pylori* colonization when HE results were unclear.

***Drug administration***

Drug doses used in *in vivo* experiments are equivalent to clinical administrations. The CAL group was treated with 49.32 mg/kg daily for 4 wk. The triple therapy group was treated daily with a suspension of lansoprazole (12.33 mg/kg), metronidazole (164.40 mg/kg), and clarithromycin (205.54 mg/kg) for 1 wk. The *H. pylori* group was given the same volume of sterile water, and the blank control group was free of any gavage. All drugs were suspended in sterile water and administrated by gastric intubation.

***Determination of H. pylori colonization and gastric inflammation***

Four weeks after inoculation, animals were deprived of feed but allowed free access to water for 24 h and then sacrificed. Stomachs were collected at the time of sacrifice, opened at the side of the greater curvature, and washed with 4 °C PBS. Half of the antral section was isolated for RUT determination at room temperature within 24 h to observe the change to pink color as positive. The second half of the antral section and partial gastric body tissue were fixed in formaldehyde for histopathology. Fixed tissues were embedded in paraffin, sectioned, and stained with HE or Giemsa to determine inflammation and degree of *H. pylori* colonization. Eradication success was determined by negative findings from both RUT and histopathology.

The degrees of *H. pylori* colonization and inflammation were scored by a pathologist blind to treatment groups using the visual analogue scale of the updated Sydney System[17]. *H. pylori* colonization was graded as: 0, no bacteria detected; +, occasional or several bacterial colonies distributed within ⅓ of the specimen length; ++, consecutive but thin bacterial colonization in the mucosal surface between ⅓ and ⅔ specimen length; +++, piles of bacterial colonies along the length of the specimen. Mononuclear infiltration in the gastric mucosa was graded as: 0, < 5 mononuclear cells in each high power field; +, few mononuclear cells restricted to superficial layer within ⅓ of the mucosa; ++, moderate mononuclear infiltration within ⅔ of the mucosa; +++, intensive mononuclear infiltration throughout the mucosa.

***Statistical analysis***

Eradication rates were compared among groups by a Fisher’s exact test using SPSS 20.0 software (IBM Corp., Armonk, NY, United States). *P* < 0.05 was considered as statistically significant.

**Results**

***In vitro***

Results of the *in vitro* inhibitory activity test indicated that there was no visible growth of bacterial colonies after 72 h incubation on the plate containing 16 mg/L CAL, thus defined as the MIC of CAL. Construction of the time-kill curve reveals continual growth of *H. pylori* colonies in blank and DMSO control groups, but marked inhibition in a dose-dependent manner with CAL (Figure 1). No *H. pylori* colonies could be detected at 24 h in the 1 and 2 × MIC CAL groups.

***In vivo***

*H. pylori* eradication rates as determined by RUT and histopathology are presented in Table 1. There was no significant difference between CAL and the triple therapy groups (*P* = 0.650). *H. pylori* colonization and inflammation scores are presented in Table 2. Images of HE staining are shown in Figure 2.

**DISCUSSION**

In China, the prevalence of *H. pylori* infection is 56.22%[18], which is higher than the global level. Bismuth-based quadruple therapy has become the preferred therapeutic regimen for *H. pylori* eradicationbecause of increasing antibiotic resistance. However, adverse effects and poor compliance necessitate the development of alternative therapies. Traditional Chinese herbs can promote relief of symptoms as well as produce anti*-H. pylori* activities. However, the active ingredient is not always obvious, and the *in vitro* efficacy does not always correlate with eradication in animal models[19].

JWC has been utilized for gastritis and peptic ulcers, with effects of regulating qi, dissipating cold, clearing heat, and resolving stasis according to Chinese medicine theory. Our previous work indicated that CAL was the active ingredient in JWC[12], which was confirmed by the *in vitro* experiments in the present study. To eliminate possible effects of Tween-80 used in the previous study, we utilized DMSO, which showed no effect on *H. pylori* growth. The MIC of 16 mg/L was sufficient to completely eliminate *H. pylori* after 24 h. The *in vivo* experiments demonstrate the clinical potential of CAL for *H. pylori* eradication, with efficacy equivalent to the triple therapy.

Despite the promising results, the eradication with CAL was not ideal, possibly be due to the acidic environment or the mucous layer of stomach, or an insufficient dose converted from clinical usage. Furthermore, the conspicuous and classic mucosal inflammation was not observed in this study, which might be ascribed to the short infection period and the application of cyclophosphamide. The conclusion that the effect of CAL rivals triple therapy should be made prudently due to the small sample size. Furthermore, the mice may have had other bacterial species present, as *H. hepaticus* has been detected in 19.2%–29.5% of SPF mice in China[20,21]. In order to eliminate the interference of other *Helicobacter* *spp.*,a PCR test should be added in future studies.

In our previous random clinical trial, the therapy containing JWC, a proton pump inhibitor, amoxicillin, and clarithromycin achieved a higher eradication ratio than triple therapy[15]. CAL is a complex compound, for which the effective composition remains undiscovered. Hence, further research to identify the active ingredients would be helpful to evaluate and improve the intensity of the effect. Nevertheless, this study confirms the *in vitro* and *in vivo* anti-*H. pylori* effects of CAL, providing experimental support for future human trials.

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**comments**

***Background***

*Helicobacter pylori* (*H. pylori*)infects more than half of the human population, causing aglobal public health issue associated with gastritis, peptic ulcer, gastric carcinoma, mucosa-associated lymphoid tissue lymphoma, and other diseases. Successful eradication of bacteria is the effective approach to cure related diseases or to improve prognosis. Antibiotic resistance has become the leading cause of treatment failure, thus new regimens or medicines are required.

***Research frontiers***

Alternative and complementary treatment approaches including phytomedicine have been focused on eradicating *H. pylori* or enhancing the bactericidal effect of antibiotics. Various basic and clinical studies have been performed to explore the anti-*H. pylori* effect of herbs and patent medicines, and some of these traditional therapies have enormous potential. Testing the bactericidal activity or the synergistic effect with antibiotics *in vitro* and *in vivo* will provide additional evidence.

***Innovations and breakthroughs***

This study demonstrates the *in vitro* and *in vivo* anti-*H. pylori* effect of *Chenopodium ambrosioides* L. (CAL), derived from the Chinese patent medicine, Jinghua Weikang Capsule. The *in vivo* bactericidal activity was reported for the first time. The effect on *H. pylori* eradication was equivalent to that of a triple therapy.

***Applications***

*Chenopodium ambrosioides* L. has been utilized for gastritis and peptic ulcers under the guidance of traditional Chinese theory. The authors’ research provides experimental evidence for further clinical study of this compound, and the possible application for treating *H. pylori* infection.

***Terminology***

The agar dilution method provides the minimal inhibitory concentration of an antimicrobial agent. The time-kill curve method indicates the bactericidal effect of a testing agent.

***Peer review***

This study investigated *in vitro* and *in vivo* the bactericidal activities against *H. pylori* strains using Chinese patent drug containing the volatile oil of CAL. Currently, there is interest in alternative treatments for *H. pylori* infection due to the high resistance to antibiotics used as the gold standard.

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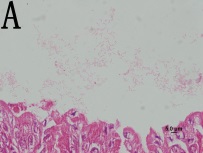
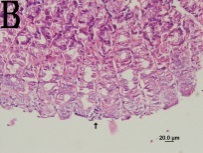
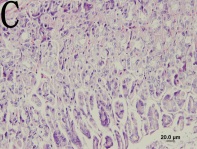
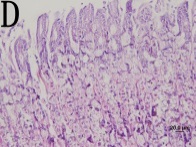
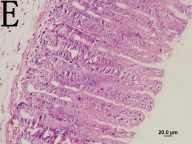
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**Figure 1 Time-kill curves of *Chenopodium ambrosioides* L.** **at different concentrations.** MIC: minimal inhibitory concentration; CAL: *Chenopodium ambrosioides* L.

**    **

**Figure 2 Histopathology of gastric mucosa.** Hematoxylin and eosin staining of gastric mucosa from mice inoculated with *Helicobacter pylori*. A: *H. pylori* colonization on the surface of gastric mucosa (100 ×); B: Mononuclear cell infiltrates (arrow; 40 ×); C: Uninfected mice exhibit a normal gastric mucosa (40 ×); D: Treatment with *Chenopodium ambrosioides* L. for 4 wk revealed no obvious inflammation (40 ×); E: Treatment with lansoprazole, metronidazole and clarithromycin for 1 wk showed no pathologic changes (40 ×).

**Table 1 Negative rapid urease test and histopathology results**

|  |  |  |  |
| --- | --- | --- | --- |
| **Group** | **RUT**  **(*n*)** | **Histopathology**  **(*n*)** | **Eradication ratio** |
| Blank control | 10 | 10 | - |
| *Helicobacter pylori* | 0 | 0 | 0/10 |
| *Chenopodium ambrosioides* L. | 6 | 5 | 5/101 |
| Triple therapy | 7 | 7 | 7/10 |

1the*P* value is 0.650, *vs* triple therapy. *n* = 10/group. RUT: rapid urease test.

**Table 2 *Helicobacter pylori* colonization and gastric inflammation scores**

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Group** | ***Helicobacter pylori* colonization** | | | | | **Gastric inflammation** | | | |
| **0** | **+** | **++** | **+++** | **0** | | **+** | **++** | **+++** |
| Blank control | 10 | 0 | 0 | 0 | 10 | | 0 | 0 | 0 |
| *Helicobacter pylori* | 4 | 5 | 1 | 0 | 7 | | 3 | 0 | 0 |
| *Chenopodium ambrosioides* L. | 5 | 5 | 0 | 0 | 8 | | 2 | 0 | 0 |
| Triple therapy | 7 | 3 | 0 | 0 | 10 | | 0 | 0 | 0 |

*n* = 10/group.