November 25, 2014

Dear Editor,
Please find enclosed the edited manuscript in Word format (file name: 14924-review.docx).

Title: Anti- *helicobacter pylori* activities of *Chenopodium ambrosioides L. in vitro* and *in vivo*

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Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 14924

The manuscript has been improved according to the suggestions of reviewers and editors:
1. The formatting-related issues have been updated.

2. Revision and responses according to the reviewers' comments:
Reviewer 1:
(1) The inhibitory activity of CAL against the growth of *H.pylori* was assessed using agar dilution method. This method determines a bacteriostatic effect. It is important that authors considered that *H.pylori* has a particular lifestyle in the presence of antagonistic molecules including antibiotics. Indeed, the curved *H.pylori* form starts rapidly a transformation in a U-shaped form followed by the establishment of a final coccoid form. It should be of interest that authors examined by transmission electron microscopy whether the Chenopodium ambrosioides L. treatment induces or not the appearance of these bacterial forms.
Response: The primary aim of this manuscript was to confirm whether CAL was bactericidal *in vitro* and *in vivo*. Under optical microscope, the coccoid form of *H.pylori* was not found in CAL and triple therapy groups. The observations on CAL/AP/JWC impacting *H.pylori* shape and gastric mucosa cells by means of scanning & transmission electron microscopies have been designed in our next approved National Natural Science Foundation of China (NSFC, No. 81473474, 2015-2018) research. These findings will be reported since we accomplish the experiments in next 4 years.

(2) On the basis of the data obtained with the agar dilution method, authors established a minimal inhibitory concentration (MIC) for Chenopodium ambrosioides L. and used this MIC (1/2×MIC, 1×MIC, and 2×MIC) for the subsequent analysis of a Chenopodium ambrosioides L.-induced bactericidal effect against *H.pylori*. The MICs for bacteriostatic and bactericidal effects are generally very different. The data in Fig. 1 beings are completed by the examination of the bactericidal effect of 1/16 MIC, 1/8 MIC, and 1/4 MIC in the objective to determine the specific MIC of the Chenopodium ambrosioides L. bactericidal effect.
Response: In our study, the designed method of time-kill curves was to test one dose firstly, based on which more doses would be tested. If the bactericidal activity was positive, then the lower doses chosen subsequently; and if it was negative, higher doses tested. The 0.5×MIC failed to be bactericidal, therefore we stopped more tests. The supplementary experiments can't be performed immediately by reason of the fund ending and lab reconstructing. This part will be conducted in the next NSFC research and the results will be included in the letter to editor or manuscript submitted to WJG or the reviewer.

(3) The determination of the concentration-dependent in vitro anti-urease activity of Chenopodium ambrosioides L. is of interest in order to improve the manuscript. A triple comparison between the MICs of in vitro anti-urease, bacteriostatic and bactericidal effects are of interest.
Response: This suggestion inspired us a lot. Actually, we have noticed that some *H.pylori* infected patients' DOB values of ^{13}C-test became lower after JWC solo treatment. Thus, the anti-urease activity of JWC may be possible. This has been our proposed research and the initial clinical data collection is in process. The studies *in vitro* will be performed as soon as we achieve more funds and data.

(4) Authors indicated that: "Jinghua Weikang Capsule (JWC), as a popular Chinese patent drug, consists of two ingredients, Chenopodium Ambrosioides L. (CAL) and Adina Pilulifera (AP)." and in the Discussion claimed that: "In our previous studies, CAL was confirmed to be active of inhibitory and bactericidal efficacy against various strains of H.pylori in vitro, while AP was negative." without adding reference(s) of publication(s). Please, authors add the references or include the in vitro and in vivo data obtained with AP in the manuscript.
Response: The reference has been added as No.12 (Article in Chinese).

(5) What is the interest of the presence of AP in JWC (claimed by authors as a compound preparation, utilized for to treat gastritis and peptic ulcer) since AP is not active against *H.pylori*?
Response: According to the former clinical studies, JWC was able to improve the bactericidal effect of triple therapy and alleviate symptoms related to *H.pylori* infection. The *in vitro* studies confirmed the anti-bacteria activity of CAL while the AP negative. We are also interested in the effects of AP and our next approved NSFC research is focused on this issue, JWC/CAL/AP + triple therapy, and inflammatory factors. And we speculated AP might influence the inflammatory reactions caused by *H.pylori*. We guarantee that as soon as we achieve some findings, the relative results will be submitted to WJG as manuscripts or to reviewer by email if it is necessary.

(6) Authors claimed: "In our previous random clinical trials, the therapy containing JWC, PPI, amoxicillin and clarithromycin achieved higher eradicating ratio than triple therapy." Please authors give the References.
Response: The reference has been added as No.15 (Article in Chinese).

(7) This statement suggests that JWC improves the efficacy of triple therapy. This enhancing effect has been reported for several human intestinal microbiota probiotic Lactobacillus strains. In order to improve their demonstration, it is of interest that authors conduct an in vitro (bactericidal effect) and in vivo (*H.pylori*-infected mice) experiments combining JWC + triple therapy.
Response: The MICs of JWC + clarithromycin and JWC + metronidazole against *H.pylori* strains were tested in our former study (reference 12th). The project of JWC + triple therapy has been designed and approved in our next NSFC research. We are glad to share the data after accomplishing the experiments.

Reviewer 2:
(1) Dose of CAL in mg/ml in in vitro studies and the dose in mg/kg body weight in in vivo studies using mice model requires explanation. The active substance of CAL is not known. In addition, CAL is the volatile oil.

Response: The dose of CAL *in vitro* study was based on the MIC tested by agar dilution method. And *in vivo* the dose was calculated on the base of clinical usage in order to detect whether the clinical dosage could be bactericidal. And in our next study approved by NSFC, we will explore more effects of the drug under different doses.

(2) The pretreatment of mice (H. pylori control group, CAL group and triple therapy group) with cyclophosphamide 200 mg/kg should be explained.
Response: In our pre-experiments, the *H.pylori* infection of mice was unsuccessful by intragastric gavage of bacteria directly or after pretreatments using alcohol, $NaHCO_3$ and antibiotics. After consulting an expert of Institute of Clinical Pharmacology Peking University, we tried immune inhibition by cyclophosphamide before intragastric gavage and *H.pylori* colonized successfully. According to other research, the immunity would recover completely in two weeks after cyclophosphamide injection with 200 mg/kg once. (XU Hai-fan, LI Han-xian. Serum Concentration of CTX and Immunofunction in Immunosuppressed Mice at Different Time. Journal of Nanhua University (Medical Edition),2001,29(6):562-564 [Article in Chinese])

(3) The assessment of gastric colonization with H. pylori and eradication was based on rapid urease test (RUT) and histological examination. In my opinion culture of bacteria should be included in this type of the study.
Response: In this study, the culture and isolation of *H.pylori* were contaminated when immediate incubation after tissue collection. The result was lack of specificity, and we had to abandon it. The RUTs and histological examination were able to be convincing. In future studies we will perform more tests on the assessment of gastric colonization with *H.pylori* after updating some facilities.

(4) It is known that mice are naturally colonized by other Helicobacters. It should be mentioned.
Response: Experimental mice are naturally infected by some other helicobacters, mainly including *H.hepaticus*, *H.bilis* and *H.rodentium*. In China, recent studies showed the occurrence of helicobacters in SPF class mice were 19.2% - 29.5% (①DING Cong, FENG Jie, XIE Jian-yun, et al. An Epidemiological Survey of *Helicobacter spp*. in Laboratory Mice and Rats in the Area around Shanghai by Two Detection Methods. CHINESE JOURNAL OF COMPARATIVE MEDICINE,2011,21(12):66-69,78; ②JI Shang-wei, WANG Song, WANG Jiang-bin, ZHANG Yong-gui. Investigation of Helicobacter hepaticus infection in various species of mice in China, 2010,30(9):597-601). This issue has been added into the discussion section. More specific tests as PCR will be applied in our future study which has been approved by NSFC. Thanks for reviewer's suggestion.

3. References and typesetting were corrected.

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,

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