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Antimicrobial Effects of Aqueous and Hydro-Alcoholic *Plantago psyllium* leaf extracts on the Experimental Infection of *Helicobacter pylori* in a Rat Model

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ABSTRACT

Background & Objective: *Plantago psyllium* has long been utilised as a medicinal agent worldwide. The current study aims to evaluate the *in vivo* antimicrobial effects of aqueous *P. psyllium* and a hydro-alcoholic extract of *P. psyllium* on *Helicobacter pylori* as well as the effects of these extracts on liver and kidney in a rat model.

Materials & Methods: A total number of 50 female Sprague Dawley rats were experimentally infected (Except for the negative control group). The rats were divided into five groups of 10. The groups were treated by Aqueous and Hydro-Alcoholic *P. psyllium* leaf extracts. Subsequently, *H. pylori* antigens which were present in the in rats' stool were measured using a serological assay. To diagnose the pathogenicity of the kidney and liver, blood samples which had been taken before and after treatment, were tested for renal and hepatic enzymes by Analyzer Electrolyte and Sysmex KX-21N.

Results: Following the amoxicillin treatment, the levels of aqueous and hydroalcoholic extracts of the treated rats were compared with those of the control group, and a significant difference was observed (P<0.05). Antigen decrease was observed in all of the groups treated with amoxicillin (P=0.0004), aqueous, and hydro-alcoholic extracts (P<0.05). Antigen decrease in the stool was observed in all groups, which indicates that treatment with the herbal extract could affect the infected rats.

Conclusion: *P. psyllium* hydro-alcoholic extract can be applied as a selective treatment for *H. pylori* infection. Hopefully, experiments suggested that the mentioned extracts could positively affect the process of disease recovery.

Keywords: Aqueous and Hydro-Alcoholic extracts, *Helicobacter pylori*, *Plantago psyllium*

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Introduction

Helicobacter pylori colonizes half of the world's population and contributes to gastritis and gastrointestinal ulcer disease increasing the risk of malignant gastrointestinal cancer (1). The onset of gastric adenocarcinoma occurs with the progression of chronic superficial gastritis to chronic gastric atrophy. Furthermore, *H. pylori* is a risk factor for gastric cancer (2). It is estimated that 14,500 patients (>65 age) pass away due to *H. pylori* infections annually (2). Antibiotics such as clarithromycin, metronidazole, amoxicillin, and tetracycline are used to treat of *H. pylori* infections. *H. pylori* resistance rate has been

estimated to range from 15% to 78%. According to previous reports, the antibiotic resistance of the mentioned bacterium against amoxicillin and other antibiotics has increased [4]. Multi-drug resistant (MDR) *H. pylori* isolates were screened >75% in Iran (3). *Plantago psyllium* is widely used as a traditional herbal medicine and a functional food in Asian counties (4). Polysaccharide, the main biological active compartment in Plantago spp., has a variety of biological effects including gastrointestinal functions and antioxidant, immunemodulatory, antitumor, and hypoglycemic activities. Variety

of biological and pharmacological functions of psyllium macromolecules have been confirmed (4). P. psyllium can be found in almost every region of the world and people have used P. psyllium to treat gastric and peptic ulcers for a very long time. This herb has had medicinal uses in Iranian traditional medicine as well (5-7). P. psyllium seeds contain 10% mucilage, which produces D-xylose, arabinose, D-glucose, and D-galacturonic acid through hydrolysis (8). In the United States, a medicine called "Metamucil" is produced from the laxative, but researchers suggest that P. psyllium is also effective in reducing cholesterol and glucose levels (9). Anti-bacterial properties of herbs against H. pylori infection were indicated in some published studies (10). Although there are some reports about P. psyllium anti-bacterial properties (11), no study has investigated H. pylori infections. Therefore, investigation of the antimicrobial effects of aqueous and hydroalcoholic extracts of P. psyllium on the infected experimental rat groups, is the main purpose of the current study.

Materials and Methods

Preparation of Aqueous and Hydro-alcoholic Extracts of *P. psyllium*

Extraction was carried out in the faculty of pharmacy under the supervision of experts as follows: fresh P. psyllium was provided from Shiraz, a city in south-central Iran. The species were matched with the German Digital Herbarium in the Faculty of Pharmacy at Shiraz University of Medical Sciences. It was found that the species had a herbarium number as B-W0298230 (9). To prepare the aqueous and hydro-alcoholic extracts of P. psyllium, the plants were dried at room temperature for five days and were then powdered. Using the percolation method, 100 g of powdered P. psyllium leaf was poured into the percolator and soaked in distilled water for 24 h. Another 100 g of powdered P. psyllium was soaked in another percolator device with one litre of 70% ethanol for 72 h. After filtering and evaporation, the obtained gelatinous extracts were reduced under pressure in the rotary device and were finally prepared as powder (12, 13).

Preparation and Culture of Bacteria

Gastric biopsy samples were taken by endoscopy from patients who referred to Namazi hospital, Shiraz. For early isolation of bacteria, agar enriched with yeast extract of Columbia culture medium (Merck, Germany) was enriched with 10% defibrinated horse blood, 10 mg/L vancomycin, 10 mg/L amphotericin B, 5 mg/L cefsulodin, and 5 mg/L trimethoprim and incubated at 37°C (14). According to previously published research, Urease test was performed on biopsy samples, which were examined for the presence or absence of *H. pylori* (15).

Confirmation of H. pylori Isolates

According to the previously published research, the isolated strains were precisely reconfirmed taking advantage of traditional biochemical tests including gram staining, urease activity, oxidase, and catalase enzymes production (14).

Taking Care of Animals

Fifty female rats of Sorague Dawly with a weight range of 200 ± 20 g were randomly divided into five groups. Rats were kept in standard conditions for relative temperature of $22\pm2\%$ and received water and food every day. Consumption doses were calculated based on previous studies and advice of traditional medicine experts (4).

Infection and Treatment

All groups, except for the negative control, received (orally) 0.5 ml of a suspension containing 2×10^7 colony-forming unit per millilitre (CFU/ml) of *H. pylori* on average every day in the morning for 7 days (4, 16). After 48 weeks of infections, groups were treated for 26 days. *P. psyllium* extracts were performed as single dosage in 300mg final concentration for experimented groups. Some study findings related to the herbal extract in diarrhea showed that higher doses (500 and 1,000 mg/kg), had antisecretory and antidiarrheal activity (**17**).

The experimental groups were as follows:

G-1: Negative control group, which was not subject to infection and treatment at all.

G-2: Amoxicillin antibiotic group, in which infection was treated with 500 mg of amoxicillin.

G-3: Aquinas extract group, in which, after infecting, the rats were treated with aqueous extract of *P. psyllium* at a concentration of 300 mg/kg of body weight.

G- 4: Hydro-alcoholic extract group, in which, after infecting, the rats were treated with hydro-alcoholic extract of *P. psyllium* at a concentration of 300 mg/kg of body weight. No cosolvents or surfactant were added to the extract.

G- 5: Positive control group, in which rats were not treated after infecting.

Biochemical Analysis of Rats' Blood in Each Group

Sampling (blood and stool) from the treated groups was immediately performed eight weeks after infections. Sera were tested in terms of renal and hepatic functions by assessing aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein (TP), creatinine, and blood urea nitrogen (BUN) according to the previously published research (18) using an Analyzer Electrolyte, Hitachi 911 (Roche Company, Switzerland) with Pars-Azmoon Commercial Kits (Pars-Azmoon Laboratories LTD, Tehran, Iran) and Sysmex KX-21N (Sysmex Corporation, Japan) according to the manufacture's manual (Fig. 2, 3). Bouin solution, ethanol, and chloroform were obtained from Sigma-Aldrich Company, USA.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was done taking advantage of polyclonal antibody-based Premier Platinum HpSA test, later HpSA (Meridian Inc., Cincinnati, USA) was performed based on the manufacturer's instructions.

Qualitative ELISA was to be performed immediately before and after the treatment, stool sampling was performed for the experimental groups to detect H. pylori antigens. Briefly, following the kit manufacturer's recommended procedure, the plates were washed with washing buffer [0.05 % (v/v) Tween 20 in phosphate buffer saline (PBS)] twice. The Plates were incubated for 2 h at 37°C. Blocking was carried out using a PBS/Tween 20 solution containing 5% bovine serum albumin (BSA) as a blocking buffer. After filing blocking and washing, 50 µl of homogenate rat stool samples were added to the wells in duplicate. The plates were incubated for 1 h at 37°C, washed three times, and incubated with HRPconjugated anti-mouse immunoglobulin (IgG) (Sigma, USA), which was diluted to 1:10,000 (as a secondary antibody) at 37°C for 2 h. The plates were then washed as described above, and the enzymatic activity was measured subsequent to adding 100 µl of tetramethylbenzidine (TMB) substrate. After 30 min of subjoining of 100 µl 2 N H₂SO4, the reaction was stopped. Finally, the optical density (OD) of each well was determined as 405 nanometres, taking advantage of an ELISA microplate reader (Anthos 2020, United Kingdom). Detailed data are given in Figure 1A.

Ethical Code

All animal experiments were done in compliance with the enlarged ethical statement and approved by the ethics committee of Shiraz University of Medical Sciences. All tests were performed complying the enlarged ethical (Human and Animal) statement (95-01-13-11069). The ethics committee approved both the human and the animal experimentation.

Results

Extraction was done based on previously published work using the species that had a herbarium number of B-

W0298230. A dried extract was then used to prepare the aqueous and hydro-alcoholic extracts (9). The experimental rat groups were infected, following a previously published procedure (4, 16).

Antibody-mediated ELISA was used to determine the *H. pylori* antigen in the homogenate rats' stool. Following the amoxicillin treatment, the levels of aqueous and hydro-alcoholic extracts of the treated rats were compared with those of the control group, and a significant difference was observed (P<0.05). A decrease in antigens was observed for all tested groups treated with amoxicillin (P=0.0004), aqueous, and hydro-alcoholic extracts (P<0.05) (Fig. 1A). After two weeks of treatment with medicine extracts, all groups showed a describing amount of *H. pylori* antigen in their stool, thus indicated the efficacy of the medicines.

Additionally, in the liver function test (LFT) assay, a significant difference (especially in aquatic extract) was observed in the levels of liver (AST and ALP) and renal damage markers, including BUN and creatinine (P<0.05). Significant differences were also seen in the levels of liver enzymes including ALT (P=0.00001), AST (P=0.00001), ALP (P=0.0005), and total protein (P<0.00001) content of the serum before and after treatment. This amount was also significant when different treated groups were compared with each other after treatment with medicine extracts. By comparing the amounts of liver and renal indicators among the treated groups, it was found that the amounts of ALP (as an example) to amoxicillin/aqueous, amoxicillin/hydro-alcoholic, and hydro-alcoholic/aqueous were significant (P<0.05) after treatment. A statistical analysis showed that blood samples especially to aquatic extract were significantly different in terms of factors that affect the liver and kidney. Furthermore, all of the findings were statistically significant when compared with positive control after treatment (Figs. 1, 2, and 3).

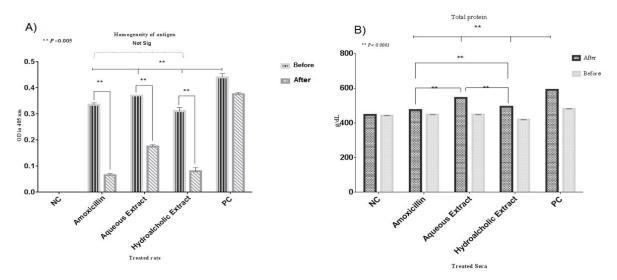


Figure 1. Difference in fecal homogeneity antigen A) and Total protein in tested groups B). The error bar is representative of the mean±sd (n=3).

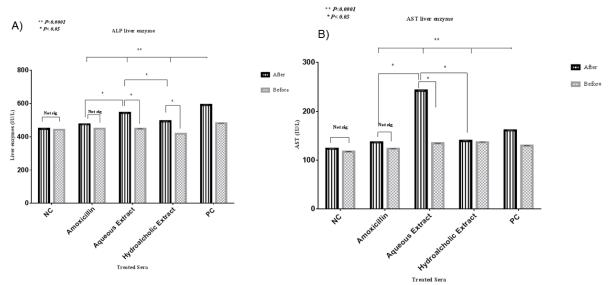


Figure 2. Biochemical analysis of liver enzymes (ALP, and AST) to tested sera in treated rat groups. A) The amount of ALP in experimental groups B) The differences of AST in studied groups. The error bar is representative of the mean±sd (n=3).

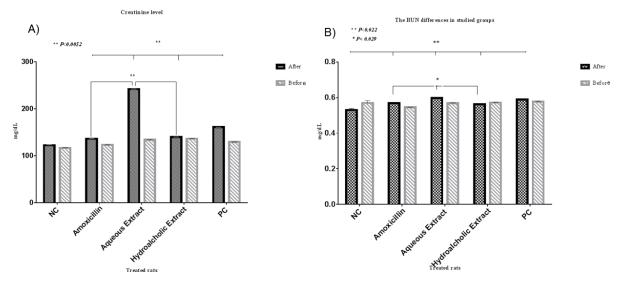


Figure 3. Analysis of biochemical factors in treated and untreated rat sera, A) the creatinine level in various groups, and B) the BUN differences in studied. The error bar is representative of the mean±sd (n=3).

Discussion

Antibiotics are often used to treat *Helicobacter* infections, and common antibiotics cause severe damage to the normal flora of the digestive system, which, in the long term, causes malnutrition and complicated diseases (19). *P. psyllium* is a valuable medicinal plant that researchers have always been interested in knowing more about. Numerous studies have been performed on the effects of this plant and its therapeutic role in constipation, diarrhea, ulcerative colitis, irritable bowel syndrome, colon cancer, diabetes, and hypercholesterolemia (20-22).

Considering the long history of the effects of *P. psyllium* on treating microbial diseases in humans and animals, in the current research, the therapeutic features of aqueous and hydro-alcoholic extracts of *P. psyllium* were investigated in rat models. The findings

showed that both extracts had significant therapeutic effects, and additional experiments demonstrated that these extracts have minor adverse effects on renal and hepatic functions. Given the emergence of MDR *H. pylori* isolates and the adverse effect of the mentioned drug of choice on liver and kidney functions, it seems appropriate to consider the use of herbal and natural ingredients to treat *H. pylori*-associated infections (3).

The protective effect of pectic polysaccharide in *P. psyllium* against systemic *Streptococcus* infections has been shown in experimental rats. This protective effect appeared to be due to the stimulation of the immune system. Other antibacterial and anti-Candida effects of pectic polysaccharide in *P. psyllium* were confirmed in another review (23). In a study investigating the antimicrobial activity of nanoparticles produced in the

examined P. psyllium, findings indicated that the highest and the lowest concentrations of inhibitors in Staphylococcus aureus were 100 mg/ml and 12.5 mg/ml, respectively. These results showed that P. psyllium has good antimicrobial functioning in the treatment of many common antibiotic-resistant infections (24). In a study conducted by Taheri et al., on the effects of hydro-alcoholic extracts of P. psyllium on S. aureus, the researchers found that these extracts had a significant effect on S. aureus bacteria in vitro (25). In the present study, the levels of fecal antigens and antibodies against H. pylori were reduced in all samples. This was also observed in Salmonella typhimurium bacteria (11). At last, although the mentioned herbal extract is suitable for H. pylori treatment, according to the obtained results especially in aquatic extract, this cocktail has an adverse effect on liver and renal functions compared to the hydroalcoholic extract.

Conclusion

Antimicrobial resistant patterns of human pathogens are essential problems, resulting in the need for novel antimicrobial resources such as plants. This study confirmed that Aqueous and Hydro-Alcoholic *P. psyllium* leaf extracts could be considered as alternative choice treatments due to the lack of renal and hepatic side effects and their proper antimicrobial effects in experimental *H. pylori* infection in a rat model. However, further research on more samples and human models are recommended.

Limitations

In the current study, herbal antibacterial effects were not measured in bacterial culture media such as agar well diffusion. Moreover, *H. pylori* was not measured in the stool samples before and after treatment because of financial restrictions. Therefore, more studies are needed to make a precise evaluation of the bactericidal effects of the herbal extract on *H. pylori*.

Abbreviations

H. pylori: Helicobacter pylori

P. psyllium: Plantago Psyllium

MDR: multi-drug resistant

CFU/ml: colony-forming unit per millilitre

AST: aspartate aminotransferase

ALT: alanine aminotransferase

ALP: alkaline phosphatase

TP: total protein

BUN: blood urea nitrogen

ELISA: Enzyme-linked immunosorbent assay

OD: optical density BSA: bovine serum albumin IgG: immunoglobulin G TMB: tetramethylbenzidine PBS: phosphate buffer saline ANOVA: analysis of variance LFT: liver function test

Declarations

Ethics approval and consent to participate: In this study, all ethics including Ethics and Consent to participate have been collected in the research. Informed consent, (written) was obtained from all participants for the 'Gastric biopsy samples'. Inbred Sprague Dawley rats were purchased from the Research Institution of Pasteur, Karaj, Iran. They were housed in standard cages (at 23-25°C and 60%–70% humidity under a 12 h light/dark cycle) with free access to standard diet and water, one week before the experiment. Euthanasia was performed using 2-5% halogenated ether which is an inhalant anesthetic, according to *Evaluation of the Aesthetics of Physical Methods of Euthanasia of Anesthetized Rats, 2011* protocol.

In this survey, Ethics and Consent to participate from their parents have been collected. All analyses were performed using multiple-group analysis of variance (ANOVA) by Graph Pad software (Chicago, IL, USA). P-value≤0.05 was regarded as statistically significant.

Consent for publication

Not applicable

Competing Interests

The authors declare no competing interests.

Authors' contributions

All authors have read and approved the manuscript. Contributions of the authors in this study were as follow:

S K: Performing laboratory tests

M S M: Designing the study, and writing the manuscript

B M: Interpretation of results and rewriting the manuscript

OKHA: Sampling

Z S: Performing laboratory tests

M M S: Performing laboratory tests

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Conflict of Interest

The authors announce that they have no conflict of interest.

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