ANTIMICROBIAL ACTIVITY OF PAEDERIA FOETIDA EXTRACT AGAINST DRUG RESISTANT HELICOBACTER PYLORI ISOLATES FROM INDIA

Silpi Chanda1, Valentina Gehlot2, Rajashree Das2, Shweta Mahant2, Kunal Das4,5, Kuldeep Singh1, Sayeed Ahmad3

1Department of Pharmacy, Jaypee University of Information Technology, Wakhnaghat, Solan, Himachal Pradesh, India 173234
2Department of Biotechnology, Amity Institute of Biotechnology, Amity University, Noida, Uttar Pradesh, India 201301
3Dept of Pharmacognosy and Phytochemistry, JamiaHamdard, New Delhi, India 110062
4Yashoda SuperSpecialityHospitaland Heart Center, Ghaziabad, Uttar Pradesh, India- 201001
5Max SuperSpecialityHospital, Patparganj, New Delhi, India-110092

ABSTRACT
Treatment failure is a major cause of concern for the Helicobacter pylori related gastroduodenal diseases like gastritis, peptic ulcer and gastric cancer. The antibacterial activity of Paederiafoetida against few clinical isolates of H. pylori in vitro was evaluated. The methanolic leaf extract showing its effectiveness in inhibiting in vitro H. pylori growth irrespective of the genetic makeup of the strains. This study provides novel insights into the therapeutic effect of P. foetida against H. pylori infection, suggesting its potential as an alternative therapy, and opens the way for further studies on identification of novel antimicrobial targets of P. foetida.

Keywords: Helicobacter pylori, Paederia foetida.

INTRODUCTION
Helicobacter pylori is a gram negative, spiral, flagellated, fastidious, microaerophilic gastric pathogen with an extraordinary ability to frequently colonize the harshly acidic milieu of the human stomach that can last for years or decades. Its infection is associated with several gastroduodenal diseases such as chronic gastritis, peptic ulcer and gastric cancer (1,2,3). In fact, H. Pyloriis the first bacterium to be classified as a group I carcinogen by the International agency for research on cancer (4,5). More than one-half of the population...
worldwide carries the *H. pylori* infection, only 10-15 % of the infected individuals develop such gastroduodenal diseases and in some developing countries like India its prevalence exceeds 90% of the population. One of the most distinctive feature of *H. pylori* is the genetic diversity between clinical isolates obtained from different geographical regions, based on their genetic characteristics and disease outcome, Indian *H. pylori* strains are genetically distinct from those from East and West Asia (6,7). There is enormous heterogeneity in the consequences of *H. pylori* infections as virulence markers of *H. pylori* are not always associated with diseases, however, more severe disease manifestations have been attributed to infection by cag pathogenesis island (*cag*+ve) isolates (8). Eradication of *H. pylori* from infected individuals remains the best choice for an effective treatment of *H. pylori* associated diseases. Several combination therapies have been formulated to eradicate this pathogen and cure or prevent associated diseases. Triple therapy gives a high eradication rate, producing a significant improvement in the status of the disease (9). However, such multiple therapy regimens have not been very successful in clinical practice, since overuse or misuse of antibacterial agents has resulted in the emergence of antibiotic-resistant strains which is the prime cause of treatment failure apart from potential side effects (10). Many studies have indicated that the prevalence of resistance varies geographically, ranging from 10 to 90% for metronidazole and from 0 to 15% for clarithromycin (11,12). In view of the incomplete cure achieved with conventional therapy because of increasingly resistant strains, undesirable side effects, noncompliance among the patients the cost of the antibiotic regimens and a few other factors contributing to ineffectiveness, hence there is an urgent need to develop new treatment strategies for *H. pylori* infection. Thus, in light of the evidence of rapid global spread of resistant clinical isolates, the need to find new antimicrobial agents is of paramount importance. Unlike synthetic drugs, bioactive natural products have a beneficial effect on the whole organism and without causing unwanted effects.

Natural products have been always a vast source of developing synthetic and semisynthetic drugs (13,14,15). The natural product extract may be considered as combinatorial libraries. As compared to synthetic molecules, Natural products provide sterically more complex and have a broader diversity of ring systems (16, 17) due to the result of a long evolutionary selection process (18). A comparison between the chemical space of natural products and synthetic drug molecules suggested that the drug and combinatorial molecules tend to include a higher number of nitrogen-, sulfur-, and halogen-containing groups. Therefore, nitrogen-, sulfur-, and halogen-containing groups could introduce in plant extract by chemical modification
These modified plant extracts may be treated as natural product-like libraries. Chemical modification of natural extract or product could be a platform for alteration of the efficacy of mother extract. Such alteration in natural chemicals may become a solution for many challenges towards the pharmaceutical industry. One such worldwide challenge is antibiotic resistance in *Helicobacter pylori*.

*Paederia foetida* belonging to the family Rubiaceae is an extensive foetid climber found in the Himalayas from Dehra Dun eastwards up to an altitude of 1800 m and also in Bihar, Orisa, Bengal and Assam. Traditionally, the prime use of the plant in different digestive problems like gastric trouble, to clean stomach, against stomach swelling, gastritis, in loose motion, diarrhoea, in indigestion, ulceration etc. Hence the plant has been chosen for the current work. In view of this our aim is to describe the evaluation of the antimicrobial activity of the plant extract and its reacted modified extract against Metronidazole (Mtz) resistant and sensitive *H. pylori* in safe and effective manner.

**MATERIALS AND METHODS**

**Collection and authentication of plant material**

Aerial part of *Paederia foetida* Linn. (Rubiaceae) was collected from the local tribal market of Agartala, Tripura. The prepared herbarium was submitted to the National Institute of Science Communication, New Delhi (authentication Ref No. NISCAIR/RHMD/Consult/2010-11/1442/40).

**Preparation of extracts**

After the pharmacognostic identification, the powdered material of leaf (1.5Kg) and stem (500gm) was extracted with methanol (4.5l and 1.5l) in a Soxhlet apparatus respectively. The extractswere collected by filtration, evaporated till become dryness on a rotary evaporator.

**Preparation of chemically modified extracts**

Plant leaf methanolic extract (100 mg) was treated with hydrazine monohydrate (1mL) in refluxing ethanol (10mL). The reaction was monitored by thin layer chromatography. The crude reaction mixture was concentrated in vacuo to yield a modified extract named as R1. Sulphonylated extract coded as R2 was obtained by treating plant extract (200mg) in dry acetone and K₂CO₃ (198mg), p-tolunesulphonylchloride (270 mg) was refluxed for 24 H. The solvent was removed in vacuo and dichloro methane was added. The organic layer was washed with water (25mL×3), brine and dried over Na₂SO₄ and concentrated in vacuo. The
brominated extract named as R3 was obtained by adding bromine (0.05 mL) at -78°C to plant extract (300 mg) in dichloromethane (15 mL). The reaction mixture was stirred for 2 hours at -5°C. Saturated thio solution (Na2S2O3) was added and the reaction mixture was stirred for 10 minutes. The organic layer was separated and the aqueous layer was extracted with dichloromethane (10mL*3). All organic layers were combined and washed with brine, dried over sodium sulphate and concentrated in vacuo.

**H. pylori strains and culture**

Patients were enrolled in this study, according to the following inclusion and exclusion criteria. Inclusion criteria included being aged between 18 and 80 years with symptoms of dyspepsia, and no previous antimicrobial therapy to eradicate *H. pylori* infection. Exclusion criteria included previous gastric surgery, any use of bismuth, antimicrobial agents, H2-receptor antagonists, proton pump inhibitors within 4 weeks prior to endoscopic examination; or any of several concomitant medical illnesses including cardiac, respiratory, renal and liver diseases. The study was approved by the ethical committee at Yashoda Super Speciality Hospital, Ghaziabad, U. P., India. *H. pylori* strains were isolated from antral mucosal biopsy specimens of patients suffering from gastro-duodenal diseases. The strains were identified on the basis of colony appearance, gram staining, and positive reactions in biochemical tests (catalase, urease and oxidase). *H. pylori* strains were revived and cultured on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, MI) supplemented with 5% horse serum (Invitrogen, NY), 0.4% IsovitaleX (Becton Dickinson, MD), trimethoprim (5µg/ml), vancomycin (8µg/ml), and polymixin B (10µg/ml). The plates were incubated at 37°C in a microaerophilic atmosphere (5% O2, 10% CO2, 85% N2) (Double gas incubator, Hera cell 150i) for 3 to 6 days. Stock cultures were maintained until use in 70°C in Brain heart infusion broth with 20% glycerol.

**Suspension Preparation**

The bacterial suspension was prepared by the direct colony method(32). The colonies were taken directly from the plate and were suspended in 5 mL of sterile 0.85% phosphate buffer saline (PBS). The turbidity of the initial suspension was adjusted by comparing with McFarland’s standard number 4 (0.4 mL 1% w/v BaCl2 × 2H2O + 99.6 mL 1% w/v H2SO4). When adjusted to the turbidity of the McFarland’s standard no. 4, the bacterium suspension contains about 12 X 10^8 colony forming units (CFU) /ml.
Determination of antimicrobial susceptibility and resistance

*H. pylori* cells growing exponentially on antibiotic free BHI agar were suspended in Phosphate buffered saline (PBS) buffer, a series of 10-fold dilutions of these cell suspensions was prepared, and 10 µl of each dilution was spotted on freshly prepared BHI agar containing various concentrations of different antibiotics (µg/ml) viz. Amoxicillin (0.125, 0.25, 1, 2), Clarithromycin (0.125, 0.25, 1, 2), Metronidazole (0.2, 0.5, 1.5, 3, 8, 16, 32, 64), Furazolidone (0.2, 0.5, 1, 2), Tetracycline (1, 2, 3, 4).

Minimum inhibitory concentration (MIC)

After 72 h incubation under microaerophilic conditions, the minimal inhibitory concentration was recorded as the lowest concentration that inhibited visible growth of organisms. Minimal inhibitory concentration (MIC) for different antibiotics was defined as Metronidazole (> 8µg /ml), Clarithromycin (>2 µg/ml), Amoxicillin (>8 µg/ml), Furazolidone (>2 µg/ml), Tetracycline (>2 µg/ml).

DNA extraction from *H. pylori* culture

The C-TAB method of Murray and Thompson (33) is used for DNA isolation and for Polymerase Chain reaction (PCR) analysis by Multiplex PCR for *H. pylori* culture.

Amplification of DNA by Polymerase Chain reaction by Multiplex PCR

PCR amplification of *H. Pylori* genes were performed for cag A, vacA s1/s2, vacA m1/m2 and cagA typing. PCR was performed in 25-µl volumes containing 2.5 pmol of primers VAG-F and VAG-R, 25 pmol of primers VA1-F and VA1-R, 10 pmol of primers cag5c-F and cag3c-R, 0.25 mM of each deoxynucleoside triphosphate, 0.9 U of *Taq* DNA polymerase, 1.5 mM of magnesium chloride (MgCl2) and were amplified under the following conditions: 3 min at 94°C for initial denaturation followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final round of 10 min at 72°C. The PCR product was finally stored at 4°C. 3% agarose gel electrophoresis was used to examined the product under gel documentation system.

Anti - *H. pylori* activity Assay

Sterile Whatman paper disks (6mm in diameter) were soaked in different samples with various concentrations and placed on the inoculated plates with 1.2 X 10⁹ colony forming unit (CFU) of *H. pylori*. The plates were kept under observation for 2 days at 37°C under microaerophilic conditions (5% O₂, 10% CO₂ and 85% N₂). All experiments were performed...
in triplicates. Pure methanol, Distilled water and DCM + Methanol were used as a negative control.

RESULTS AND DISCUSSION

A total of eight H. pylori isolates were obtained from antral biopsy specimens. Endoscopic evaluation of the patients revealed 3 subjects suffered with Gastroesophageal reflux disease (GERD), 2 with Non-erosive reflux disease (NERD) and one have Duodenal ulcer. Out of the eight strains, resistance to metronidazole was found in one isolate (H. pylori 2).

We found anti H. pylori activity in methanolic extract of P. foetida against eight different H. pylori strains (table 1& 2) including one metronidazole resistant strain isolated from North India and also evaluate the activity of its modified product in Metronidazole sensitive strain (H. pylori 1) and Metronidazole resistant strain (H. pylori 2).

Table 1: Concentration of Methanolic extracts of P. foetida and zone of inhibition in different strains.

<table>
<thead>
<tr>
<th>H. pylori Strain No.</th>
<th>In µg/ml (concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>6mm</td>
</tr>
<tr>
<td>3</td>
<td>6mm</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>6mm</td>
</tr>
<tr>
<td>6</td>
<td>6mm</td>
</tr>
</tbody>
</table>

Table 2: Zone of inhibition with different concentration of methanolic extract of P. foetida leaf.

<table>
<thead>
<tr>
<th>Reactions</th>
<th>Concentration in µg/ml</th>
<th>H. pylori 1 (Metronidazole sensitive strain)</th>
<th>H. pylori 2 (Metronidazole resistant strain)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>130.970</td>
<td>7mm</td>
<td>No zone of inhibition</td>
</tr>
<tr>
<td>R2</td>
<td>130.220</td>
<td>8mm</td>
<td>6mm</td>
</tr>
<tr>
<td>R3</td>
<td>143.300</td>
<td>6mm</td>
<td>7mm</td>
</tr>
<tr>
<td>P. foetida leaf</td>
<td>100</td>
<td>11mm</td>
<td>11</td>
</tr>
<tr>
<td>P. foetida stem</td>
<td>100</td>
<td>13mm</td>
<td>13</td>
</tr>
</tbody>
</table>
DISCUSSION

Treatment of *H. pylori* infection by eradication with a proton pump inhibitor based triple therapy is presently used. Though it has a success rate of 80 to 90%, problems like treatment failure, contradictions for some patients, and rapidly emerging drug resistance in *H. pylori* strains during treatment with various antibiotics is a major obstacle for successful eradication therapies. For eradicating antibiotic-resistant *H. pylori* strains, there is an increasing search for safe and effective nonantibiotic compounds that inhibit *H. pylori* growth. In the Indian traditional medical system, a number of plants and plant products are known to possess potent medicinal properties, suggesting their usefulness in treating. Recent studies have shown that mother leaf and stem extract of *Paederia* possesses anti-*H. pylori* potential. This prompted us to explore its antimicrobial potential against Indian *H. pylori* strains that are geographically distinct from East Asian and Western strains. Moreover, a majority of the Indian population harbors *H. pylori* and quite a number of them suffer from *H. pylori*-associated gastrointestinal diseases.
In the present study, we have primarily shown that mother extract and its reacted products(R1, R2, R3) potentially inhibited the growth of all the *H. pylori* strains in vitro that were isolated from infected patients suffering from gastrointestinal disorders. It is noteworthy that one of the *H. pylori* strains were metronidazole (MIC>8µg/ml) resistant. So, our results suggest that the methanolic extract of Paederia leaf and stem acts through mechanisms distinctly different from the mode of action of these antibiotics for inhibition of *H.pylori* growth. Overall, this study provides novel insights into the therapeutic potential of Paederia extract against *H. pylori* infections, although further studies are required to extrapolate its effect on humans.

REFERENCES


