Anti-bacterial activity of herbal extracts, EuMiL® and antibiotics against Helicobacter muridarum

Vimal S.K.¹, Sharma D.¹, Bhatnagar M.¹

¹Animal Biotechnology Laboratory, Dept of Zoology Univ. College of Science, M.L.S. University, Udaipur, India

* Correspondence to : Dr. Maheep Bhatnagar, Department of Zoology , University College of science, M.L.S. University, Udaipur-313001, India, phone +91 9414165750, Fax- 91-294-2425010, email- mbhatnagar@yahoo.com

INTRODUCTION

Peptic ulcer disease (PUD) encompassing both gastric and duodenal ulcer, is the most prevalent gastrointestinal disorder[1]. There are two main approaches for treating peptic ulcer. The first deals with reducing the production of gastric acid and the second with re-enforcing gastric mucosal protection and second through development of proton pump inhibitors, histamine receptor blockers, and drugs affecting the mucosal barrier and prostaglandin analogs[2,3]. But clinical evaluation of these drugs showed development of tolerance and incidence of relapses, thus an indigenous drug possessing fewer side effects is the major thrust area of the present day research, aiming for a better and safer approach for the management of PUD.

Several plant species like Asparagus racemosus, Ocimum sanctum, Emblica officinalis, Centella asiatica, and Withania somnifera, have shown both antibacterial and antiulcer activities. Pepticare[11] and EuMil[12], a antibacterial polyherbal formulation, based on classical Ayurvedic literature comprises of standardized extracts of Withania somnifera, Ocimum sanctum, Asparagus racemosus and Emblica officinalis has been used commonly by ayurvedic practitioners for Gastric diseases. All these herbs mentioned above

Keywords: Ethanopharmacology, Phytochemicals, Traditional medicine, Helicobacter Sp, peptic ulcer, antiulcer.

ABSTRACT

Aims
The aim of this research was to investigate the anti-bacterial activity of herbal extracts of Asparagus racemosus Wild (AR), Centella asiatica Linn (CA), Convolvulus pluricaulis (CP), Emblica officinalis (EO) and Withania somnifera Dunal (WS), a polyherbal drug EuMil® and antibiotics -Amoxycillin, Metronidazole, Oxy-tetracycline, Roxithromycin and Tinidazole against Helicobacter muridarum a species of helicobactor present in rodents.

Materials and methods
H.muridarum was isolated from gastric mucosa of control and experimental swiss albino mice on selective media and identified by standard methods. Anti-bacterial activity was assayed by Kirby-Bauer Cup-well agar diffusion method at 5 mg/ml concentration.

Conclusions
Treatments of extracts of AR, WS, CP, CA and also EuMil® were found to be anti-bacterial and were inhibitory to H. muridarum. C. pluricaulis whole plant extract, EuMil® and Oxytetracycline showed the highest inhibitory activity against H. muridarum.

Significance and Impact of study
The study signify the importance of these plants as an alternative anti-ulcer and healing agents.
are claimed to increase the resistance of body against infection and other external and internal factors tending to perturb the homeostasis.

This study was thus carried out with the aim to evaluate the anti-ulcer properties with particular emphasis to anti-bacterial and inhibitory action of AR, CA, CP, EO, OS and WS extracts, EuMil® and certain antibiotics against *Helicobacter muridarum*

**MATERIALS AND METHODS**

All experiments carried out were cleared by institutional ethical committee. CPCSEA (India) Authorization is #: 973/ac/06.

**Collection of plant material**

The plant materials of CA and CP were collected from Botanical Garden, Univ. College of Science, Udaipur. The methanolic extracts of AR, WS, EO, OS and poly herbal formulation EuMil® was purchased from Envin Bioceutical Pvt. Ltd., Saharanpur (U. P.).

**Preparation of plant extracts**

Methanolic extract of CA and CP were prepared by reflux method in Soxhlet apparatus. The powder of plant parts was extracted with 100% methanol in 1:7 ratios. The process was repeated till complete extraction took place. Extracted plant material was vacuum dried and placed in hot air drier. Dried extract was stored in air-tight jar and was placed in a refrigerator.

**Antibiotics**

The antibiotics were purchased from authorized medical shop and stored in refrigerator.

**Preparation of stock solutions**

Stock solution of extracts as well as EuMil ® was prepared in 1:1 ratio with 50%DMF (N, N di-methyl formamide) and sterile distilled water. The stock solution of antibiotics was prepared in sterile distilled water. Each stock solution contained 100 mg extract or antibiotics per milliliter.

**Test bacterium**

Helicobacter sp used in the study was isolated from the biopsy samples taken from alcohol induced gastric mucosa of Swiss albino mice. The cultures were identified on the basis of gram staining, culture characters and biochemical tests[13,14]. As per published report[15], the bacteria species was identified as *Helicobacter muridarum* and authenticated by a microbiologist of the university.

**Culture medium and inoculum development**

Brucella blood agar base (HiMedia) with 10% defibrinated sheep blood was used to culture *H. muridarum*. The inoculum was developed by sub-culturing *H. muridarum* on Brucella blood agar. Inoculated media plates were incubated at 37°C for 48 hours under microaerophilic conditions in a CO₂ incubator. 4–5 colonies of this fresh culture was added in 10 ml sterile distilled water and turbidity was adjusted to 0.5 McFarland opacity standards contained 1.5x10⁶ cells/ml.

**Experimental groups**

Four experimental groups were - Group 1-six plant extracts (sub groups of AR, WS, CA, CP, OS, EO treatment each); Group 2-EUMIL®; Group 3-Antibiotics (sub groups of amoxycillin, metronidazole, oxy-tetracycline, roxithromycin and tinidazole each) and Group 4-Control group.

**Assay of anti-microbial activity**

Antimicrobial activity was assayed by Kirby-Bauer cup-well agar diffusion method. 30 ml culture medium was dispensed in respective petri plates and inoculated with 0.1 ml fresh culture[16]. Inoculum of *H. muridarum* was spread onto the surface of Brucella blood agar (BBA) plates using a sterile glass spreader. 8mm wide cup-well was bored in each petri plate and was filled with 50¼l of stock solution to give 5mg/ml final concentration. Each sample was assayed in triplicate. Culture control and DMF control were also maintained along with test samples. All the inoculated media plates were incubated for 48 hours at 37°C under microaerophilic conditions in a CO₂ incubator. The antibacterial activity was interpreted from the zone of inhibition measured to nearest in millimeter (mm).

**RESULTS**

Zones of inhibition produced by Herbal Drugs, EUMIL® and antibiotics against *Helicobacter muridarum* have been shown in Figure1 (A-H).

**Anti-microbial activity of plant extracts.**

90mm growth was observed for control. Out of 6 herbal extracts in group1, the methanolic extracts of whole plant of CA and CP produced 25mm and 27mm zones of
Figure 1. Zones of Inhibition Produced by Herbal Drugs, EUMIL® and Antibiotics against Helicobacter muridarum.
A. Inhibition by a-Asparagus racemosus; b-Withania somnifera
B. Inhibition by a-Convolvulus pluricaulis; b-Centella asiatica
C. Inhibition by a-Emblica officinalis, b-EUMIL
D. Inhibition by a-Ocimum sanctum; b-Oxytetracycline
E. Inhibition by a-Metronidazole; b-Amoxycillin
F. Inhibition by a-Roxithromycin; b-Tinidazole
G. Positive control (medium plus test bacterium)
H. Negative control (medium plus DM)
inhibition. 25.67 mm zone of inhibition was produced by methanolic extracts of AR as well as WS roots. 12.34 mm zone of inhibition was formed by methanolic extract of EO as well as OS. Maximum zone of inhibition was produced by extract by CP. AR, CA and WS extracts showed similar inhibitory action. Extracts of EO as well as OS showed minimal inhibition as compared to other extracts. Results are summarized in Table-I.

**Anti-microbial activity of EuMil® and antibiotics**

Poly herbal formulation- EuMil® produced 28 mm zone of inhibition. While various antibiotics amoxycillin, metronidazole, oxy-tetracycline, roxithromycin and tinidazole produced 18.34, 29mm, 38mm, 29.34mm and 34.67mm zone of inhibition respectively. Maximum zone of inhibition was observed with oxy tetracycline followed by tinidazole. Roxithromycin and metronidazole were comparable in antibacterial activity. Amoxycillin showed lowest inhibition as compared to the other antibiotics. Overall highest inhibitory activity was demonstrated by C. pluricaulis, EuMil® and oxy tetracycline. 90 mm growth in culture plate was observed in positive control group (Table.I).

**DISCUSSION**

*Helicobacter muridarum* is a helical organism harboring gastric region of the rodents. The bacteria are microaerophilic, nutritionally fastidious, and physiologically similar to *Helicobacter pylori* but both could be differentiated by their unique cellular ultra structure[17]. Infection of Helicobacter sp is related with diseases of the digestive system, especially PUD[18]. Comparative study of antibacterial activity of herbal extracts of AR, WS, CA, CP, OS, EO and EuMil® as well as antibiotics have shown interesting results. Methanolic extracts of all the plant extracts showed significant inhibition of bacterial activity. Though maximum inhibition was shown by extract of CP. The inhibition produced by AR, WS and CA extracts was comparable. Extracts of EO as well as OS showed minimal inhibition. EuMil® also produced significant inhibition comparable to that observed for herbal extracts. In case of antibiotics, maximum zone of inhibition was observed with Oxytetracycline followed by Tinidazole. Roxithromycin and Metronidazole produced comparable inhibition. Amoxycillin showed minimal inhibition. Overall highest inhibitory activity was demonstrated by CP, EuMil® and Oxytetracycline

**Table I: Antibacterial activity against Helicobacter muridarum (at 5mg/ml)**

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Name of agents</th>
<th>Plant part used</th>
<th>Extract</th>
<th>Zone of inhibition (mm ±SD) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1; Herbal extracts</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td><em>Asparagus racemosus</em></td>
<td>Root</td>
<td>Methanolic</td>
<td>25.67±0.58</td>
</tr>
<tr>
<td>2.</td>
<td><em>Centella asiatica</em></td>
<td>Whole plant</td>
<td>„</td>
<td>25.00±1.0</td>
</tr>
<tr>
<td>3.</td>
<td><em>Convolvulus pluricaulis</em></td>
<td>Whole plant</td>
<td>„</td>
<td>27.00±1.0</td>
</tr>
<tr>
<td>4.</td>
<td><em>Emblica officinalis</em></td>
<td>Fruits</td>
<td>„</td>
<td>12.34±0.58</td>
</tr>
<tr>
<td>5.</td>
<td><em>Ocimum sanctum</em></td>
<td>Whole plant</td>
<td>„</td>
<td>12.34±0.58</td>
</tr>
<tr>
<td>6.</td>
<td><em>Withania somnifera</em></td>
<td>Root</td>
<td>„</td>
<td>25.67±0.58</td>
</tr>
<tr>
<td>Group 2; Herbal Drug</td>
<td></td>
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<tr>
<td>7.</td>
<td>EuMil®</td>
<td></td>
<td></td>
<td>28.00±1.0</td>
</tr>
<tr>
<td>Group 3; Synthetic drugs (Antibiotics)</td>
<td></td>
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<tr>
<td>8.</td>
<td>Amoxycillin</td>
<td>-</td>
<td>-</td>
<td>18.34±0.58</td>
</tr>
<tr>
<td>9.</td>
<td>Metronidazole</td>
<td>-</td>
<td>-</td>
<td>29.00±0.58</td>
</tr>
<tr>
<td>10.</td>
<td>Oxy-tetracycline</td>
<td>-</td>
<td>-</td>
<td>38.00±1.0</td>
</tr>
<tr>
<td>11.</td>
<td>Roxithromycin</td>
<td>-</td>
<td>-</td>
<td>29.34±1.0</td>
</tr>
<tr>
<td>12.</td>
<td>Tinidazole</td>
<td>-</td>
<td>-</td>
<td>34.67±1.0</td>
</tr>
<tr>
<td>Group 4; Control group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>Positive control</td>
<td>-</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td>14.</td>
<td>Negative control</td>
<td>-</td>
<td>-</td>
<td>− − − −</td>
</tr>
</tbody>
</table>

± = Standard deviation, * = 8mm well diameter included in zone of inhibition, ++++ = 90mm growth all over the culture
Ulcer healing is a complex process that involves combination of wound retraction and re-epithelialization. It also involves other factors, such as, growth factors and angiogenesis. Several drugs such as antibiotics and general inhibitors, channel blockers are used for treatment but each have varying side effects. Drugs of plant origin could be alternative therapy for PUD. As observed in earlier study carried out in this laboratory, root extracts of AR and WS were effective not only in reducing the ulcer and acid secretions but were also effective in increasing the gastric protective secretions. Earlier studies on these drugs have also shown antibacterial activity of AR, OS, EO, CP, CA, WS. The exact mechanism of the antibacterial action of these plants species is not known, but a hypothetical diagram (Figure 2), to demonstrate the possible mechanism of anti-bacterial and anti-ulcer activity of polyphenols, flavonoids etc., present in these plants has been included. Ach release from post ganglionic vagal fibers stimulate gastric acid secretion directly through M3 sub type receptors located on the basolateral membrane of the parietal cells. Polyphenols by inhibiting Ach synthesis might regulate the excess synthesis of acid secretion. Polyphenols also stimulate inflammatory reactions. As Helicobacter bacteria is specifically adapted to live deep in the mucus layer close to epithelium and able to survive in acid environment, they may be phagocytosed by the phagocytic cells stimulated due to inflammatory reaction. Besides, Polyphenols and flavonoids are also known to increase the blood flow and stimulate the secretion of mucus, bicarbonate and NO. NO also plays the acid secretion inhibitory role. Polyphenols and flavonoids also reduce ulcer area, increase mucus secretion, and helpful in scavenging free radicals.

Antimicrobial activity of the medicinal plants could be attributed to the presence of secondary metabolites such as phenolic compound. In many cases these substances serve as plant defense mechanisms against predatory microorganisms, insects and herbivores. Substance like polyphenols e.g., Catechol and Pyrogallol are toxic to pathogenic microorganisms, due to enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins. Quinones target the microbial cell surface adhesins; cell wall polypeptides and membrane bound enzymes. Flavones, flavonoids and flavonols are known to be synthesized by plants in response to microbial infection. Their antimicrobial nature is probably due to their ability to form complexes with bacterial extra cellular and soluble proteins which then bind to bacterial cell wall. Lipophilic flavonoids may also disrupt microbial membranes Human physiological activities such as stimulation of phagocytic cells, host mediated tumor activity and wide range of anti-infective actions have been assigned to tannins. Tannins have the ability to inactivate microbial adhesions, enzymes.

Figure 2: Hypothetical diagram to demonstrate the possible sites of inhibitory action of various constituents of plant extracts on Helicobacter muradirum and ulcerative gastrointestinal membrane.
and transport proteins on cell envelope and lipophilic terpenoids and essential oils are also speculated to be involved in membrane disruption[31,32]

In conclusion, study thus demonstrate that C. pluricaulis whole plant extract, EuMil® and Oxytetracycline shows the highest inhibitory activity against H. muridarum. Data presented may serve as background for future studies on Helicobacter pylori. Our results fortify importance of these plants as an alternative anti-ulcer and ulcer healing agents.

ACKNOWLEDGEMENT

Authors are thankful to UGC for providing UGC-SAP program to the department and to DST Rajasthan for a Minor research grant to SKV.

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