In vivo Anti-helicobacter pylori activity of methanolic extract of Symplocos racemosa ROXB

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ABSTRACT
Helicobacter pylori leads to chronic gastritis, peptic ulcer disease and gastric cancer. With increasing issues of antibiotic resistance and changing epidemiology of this pathogen, new approaches are needed for effective management. This attempt focuses on the antulcer effect of Methanolic extract of Symplocos racemosa (SRME) against H. Pylori. Seven groups of animals (Wistar rats) were made for determination of antiulcer activity of SRME extract against H. Pylori. Chronic ulcers were induced by indomethacin & H. pylori. Group 1 was vehicle control, group 2 was treated with standard drugs Clarithromycin, Amoxicillin & Omeprazole (CAO). Group 3, 4, 5, & 6 received 100mg, 200mg, 400mg of SRME & 400mg + CAO/kg/day respectively. Group 7 was kept as healthy control. Animals were infected by H. Pylori. Presence of H. pylori in antrum tissue was confirmed by rapid urease test. Genomic DNA of H. pylori from pyloric antrum was isolated by phenol chloroform C-TBA method. After 4 weeks of administration of indomethacin & H. pylori, infection status was determined by DNA amplification. Gel image of amplified DNA revealed amplification of 16s rRNA gene of H. Pylori. Infection status of H. pylori was also determined using PCR. Gastric tissue of animals in vehicle treated group 100% animals were detected positive for 16s rRNA at the end of treatment of 4 weeks. Groups 3, 4, 5, & 6 showed dependent response after treatment period of 4 weeks.

KEY WORDS: Antiulcer, H. Pylori, DNA amplification, Symplocos racemosa

INTRODUCTION:
Gastric hyperacidity and gastro duodenal ulcer is a very common global problem today. It is now generally agreed that gastric lesions develop when the delicate balance between some gastro-protective and aggressive factors are lost. Major aggressive factors are acid, pepsin, Helicobacter pylori and bile salts. Defensive factors mainly involve mucus bicarbonate secretion and prostaglandins. Hyper secretion of gastric acid is a pathological condition, which occurs due to uncontrolled secretion of hydrochloric acid from the parietal cells of the gastric mucosa through the proton pumping H+K+ATPase. Infection of the stomach mucosa with helicobacter pylori – a Gram-negative spiral-shaped bacterium – is now generally considered to be a major cause of gastro-duodenal ulcer.

H. pylori were the first isolated microaerophilic gram-negative bacteria from the gastric mucosa of gastritis patients by Marshall and Warren in 1980s. It is a worldwide common infection with prevalence rates in the general population ranges from not only 30-40% in United States, 80-90% in South America and 70-90% in Africa but also in developing countries like India, China from the age of teenagers 20% to 50-60% of elderly subjects.

According to the statistics, it causes peptic ulcer disease approximately one in six (17%) persons and each year 1% to 2% of these will experience a major or life threatening complication, such as bleeding or gastric outlet obstruction. H. pylori is such a threat that the World Health Organization’s (WHO) International Agency for Research into Cancer (IARC) in 1994 has classified as a “Class-I-Carcinogen”.

Prostaglandins are involved in promoting the defense mechanisms of the stomach, and H. pylori may promote gastric mucosal prostaglandin secretion by up to 50% to maintain its preferred environmental conditions. Because prostaglandin levels in the gastric mucosa are decreased in elderly patients, ageing is associated with a diminished epithelial cell turnover rate and a reduced capacity to repair the gastric mucosa. Advanced age is therefore a major risk factor for complicated peptic ulcer disease. According to an estimate by the World
Health Organization (WHO), half of the world’s population is infected with *H. pylori*, but the infection has no detectable symptoms in most cases. However, over the past two decades, there has been a decrease in reported *H. pylori*-related peptic ulcer disease. This decrease is due to early detection using several sophisticated diagnostic tools and early treatment of the infection.\(^7\)

*H. pylori* produces a variety of enzymes and is characterized by high urease activity. Urease breaks urea into bicarbonate and ammonia, which help to neutralize gastric hydrochloric acid (HCl) and protect the bacterium in the acidic environment of the stomach. Hydroxide ions generated by the equilibration of water and ammonia may contribute to gastric mucosal epithelium damage. Conversely, *H. pylori* infection reduces epithelial cell bicarbonate secretion, which leads to excessive diffusion of HCl into the mucosa, causing damage of the gastro-duodenal lining and leading to ulcer formation. It appears that *H. pylori* infection activates the vago-vagal reflexes (gut-brain axis) in the gastroduodenal mucosa that damage the mucosal cells directly and enhance the secretion of gastric HCl, which ultimately leads to ulcerogenesis.\(^8,9\)

To date, the most effective therapies of *H. pylori* infection require a minimum of two antibiotics in combination with a gastric acid inhibitor. Both Triple Therapy (levofloxacin/Clarithromycin + amoxicillin + proton pump inhibitor) and Bismuth Quadruple Therapy (bismuth + tetracycline + metronidazole + proton pump inhibitor) are well known for *H. pylori* eradication as well as for *H. pylori*-induced gastropathy prevention. Complete eradication of *H. pylori* infection improves symptoms, including dyspepsia, gastritis and peptic ulcers, and may prevent gastric cancer. However, these treatments may cause nausea, drug resistance, infection recurrence, stomach upset and diarrhea. Rising levels of acquired antimicrobial resistance necessitate the search for an effective *H. pylori* infection prevention strategy. Alternatively, there is a growing interest in and need to find non-toxic, safe and inexpensive anti-ulcer formulations from medicinal plants.

The medicinal properties of folk plants are attributed mainly to the presence of natural antioxidants (mainly polyphenols and flavonoids). Flavonoids and other polyphenols present in the plant materials are beneficial for human health. Several mechanisms may account for their antioxidant activity. Flavonoids and polyphenols are efficient in trapping superoxide anion (O2-), hydroxyl (OH·), peroxyl (ROO·) and alcohoxyl (RO·) radicals, decreasing acid mucosal secretion, inhibiting the production of pepsinogen, promoting gastric mucosa formation and decreasing ulcerogenic lesions.\(^10\) Recent studies have suggested that *H. pylori* infection can be suppressed through the use of medicinal plants. *Symlocos racemosa* is a medicinal plant. Its bark is used to treat various ailments. The chemical constituents of the *Symlocos racemosa* bark led to the isolation of two new phenolic glycosides, Symconoside A and Symconoside B.\(^11\) The important chemical constituents of *Symlocos racemosa* are flavonoids, tannins, loturine, loturidine, and colloturine. *Symlocos racemosa* (Fam. symlocaceae) is a widely used ayurvedic remedy for various ailments. It is also known as lodhra and is used as a single drug or in multicomponent preparations. It possesses cardiotonic, antipyretic, antihelminthic and laxative properties. It is beneficial in bilious fever, urinary discharge, blood troubles, burning sensations, leukoderma, and jaundice. Lodhra bark is acrid, digestible, and astringent to bowels. It is useful in treatment of fever, eye diseases, for spongy gums and bleeding. It cures diseases of the blood, leprosy, dropsy and liver complaints.\(^12\) The study indicates that the methanolic extract of *Symlocos racemosa* exhibited in vitro antibacterial activity against *H. Pylori*. Methanolic extract of *Symlocos racemosa* possess good quantity of Total Phenolic content & Total flavonoid content.\(^13\)

### MATERIALS AND METHODS

1. **Acute oral toxicity test:** The acute oral toxicity study for SRME was carried out according to OECD guidelines 423. Swiss albino mice were fasted overnight, water also being withheld. The SRME was administered at a dose of 2000 mg/kg. Animals were observed individually during the first 30 minutes and periodically during 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total 14 days. (Animal ethical committee No.(Ref/ACP/IAEC/11-12/12-05).

2. **Determination of Antibacterial activity against *H. Pylori* (in-vivo):** Seven groups of animals (Wistar rats) were made for determination of antiulcer activity of SRME extract against *H. Pylori*. Animals were made for determination of antiulcer activity of SRME extract against *H. Pylori*. The treatment regimen as follows:

- **Animal groups (n=6):** The treatment regimen as follows:
  - **Group 1:** Vehicle control (VC): Ulcerated non infected Vehicle treated.
  - **Group 2:** Standard drug treated group (CAO): (clarithromycin 25 mg/kg + amoxicillin 50 mg/kg + omeprazole 20 mg/kg) p.o.
  - **Group 3:** SRME treated group: (SRME 100 mg/kg/day) p.o.
  - **Group 4:** SRME treated group: (SRME 200 mg/kg/day) p.o.
  - **Group 5:** SRME treated group: (SRME 400 mg/kg/day) p.o.
  - **Group 6:** SRME treated group: (SRME 400mg/kg/day) + CAO (clarithromycin 25 mg/kg + amoxicillin 50 mg/kg + omeprazole 20 mg/kg) p.o.
  - **Group 7:** Healthy Control (HC): Nonulcerated non infected Vehicle treated.
2. Induction of unhealed ulcers: 15, 16

The rats were fasted for 24 hours before the induction of ulcers. The rats were anesthetized with ketamine (60 mg/kg i.p.). An epigastric incision was made through midline and stomach was exposed. 0.3 ml of a 20% solution of acetic acid was injected into the sub serosal layer of the glandular portion of the stomach with the aid of a tuberculin syringe. Subsequently stomach was re-internalized; the abdomen was closed and sutured. The animals were maintained in individual cages with meshed bottom to prevent coprophagy. The size of the mesh (4 x 4 mm) allowed feces to fall to the floor of the cage below the mesh. After the induction of ulcers, five days were required for the ulcers to develop fully. The fifth day after ulcer induction was considered day 0. These ulcerated animals were administered indomethacin 1mg/kg/day p.o. for 4 weeks to produce unhealed ulcers. High mortality of rat after subcutaneous indomethacin necessitated change of route from subcutaneous to per oral. Thus a modification was made in the original protocol. Unhealed ulcers were produced after oral administration of indomethacin. To infect the pyloric antrum tissue of the animals with H. pylori, a broth of H. pylori (1 ml p.o.) was administered three times a week for four weeks. During this period indomethacin administration was uninterrupted.

H. pylori was resurrected from the cryopreserved stage onto brucella blood agar culture plates using fresh sheep blood and blood agar media. H. pylori was grown in microaerophillic conditions in a desiccator which was maintained at 37°C in an incubator. Thereafter, the bacterial colonies were scraped from the culture plates and transferred aseptically into H. pylori broth consisting of brucella broth and fetal calf serum in laminar air flow. The bacterial broth administered to rats was adjusted to Mc-Farland turbidity standard 1 using brucella broth and 1mL of 1% tween 80 solution was administered 3 times a week for four weeks. The dose of piperine (10, 20 and 40 mg/kg p.o) was selected on the basis of the pilot study performed on the unhealed ulcer induced rats.

At the end of the treatment regimen of four weeks, after 24 hour fasting, the rats were euthanized under deep ether anesthesia. A midline incision was performed. The stomachs were rapidly removed and opened along the greater curvature. It was washed with normal saline and each stomach was photographed using a crystal clear display (CCD) scanner at a magnification of 2400 dots per inch (DPI). The lesion was localized and measured along the external (comprising the regenerative tissue) and internal (only exposed sub mucosa) borders for area determination. The fundic portion of each stomach was excised. The contents were washed off and fundic area was excised off. The pyloric antrum area of stomach was selected and used for all investigations as it has been previously investigated that H. pylori is favorably harbored in this region. The image of each excised stomach was captured at a magnification of 2400 D.P.I and then it was processed for nucleic acid extraction. RUT, biochemical studies and western blot to determine the H. pylori infection status and gene expression studies.

The pyloric antrum region of each stomach was divided into three parts in each group of animals. Out of the six animals in each group, the excised stomachs of three animals were utilized for mitochondrial assays, RUT (Rapid Urease Test) and gene expression studies. The rest of the three animals were used for nucleic acid extraction to be further used for infection status determination, reverse transcriptase PCR.

3. Preparation of genomic DNA for PCR 17

DNA isolation from pyloric antrum tissue was performed according to phenol chloroform C-TAB method. Briefly, 200 mg of the pyloric antrum tissue sample was suspended in 250 µL of digestion buffer II {0.1M NaCl, 0.01M Tris-HCl (pH 8.0), 0.25M EDTA (pH 8.0), 1% SDS} containing 100µg/ml of proteinase k (Vivantis, India). To this, 250 µl of digestion buffer I {0.1M NaCl, 0.01M Tris-HCl (pH 8.0), 0.25M EDTA (pH 8.0)} was added and incubated at 56°C overnight. DNA was extracted with an equal volume of phenol chloroform and precipitated with 0.6 volume iso propanol. The DNA pellets were washed thrice with 80%, 75% and 70% ethanol, respectively, and finally re suspended in 100µl of sterile water for injection. All the steps were performed in aseptic conditions to minimize contamination. The DNA was extracted and preserved at -20°C until amplification was performed.

4. Amplification of virulent and non-virulent genes of H. pylori 18, 19

H. pylori specific genes were amplified in a programmable thermal
cycler. The template DNA (1 µL) was added to 19 µL of the reaction mixture containing PCR buffer (50 µmol KCl, 10 µmol Tris-HCl (pH 8.3), 1.5% [v/v] Triton X-100), 1.5 µmol MgCl₂, 200 µmol concentrations of each dNTP, 10 pmol of each primer (forward and reverse), and 1 U of Taq polymerase.

PCR amplification was carried out, which included initial denaturation at 95°C for 5 minute, 40 cycles with 1 cycle consisting of 30 seconds at 94°C, 30 seconds at 52°C, 1 minute at 72°C. The final cycle included a 10 min extension step to ensure full extension of the PCR products. Amplification was carried out in a thermocycler (Eppendorf, USA). The PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide for visualization. The DNA of H. pylori (type ATCC 26695) served as a positive control. Water instead of DNA template was used as a negative control. At each amplification, H. pylori DNA obtained from a fully sequenced standard strain (ATCC 26695) was used as a positive control, while sterile water for injection instead of DNA served as a negative control. The products were analyzed by agarose gel electrophoresis and the image of the gel was captured using gel documentation. The DNA isolated from all the samples were amplified to get a particular base pair fragment corresponding to the specific H. pylori gene. The annealing temperature was optimized according to melting point (Tm) of a particular primer pair and was unique for each gene.

5. Rapid Urease test:

Weighed quantity of the excised pyloric antrum tissue (50 mg) was immersed in 5 mL RUT solution. The color change was recorded. The color changed from yellow to pink in one hour if H. pylori was present in the pyloric antrum tissue.

RESULTS AND DISCUSSION:

1. Acute toxicity Study:

SRME administered at a dose of 2000 mg/kg did not show any signs or symptoms of toxicity or mortality during the observation period. The starting dose was selected as 1/10th and 1/5th of 2 000 mg/kg. SRME administered at a dose of 2000 mg/kg did not show any signs or symptoms of toxicity or mortality during the observation period. The starting dose was selected as 1/10th and 1/5th of 2000 mg/kg.

2. PCR Amplification of virulent and non-virulent genes of H. pylori:

Figure no. 1 showing the successful amplification of 534 base pair amplicon corresponding to 16S rRNA gene of H. pylori. Lane 1: 100 base pair ladder, Lane 2: Vehicle Control, Lane 3: SRME 100 mg/kg p.o. treated group, Lane 4: SRME 200 mg/kg p.o. treated group, Lane 5: SRME 400 mg/kg p.o. treated group, Lane 6: CAO treated group, Lane 7: CAO + SRME 400 mg/kg p.o. treated group Lane 8: Healthy control.

2. Determination of H. pylori infection status determination using polymerase chain reaction

The gastric tissue of animals in the vehicle treated group six out of six (100%) animals were detected positive for 16S rRNA gene of H. pylori at the end of treatment period of 4 weeks.

In the SRME 100 mg/kg treated group having 6 rats (83.33%) were detected positive for 16S rRNA gene at the end of treatment period of 4 weeks.  In the SRME 200 mg/kg treated group, group two out of six (33.33%) animals were detected positive for all the H. pylori genes at the end of treatment period of 4 weeks.  In the SRME 400 mg/kg treated group, group one out of six (16.66%) animals was detected positive 16 sRNA gene of H. pylori at the end of treatment period of 4 weeks. In the animals treated with piperine (SRME 400 mg/kg +CAO), (CAO) and healthy control group, none of the animals were detected positive for 16S rRNA gene of H. pylori at the end of treatment period of 4 weeks.

3. Rapid Urease Test:

Weighed quantity of the excised pyloric antrum tissue (50 mg) was immersed in 5 mL RUT solution. The color change was recorded. The color changed from yellow to pink in one hour if H. pylori was present in the pyloric antrum tissue.
CONCLUSION:
Elimination of *H pylori* has been a major objective of treatment strate-
gies worldwide. Unfortunately, none of them have been able to
achieve 100% eradication rates. The main reason underlying this fail-
ure could be the rapidly emerging resistance among many strains of
*H pylori* towards various antibiotics. Flavonoids and other
polyphenols present in the plant materials are beneficial for human
health. Methanolic extract of *Symplocos racemosa* rich in Flavonoid
and polyphenolic compounds. It can be concluded from the results
that methanolic extracts of *Symplocos racemosa* used in present in-
vestigation possess significant antibacterial activity against the tested
*H. Pylori*. The results of in vivo test by the determination of *H. pylori*
infection status determination using polymerase chain reaction
method exhibited dose dependent activity comparable to standard
clarithromycin, amoxicillin & omeprazole. Overall, our study revealed
that at a high concentration *Symplocos racemosa* extract can exert
antimicrobial activity against oral bacteria and *H. pylori* isolated from
duodenal ulcer. In conclusion, methanolic extracts of *Symplocos racemosa* (SRME) possess considerable antibacterial activity
against *H. pylori*. The potential of *Symplocos racemosa* in the pre-
vention or treatment of *H. pylori* infection is worth further extensive
evaluation. In addition, the in vivo studies also proved to be highly
efficient in terms of dosage, tolerability and curing active *H pylori*
infection. Future studies will assess the mechanism by which these extracts effect the survival of *H pylori*.

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