

Comparative evaluation of the efficacy of *Cardiospermum halicacabum* Linn. on Indomethacin, Pylorus ligation and *Helicobacter pylori* induced gastric ulcer in rats

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Abstract

Helicobacter pylori infection, excessive consumption of alcohol and the prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs) are the major factors that cause peptic ulcer. Previous studies from our lab revealed the potency of the ethanol extract of the plant, *Cardiospermum halicacabum* Linn. against ethanol induced gastric ulcerogenesis in rats. The present study intended to compare the antiulcer activity of the ethanol extract of *C. halicacabum* against gastric ulcers, induced by three different agents, namely; indomethacin administration, pyloric ligation and *Helicobacter pylori* infection in Wistar rats. Experimentally, gastric ulcer was induced in rats by oral administration of indomethacin, pyloric ligation and infection with *H. pylori* (2.0×10^8 CFU/ml) in three separate settings. Gastric protection by *C. halicacabum* was evaluated by measuring the ulcer index, gastric glutathione level, alkaline phosphatase activity and histopathological examination. Ethanol extract of *C. halicacabum* in a concentration dependent manner (200-600 mg/kg) inhibited gastric ulcers induced by oral administration of indomethacin (48 mg/kg). The active extract administration reduced the ulcer index and alkaline phosphatase activity showing the protective role of *C. halicacabum* against indomethacin induced gastric ulceration in rats. Similarly, *C. halicacabum* ethanol extract reduced gastric acid secretion in pylorus ligated rats. However, the extract did not show much healing of gastric ulcer, induced by *H. pylori*. Omeprazole and triple therapy drugs were used as standards for indomethacin and *H. pylori* induced gastric ulcers, respectively. When comparing the effectiveness of *C. halicacabum* against the three modes of ulcer induction, the alcohol extract of *C. halicacabum* showed better antiulcer activity against ulcers induced by indomethacin and pylorus ligation dose dependently. However, the same extract failed to show any anti *H. pylori* activity when monitored both *in vitro* and *in vivo*.

Key words : *Cardiospermum halicacabum* Linn., ethanol extract, *H. pylori*, indomethacin, pyloric ligation, omeprazole

1. Introduction

Gastric and duodenal ulcers affect a large proportion of the world population and are induced by several factors such as stress, alcoholism, drugs, bacterial infection, smoking and nutritional deficiencies (Nash *et al.*, 1994; McGuigan, 1991). A peptic ulcer is an open sore. They are usually found in the lining of the stomach, esophagus, or upper small intestine. Ulcers that occur in the stomach are called gastric ulcers. Ulcers that occur in the upper area of the small intestine, the duodenum, are called duodenal ulcers. It has been demonstrated that the pathophysiology of peptic ulcer disease involves an imbalance between offensive (gastric acid and pepsin secretion, active free radicals and oxidants, leukotrienes, endothelins, and exogenous factors such as ethanol or nonsteroidal anti-inflammatory drugs (NSAIDs) and *H. pylori*) and defensive factors (gastric mucus, bicarbonate, normal blood flow, prostaglandin 'PG'),

nitric oxide (NO), and antioxidant enzymes such as catalase and glutathione (GSH) (Tytgat, 2000; Basil and Howard, 1995). Numerous approaches have been used to combat gastric ulcers including the control of gastric acid secretions, H^+ / K^+ ATPase activity, *etc.*, in an attempt to reverse mucosal damage and inflammation (Woo *et al.*, 1998).

Indomethacin, a NSAID, widely used to treat arthritic diseases, causes gastric lesions (Langman *et al.*, 1999). It is suggested that indomethacin induces gastric damage by inhibiting release of protective factors like Cyclooxygenase 1 (COX-1), prostaglandin E2 (PGE2), bicarbonate and mucus, thus, increasing acid secretion (Mitchell *et al.*, 1995). The development of gastric lesions by the administration of indomethacin has been implicated to be due to the inhibition of prostaglandin biosynthesis (Whittle, 1981). Pylorus ligation alters the function of cytoprotective prostaglandin through the accumulation of gastric juice and, thereby, induces gastric ulcer (Singh *et al.*, 2008). In addition to the long term use of NSAIDs, *H. pylori* infection is a prevailing risk factor for peptic ulcer disease (Pahwa *et al.*, 2010). *H. pylori* colonization of the human stomach became first associated with human disease when Marshall and Warren succeeded in culturing the bacterium in 1983 (Warren and

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Marshall, 1984). These organisms are spiral, microaerophilic, gram negative bacteria which can induce gastric inflammation (Goodwin *et al.*, 1987); cytotoxins of this organism activate NF- κ B (Liu and Schubert, 1997). Further, *H. pylori* toxins are an important risk factor for chronic ulcer (Fallone *et al.*, 2000). *H. pylori* remain present between the mucous layer and the gastric epithelium and are strategically designed to live within the aggressive environment of the stomach. Initially, *H. pylori* resides in the antrum but over time migrate toward the more proximal segments of the stomach (Das *et al.*, 1997).

Plants have the ability to synthesize numerous compounds for their biological functions. Many of these phytochemicals derived from plants, have been used to treat human diseases, since the dawn of medicine. Roughly 50% of new chemical entities introduced during the past two decades are from natural products. Recent technological advances have renewed interest in natural products in drug discovery. There are various medicinal plants and their extracts containing active chemical constituents that have significant antiulcer activity in *in vivo* experiments on animal models. In this context, extract or active principles from plants could serve as leads for the development of new safe and effective drugs (Gonzales *et al.*, 2000).

C. halicacabum Linn. (Sapindaceae) is a medicinal herbaceous climber, widely distributed in tropical and subtropical regions of Asia. Toxicological and pharmacological studies carried out by various investigators revealed its analgesic, antipyretic, antiarthritic, anti-inflammatory, antimalarial, antimicrobial, antidiarrhoeal, antidiabetic, antioxidant, hepatoprotective and nephroprotective properties (Asha and Pushpangadan, 1999; Gopalakrishnan *et al.*, 1976; Paakkari, 1994; Sadique *et al.*, 1987; Sheeba and Asha, 2006, 2009; Ghosh and Sil, 2007; Das *et al.*, 1997; Venkat Rao *et al.*, 2006; Pratheesh Kumar and Girija Kuttan, 2010).

The present study was undertaken to evaluate the gastroprotective effect of *C. halicacabum* and compare its potency against acute (indomethacin, pylorus ligation) and chronic (*H. pylori* infection) models of ulcerogenesis in rats.

2. Materials and Methods

2.1 Materials

2.1.1 Reagents

Indomethacin, omeprazole, clarithromycin, metronidazole, 5'5'-dithio-bis-2-nitrobenzoic acid (DTNB), thiobarbituric acid (TBA), deoxyribose, trichloro acetic acid (TCA), curcumin, hexadecyl trimethyl-ammonium bromide, antibiotics such as polymyxin B, vancomycin, amphotericin B and trimethoprim were purchased from Sigma. PGE₂ enzyme immunoassay kit (EIA) was purchased from Cayman Chemical Company, tritiated arachidonic acid from American radiolabeled chemicals, FBS from GibcoBRL, Brain Heart Infusion (BHI) broth and agar-agar from Hi Media. All other chemicals and reagents used were of analytical grade.

2.1.2 Plant material and preparation of active extract

Fresh plants were collected from Ayurveda Research Center, Thiruvananthapuram, Kerala during the month of September. Mrs. Padmaja, Taxonomist, Regional Research Institute (RRI), Poojapura,

Kerala, identified the plant material and a voucher specimen (Ethno 10) was deposited at the Institute's herbarium. The whole plant was cleaned, dried under shade at room temperature and powdered. 10 g of the dried plant powder was used for Soxhlet extraction with 400 ml of alcohol for 48 h. The dried plant material wrapped in a cotton cloth was loaded into the main chamber of the Soxhlet extractor. The extractor was then placed onto a round bottom flask containing alcohol. The apparatus was then attached to a condenser. Soxhlet extraction was carried out at the boiling point of alcohol – 78.37°C. The extraction was continued until a drop of solvent from the siphon tube when evaporated does not leave a residue. The extract was then collected and the solvent evaporated under vacuum under reduced temperature and pressure in a rotary evaporator (yield of ethanol extract was 10% (w/w)).

2.1.3 Test animals

Charles Wistar rats (120-160 g) of either sex were maintained in a 12 h light/dark cycle at a constant temperature 25°C with free access to feed (Sai Durga Feeds and Foods, Bengaluru) and water. All animals were fasted prior to all assays and were allocated to different experimental groups each of 6 rats. Moreover, the animals were kept in specially constructed cages to prevent coprophagia during the experiment. All experiments were carried out according to the guidelines for care and use of experimental animals and approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) IAEC/8/VVA/2005.

2.2 Induction of gastric ulcer in rats

2.2.1 Indomethacin induced gastric ulcer

Gastric damage was induced in rats by oral administration of indomethacin at the dose of 48 mg/kg (Snehasikta *et al.*, 2005). The rats were divided into six groups, each containing six animals and fasted for 24 h. but water was given *ad libitum*. The first group received distilled water only and the second group received indomethacin orally. The third, fourth and fifth groups were given indomethacin and ethanol extract of *C. halicacabum* at a dose of 200, 400 and 600 mg/kg, respectively. The sixth group received indomethacin and 20 mg/kg of standard antiulcer drug, omeprazole (Kinsey *et al.*, 2011; Arun and Asha, 2008; Lind *et al.*, 1983). The drugs were administered orally 30 min prior to the oral administration of indomethacin. The animals were anaesthetized 6 h. later with ether and stomachs were incised along the greater curvature and ulceration was scored. The gastric mucosa was subjected to biochemical tests.

2.2.2 Pylorus ligation induced gastric ulcer

Rats were divided into four groups and fasted for 24 h. The first group received the vehicle only. The second, third, fourth groups received the ethanol extract of *C. halicacabum* at different doses (200, 400 and 600 mg/kg) one hour before pyloric ligation (Shay *et al.*, 1954). The rats were anaesthetized, the viscera were opened, and pyloric region of the stomach was located and ligated without internal injury. The animals were deprived of both food and water after post operative period. The animals were sacrificed 4 h. after pyloric ligation and the gastric juice was collected and centrifuged for 10 min. at 4000 rpm for removing the solid particles. The supernatant was used for further analysis.

2.2.3 *H. pylori* induced gastric ulcer

2.2.3.1 *H. pylori* culture

Cag A- and Vac A-positive standard strains of *H. pylori* were purchased from American Type Culture Collection (ATCC 43504) Rockville, MD. The bacteria were grown in BHI broth containing 10% FBS at 37°C under a microaerophilic atmosphere for 5 days. Plate culture was done in BHI agar containing 10% FBS at same conditions described here.

2.2.3.2 *H. pylori* inoculation in experimental rats

The rats (50-80 g) were divided into seven groups of five animals each and kept in cages with regulated temperature (approximately 20-22°C), humidity (approximately 55%), and light/dark cycle (12/12 h.). The rats were deprived of food for 24 h. before and 4 h. after *H. pylori* inoculation, but were otherwise afforded free access of food and water. *H. pylori* (2.0×10^8 CFU/ml) were orally inoculated into each animal at a dose of 1.0 ml/animal on three successive occasions within a five day period. Animals of the uninfected groups were administered broth medium alone.

2.2.3.3 Experimental design

In *H. pylori*-infected animals, drug treatment was started 3 months after inoculation. The groups were given *C. halicacabum* ethanol extract at different doses (200, 400 and 600 mg/kg), Omeprazole (20 mg/kg) and Triple therapy drugs: Omeprazole (20 mg/kg), Clarithromycin (50 mg/kg) and Metronidazole (10 mg/kg) for 2 weeks. The extract and drugs were suspended in Tween 80. The control rats were given the vehicle only. After 15 days of drug treatment, the rats were sacrificed on the 16th day with an overdose of ether. The stomach of each animal was removed, opened along the greater curvature and examined for ulcerative lesions. The mucosal tissues were used for further experiments.

2.3 Effect of ethanol extract of *C. halicacabum* against indomethacin induced gastric ulcer in rats

2.3.1 Estimation of alkaline phosphatase (ALP)

The excised stomach tissue, immersed in 4 ml of the buffer solution, was ground in a mortar and then centrifuged for 10 min. The ALP activity was measured from the supernatant, using the method of Kind and King (1954).

2.3.2 Glutathione (GSH) assay

The mucosa of glandular stomach was removed by scraping with a blunt knife and 10% homogenate was prepared. Reduced glutathione (GSH) in the gastric mucosa was determined by Ellman's reaction using 5'5'-dithio-bis-2-nitrobenzoic acid (DTNB) as described. 10% stomach mucosal homogenate was prepared in 30 mM KCl.

The proteins were precipitated by adding 125 µl of 25% trichloroacetic acid (TCA) in 0.5 ml of homogenate and kept in ice for 5 min. It was further diluted with 0.6 ml of 5% TCA and centrifuged at 9000 x g for 10 min and the supernatant was collected. The supernatant was taken for GSH estimation, using freshly prepared 6 mM DTNB solution and 0.2 M phosphate buffer (pH 8.0). The reaction mixture was incubated for 10 min at room temperature. The intensity of the yellow color formed was read at 412 nm in a spectrophotometer (Moron *et al.*, 1979). Reduced GSH was used as the standard.

2.3.3 Histopathological studies

Two animals from each group were sacrificed and the stomach was isolated, washed with saline and preserved in 10% formaldehyde solution for histopathological studies. The tissues were dehydrated, embedded in paraffin wax and then sectioned by using a microtome. The 5 µm thick sections of the stomach, stained with haematoxylin and eosin, were assessed for histopathological changes and photomicrographs were taken using Leica microscope with a magnification of 200 X.

2.4 Effect of ethanol extract of *C. halicacabum* against pylorus ligation induced gastric ulcer

2.4.1 pH of gastric secretions

The pH of the gastric juice was measured using pH paper strips of varying ranges. The color of the pH paper after procedure was matched with standard scale and pH was recorded for different groups of animals.

2.4.2 Determination of total acidity

1 ml of gastric juice was pipetted into a conical flask. 2-3 drops of phenolphthalein solution was added and titrated with 0.01 N NaOH (which was previously standardized with 0.01 N HCl) until a definite red tinge reappeared. The volume of NaOH was noted. The volume corresponds to total acidity. Units were expressed as mEq/l:

$$\text{Acidity} = \frac{\text{Volume of NaOH} \times \text{Normality of NaOH} \times 100}{0.1}$$

2.5 Effect of ethanol extract of *C. halicacabum* on gastric ulcer induced by *H. pylori*

2.5.1 Quantification of viable *H. pylori*

The stomach of each animal was homogenized in phosphate buffered saline (PBS) of pH 7.6 with a tissue homogenizer and then serially diluted with the same buffer. Aliquots (0.1 ml) of the dilutions were applied to BHI agar plates containing 10% FBS, 2.5 µg/ml amphotericin B, 9 µg/ml vancomycin, 0.32 µg/ml polymyxin B, 5 µg/ml trimethoprim and 50 µg/ml 2,3,5-triphenyltetrazolium chloride for preventing the growth of other bacteria. The plates were incubated at 37°C under a microaerophilic atmosphere for 7 days. All colonies were either black or gold. The number of colonies was determined and the viable *H. pylori* count was expressed as CFU/stomach.

2.5.2 Measurement of myeloperoxidase (MPO) activity

Myeloperoxidase (MPO) activity of gastric tissue, a marker of neutrophil infiltration (Krawisz *et al.*, 1984), was assayed by the method of Bradley *et al.* (1982). Gastric tissue (50 mg) was extracted from each stomach, homogenized in 1.0 ml of 50 mM phosphate buffer (pH 6.0), containing 0.5% hexadecyltrimethyl-ammonium bromide and then subjected to freeze thawing three times. The homogenates were centrifuged at 1,600 g for 10 min at 4°C. An aliquot (5 µl) of each supernatant was mixed with 145 µl of phosphate buffer (pH 6.0), containing 0.167 mg/ml o-dianisidine dihydrochloride and 0.0005% H₂O₂, and the change in the rate of absorbance at 450 nm was measured with a microplate reader.

2.5.3 Determination of PGE₂ synthesis

The gastric tissue was homogenized in 1 ml 0.1 M phosphate buffer (pH 7.4), containing 1 mM EDTA and 10 µM indomethacin. Indomethacin was added to the buffer to inhibit excessive COX-2 activity. The homogenates were centrifuged at 9,000 rpm for 40s. Supernatant was collected and the amount of PGE₂ in the supernatant was determined by enzyme immunoassay (PGE₂ EIA kit, Cayman chemical). PGE₂ production was expressed as nanograms of PGE₂ per gram tissue per min.

2.5.4 Histopathological observation

For studying the histopathological changes in *H. pylori* infected gastric mucosa, the tissues were fixed in buffered formalin and then embedded in paraffin. Subsequently 4 µm thick paraffin samples were prepared and stained with Giemsa, hematoxylin and eosin (H and E).

2.5.4.1 Detection of *H. pylori* 16S rRNA by polymerase chain reaction (PCR)

For DNA extraction, TRIzol reagent was used according to the manufacturer's instructions (Gibco BRL). The samples from gastric mucosa were homogenized in 250 µl of TRIzol reagent for 15-20s. After 5 min of incubation at room temperature, 50 µl of chloroform was added, followed by centrifugation at 12,000 x g for 15 min, which led to the separation of the sample solution into an aqueous and an organic phase. After removal of the aqueous phase, DNA was extracted from the samples by precipitation with 75 µl of 100% ethanol. Following the precipitation and series of washes, DNA was dissolved in 8 mM NaOH. The sample was precipitated again with ethanol and dissolved in TE buffer. The concentration of DNA was estimated by absorbance at 260 nm. DNA samples were stored at -80°C.

Polymerase chain reaction (PCR) was performed as described earlier (Aly *et al.*, 1985). Briefly 2 µl of DNA was amplified in a 50 µl reaction volume containing 2 U Taq DNA polymerase, dNTPs 200 µM each, 1.5 mM MgCl₂, 5 µl 10 X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3) and primers for *H. pylori* 16S rRNA used at a final concentration of 1 mM. The PCR mixture was amplified in a DNA thermal cycler (BioRad). Amplification consisted of initial denaturation at 94°C for 2 min, followed by denaturation for 45 s, primer annealing at 61°C for 45 s and extension for 2 min at 72°C. The samples were amplified for 35 cycles, with the final extension step at 72°C for 7 min. The nucleotide sequence of the primers was based on the sequences of the published cDNAs (Zayachkivska *et al.*, 2005). The sense primer of 16SrRNA 5'-TCAGCCTATG TCCTATCAGC-3' and the antisense primer of 16S rRNA 5'-CAGTAATGTTCCAGCAGGTC-3' yield the 600 bp product. The primers were synthesized by Sigma Genosys. As a positive control for *H. pylori* primers, DNA extracted from pure *H. pylori* culture was amplified with the same primer set. PCR products were detected by electrophoresis on a 1.5% agarose gel containing Ethidium Bromide (EtBr). Location of predicted products was confirmed by size comparison of band using 100 bp ladder (Supplementary Figure 1).

2.5.4.2 Sequencing of PCR amplified product

For the preparation of DNA for sequencing, about one tenth volume of 3 M sodium acetate pH 5.2 and double the volume of ice cold ethanol were added to the PCR amplified sample. It was incubated in ice for 30 min and later centrifuged at 13,000 rpm for 30 min at 4°C. The obtained pellet was washed in 70% ethanol. The pellet was dried and resuspended in minimum volume of sterile water and incubated for 37°C for 10 min. From the sample, 2 µl was taken for finding out the DNA concentration.

The sequencing reaction was set for 10 µl reaction containing 5 µl of DNA. The sequencing mix contained 1.0 µl of ABI 310 Big Dye sequencing mix, 3 picomoles of either sense or antisense primer of 16 S rRNA and 1.4 mM MgCl₂ and the rest was sterile water. Sequencing reactions were carried out in a PCR machine (iCycler, BioRad) for 25 cycles with the following reaction conditions: denaturation at 96°C for 30 s, annealing at 54°C for 1 min and extension at 60°C for 4 min. The ramping rate of the PCR machine was adjusted to 1°C/second during the sequencing PCR. Post reaction cleanup of the sequencing PCR products was done as per the manufacturer's instructions. 10 µl of the PCR product was mixed well with 10 µl of nuclease free water, 1 µl 0.25 M EDTA and 2 µl of 3 M sodium acetate of pH 5.7 and after the addition of ice cold ethanol, the mix was kept in room temperature for 15 min. The reaction mix was centrifuged at 13,000 rpm for 20 min at room temperature. The pellet was washed with 70% ethanol. Air dried pellets were resuspended in Template Suppressor Reagent (TSR), heat denatured at 95°C, loaded and analyzed on an ABI 310 automated DNA sequencer.

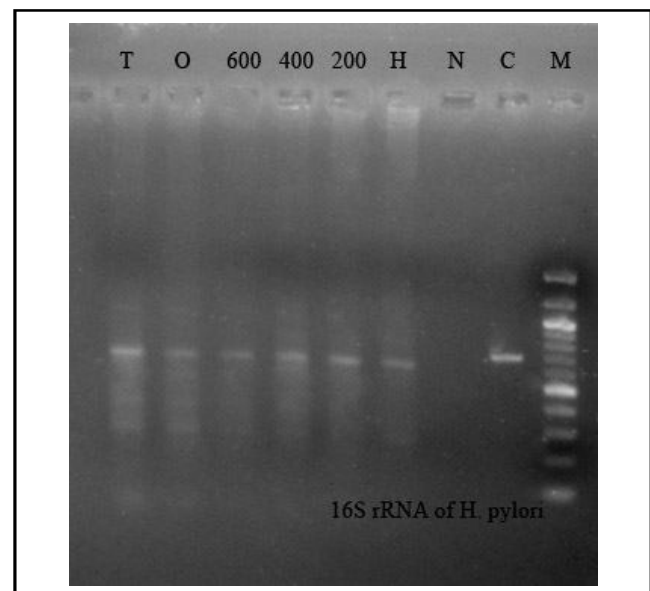


Figure 1: (Supplementary Figure 1) : Representative analysis of 16S rRNA expression of *H. pylori* in the gastric mucosa of different treatment groups. C-*H. pylori* culture DNA; N-normal gastric mucosa; H-*H. pylori* infected control mucosa; 200, 400 and 600 - *C. halicacabum* ethanol extract treated groups; O-omeprazole treated group; T-Triple therapy treatment group, M-Marker 100 bp ladder.

2.5.4.3 MIC measurements

The minimum inhibitory concentration (MIC) was detected by agar dilution method (Dixon, 1994). 1 ml of *C. halicacabum* ethanol extract in sterile water (possibly less amounts of DMSO were used for an acceptable intermiscibility) was separately added into petri dishes containing 8 ml of unsolidified brain heart infusion agar base supplemented with 10% FBS. Final concentrations of the ethanol extract in the medium were set to be 20, 15, 10, 5 mg/ml with DMSO concentration lower than 1%. *H. pylori* cultures in BHI broth at approximately 5×10^7 CFU/ml (0.1 ml) was inoculated into the surface of the sample-supplemented agar plates, followed by incubation at 37°C for 3 days in microaerophilic conditions in a bacterial incubator. MIC was defined as the lowest concentration at which no microbial growth could be detectable. Clarithromycin was co assayed as a positive reference at a concentration of 20 µg/ml

2.6 Statistical analysis

The results are expressed as Mean \pm SD. The total variation and difference among means were analyzed through one-way-analysis of variance (ANOVA), followed by *Tukey post-hoc* analysis and *p*-values less than or equal to 0.05 were considered significant.

3. Results

3.1 Effect of *C.halicacabum* extract (CHE) against indomethacin induced gastric ulcer

3.1.1 Effect of CHE extract on ALP and GSH activity

Administration of indomethacin resulted in gastric lesions, increase in alkaline phosphatase activity and reduction in gastric glutathione levels (Figures 1A, 1B and 1C). The ethanol extract of *C. halicacabum*, when administered 30 min prior to indomethacin, brought about appreciable changes. The extract significantly reduced the ulcer index and ALP activity when compared to untreated indomethacin control. The reference drug omeprazole was found to be better than the CHE extract in reducing the ALP activity. Indomethacin administration reduced GSH in gastric mucosa compared to normal rats but in the extract treated groups, the level of GSH increased distinguishably in gastric mucosa in a dose dependant manner (1C).

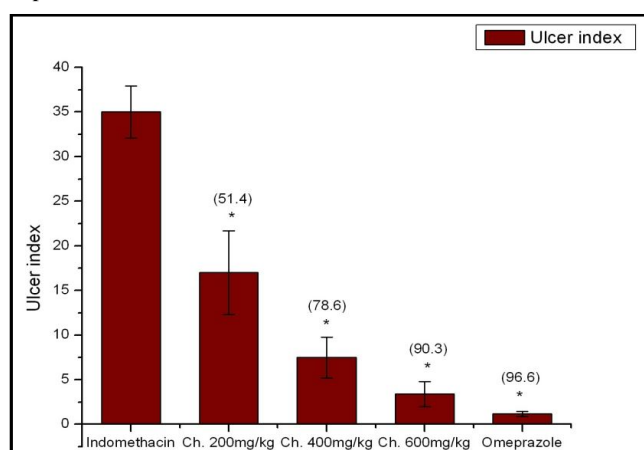


Figure 1(a): Effect of ethanol extract of *C. halicacabum* on gastric ulcer induced by indomethacin in rats. Values are expressed as Mean \pm S.D., n = 6. Values in parantheses represent percent protection. * $p \leq 0.05$ (compared to indomethacin treated control); Ch-*C. halicacabum* ethanol extract.

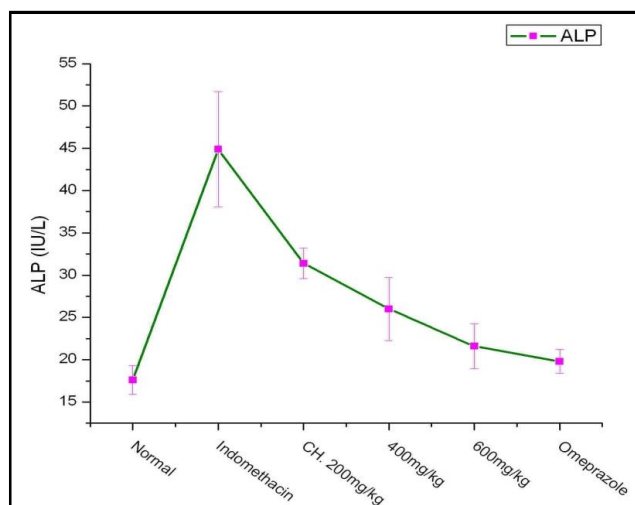


Figure 1(b): Effect of ethanol extract of *C. halicacabum* on ALP activity in gastric mucosa of rats in indomethacin induced gastric ulcer. Values are expressed as Mean \pm S.D., n = 6. Ch-*C. halicacabum* ethanol extract.

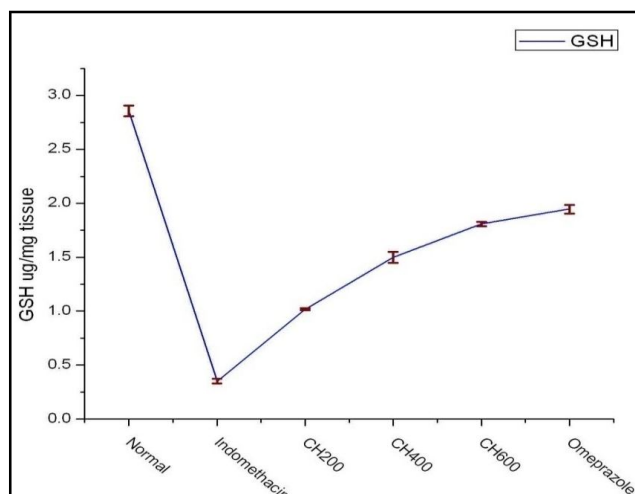


Figure 1(c): Effect of ethanol extract of *C. halicacabum* on GSH level in gastric mucosa of rats in indomethacin induced gastric ulcer. Values are expressed as Mean \pm S.D., n = 6. Ch-*C. halicacabum* ethanol extract.

3.1.2 Histopathology of the gastric mucosa

Histopathological studies further confirmed that pretreatment with the extract inhibited indomethacin induced ulcer, congestion, oedema, hemorrhage and necrosis in gastric mucosa. In reducing congestion and hemorrhage, the extract's efficacy was comparable to that of omeprazole, whereas this standard drug was found to be better than the extract in normalizing oedema and necrosis (Figures 2 A-F).

3.2 Effect of the CHE extract against pylorus ligation induced gastric ulcer

3.2.1 Effect on gastric acid secretion and pH of the gastric juice

C. halicacabum ethanol extract reduced gastric acid secretion in pylorus ligated rats. In CHE extract treated animals, the total acidity in gastric juice was significantly reduced as compared to the normal untreated rats. The pH of the gastric juice in normal animals was

3.10 ± 0.16 whereas in extract treated animals pH was 6.33 ± 0.02 and was comparable with that of omeprazole control (6.66 ± 0.06) (Table 1).

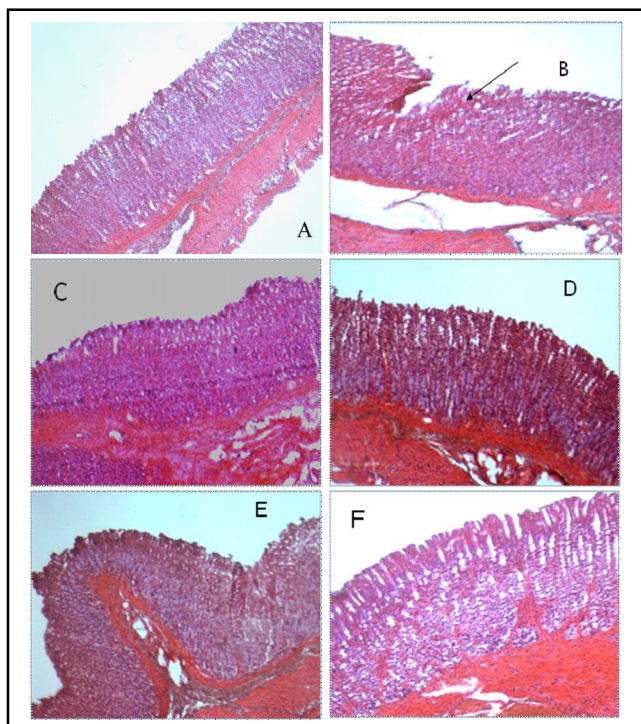


Figure 2: Microscopic observation of gastric mucosa of indomethacin treated rats stained with hematoxylin and eosin (200X) (A) Normal control. (B) Indomethacin control. Note the lesion in the mucosal Layer. (C) *C. halicacabum* ethanol extract (200 mg/kg). (D) *C. halicacabum* ethanol extract (400 mg/kg). (E) *C. halicacabum* ethanol extract (600 mg/kg). (F) Omeprazole (20 mg/kg).

Table 1: Effect of *C. halicacabum* (mg/kg. po.) on gastric juice pH and total acidity in pylorus ligated rats

Type	pH	Total acidity (meq/l)
Normal	3.10 ± 0.16	36.27 ± 0.43
<i>C. halicacabum</i> extract (200 mg/kg)	$5.65 \pm 0.09^*$	$25.25 \pm 0.58^*$
<i>C. halicacabum</i> extract (400 mg/kg)	$6.11 \pm 0.07^*$	$17.66 \pm 0.42^*$
<i>C. halicacabum</i> extract (600 mg/kg)	$6.33 \pm 0.02^*$	$13.66 \pm 0.33^*$
Omeprazole (20 mg/kg)	$6.66 \pm 0.06^*$	$12.26 \pm 0.14^*$

Values are expressed as Mean \pm S.D., n = 6 in each group

* $p \leq 0.05$ (compared to normal control).

3.3 Effect of plant extract on inhibiting the growth of *H. pylori*

3.3.1 Colonization of *H. pylori* in the antral mucosa

H. pylori was detected in the antrum of *H. pylori* inoculated rats but was undetectable by bacterial culture in uninoculated rats, i.e.,

vehicle treated rats. Viable colonies were found more in *H. pylori* infected group without any treatment. The number of viable colonies was remarkably reduced in extract treated and positive control groups. At 600 mg/kg level of the extract, the antibacterial effect was comparable to that of omeprazole (20 mg/kg). Least number of viable colonies was found in triple therapy (Figure 3A-D). Colonization levels of *H. pylori* in the antral gastric mucosa were quantified (Table 2).

Table 2: Quantification of viable *H. pylori* $p \leq 0.05$ (compared to *H. pylori* control group)

Type	Number of Colonies (CFU/mg mucosa)
Normal control	0
<i>H. pylori</i> control	1050 ± 25
Ch. 200 mg/kg	$410 \pm 10^*$
Ch. 400 mg/kg	$54 \pm 50^*$
Ch. 600 mg/kg	$35 \pm 12^*$
Omeprazole (20 mg/kg)	$40 \pm 7^*$
Triple therapy	$5 \pm 3^*$

Colonization levels of *H. pylori* in the antral gastric mucosa. Ch-*C. halicacabum* ethanol extract; data represented as Mean \pm SD. for 6 animals. * $p \leq 0.05$ (compared to *H. pylori* control group).

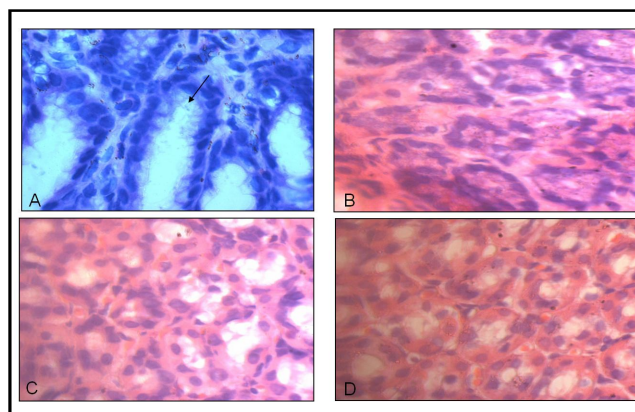


Figure 3: A, Histological appearance the *H. pylori* infected rat gastric mucosa (400X, Geimsa stain). Note the *H. pylori* organism present in the gastric mucosa. B, *H. pylori* infected control. C, Normal rat gastric mucosa. D, *H. pylori* infected gastric mucosa with triple therapy

3.3.2 Effect on MPO and PGE₂ levels in gastric mucosa

In the normal control group, the MPO activity in the gastric mucosa was negligible ($0.170 \pm 0.006 \mu\text{mol H}_2\text{O}_2/\text{min/g tissue}$). In *H. pylori* infected groups, the MPO level was significantly raised ($0.316 \pm 0.012 \mu\text{mol H}_2\text{O}_2/\text{min/g tissue}$) compared with that in the normal rat gastric mucosa. In extract treated gastric mucosa, the MPO activity was significantly higher than that of the normal groups ($0.24 \pm 0.014 \mu\text{mol H}_2\text{O}_2/\text{min/g tissue}$) but reduced when compared with *H. pylori* infected group. Similar was the case of omeprazole and triple therapy groups ($0.227 \pm 0.003 \mu\text{mol H}_2\text{O}_2/\text{min/g tissue}$ and $0.200 \pm 0.008 \mu\text{mol H}_2\text{O}_2/\text{min/g tissue}$). In both the cases, the values were not significant when compared with the normal rat gastric mucosa (Figure 4).

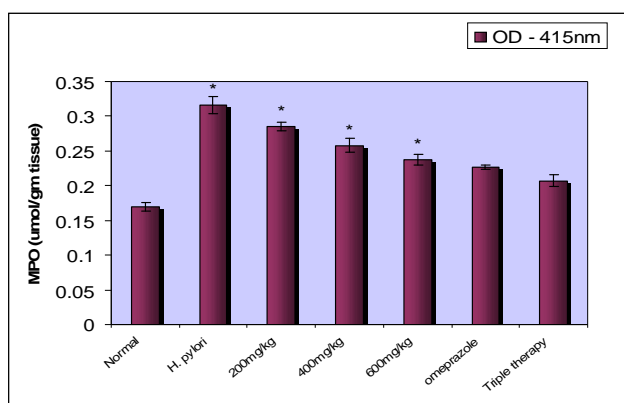


Figure 4: Myeloperoxidase activity in normal and *H. pylori* infected gastric mucosa of various treatment groups. Data are presented as Mean \pm SE for 6 animals. * $p \leq 0.05$ (compared to normal control group). 200, 400, 600 mg/kg- *C. halicacabum* ethanol extract treated groups.

In the normal gastric mucosa, PGE_2 was 15.89 ± 1.39 (pg/ml) and this was remarkably increased in *H. pylori* infected gastric mucosa (41.43 ± 1.53). Triple therapy and omeprazole treated rat mucosa showed significant reduction (21.56 ± 0.84) when compared with *H. pylori* control group. The extract treated group also showed increase in PGE_2 levels, compared to normal control groups and PGE_2 levels in the extract treated groups were not significantly different from those in the *H. pylori* control group (Figure 5).

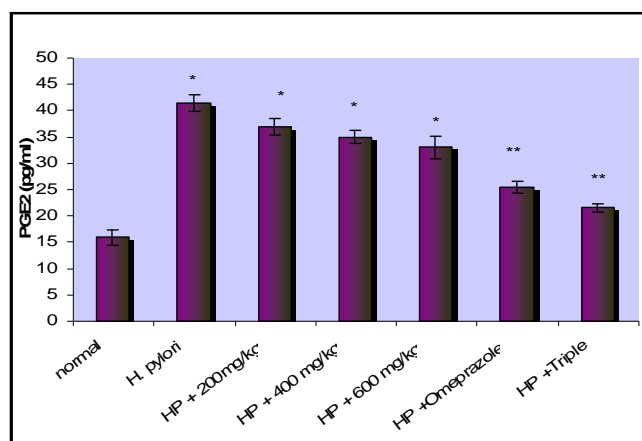


Figure 5: Amount of prostaglandin E_2 in the gastric mucosa of normal rat and different *H. pylori* treatment groups of rat. Data represented as Mean \pm SE; n=6 animals. * $p \leq 0.05$ (compared to normal control group) ** $p \leq 0.05$ (compared to *H. pylori* only group). HP- *H. pylori*; 200, 400, 600 mg/kg- *C. halicacabum* ethanol extract

3.3.4 Histopathology of gastric mucosa infected with *H. pylori* before and after the CHE extract treatment

Macroscopically, oedematous thickening of gastric mucosa was observed in *H. pylori* infected rats, especially in the fundus near antrum. None of the groups showed macroscopic lesions. Histologically, *H. pylori* were found in the gastric mucosa of the infected groups (Figure 6A). The infiltration of neutrophils and

mononuclear cells in the lamina propria and hyperplastic changes were observed in the infected animals (Figure 6 B). The extract treated groups did not show significant pathological changes of gastro-protection when compared with control animals.

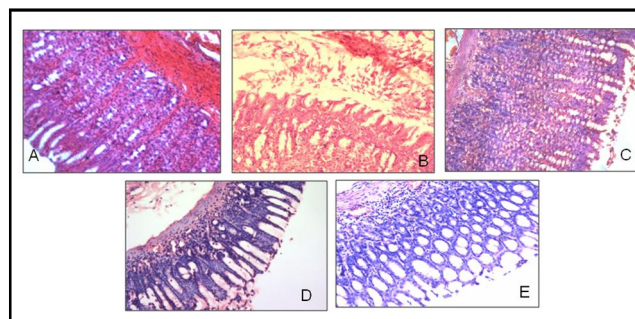


Figure 6: Histopathological observation of the gastric mucosa infected with *H. pylori* with different treatment groups (H and E staining; 400X) (A) Normal gastric mucosa, (B) *H. pylori* infected gastric mucosa showing neutrophil infiltration, (C) infected gastric mucosa treated with the extract at a dose of 400 mg/kg showing cytolysis, (D) infected mucosa treated with the extract at a dose of 600 mg/kg showing atrophic condition (E) infected mucosa treated with omeprazole showing neutrophil infiltration

3.3.5 In vitro anti-*H. pylori* activity of *C. halicacabum* ethanol extract

Even at the highest concentration of the extract tested (20 mg/ml), *H. pylori* colonies were found. Clarithromycin showed MIC at a concentration of 20 μ g/ml.

4. Discussion

In the previous century, there has been an upsurge in research on medicinal plants and their diverse beneficial effects against multitude of diseases. This deviation towards drug discovery from plant sources owe to their widespread availability, lesser side effects, traditional background of efficacy, etc. Plenty of reports from across the globe approve of the utilization of plant extracts against gastric ulcers. Through the study, it was established that *C. halicacabum* ethanol extract is able to block indomethacin and pylorus ligation induced gastric lesions in Wistar rats.

When gastric ulcer was produced by dosing with indomethacin, its reversal by *C. halicacabum* ethanol extract was assessed by analyzing GSH and ALP activity. Observation of histopathological changes in rat gastric mucosa affirmed the outcomes of these assays. The ALP activity is identified as a marker for neutrophil infiltration to the site of injury (Tengrup *et al.*, 1981). Treatment of rats with indomethacin led to a decrease in GSH in gastric mucosa compared to normal rats. But in the extract treated groups, the level of GSH increased distinguishably in gastric mucosa in a dose dependent manner. On the other hand, the levels of ALP were decidedly reduced in the mucosal tissue upon treatment with CHE extract. The reduction of ALP activity and increase of GSH activity by this extract suggests the basis of its antiulcerogenic activity.

Reactive oxygen species (ROS) has been proposed to be a significant aspect that worsens the mucosal damages caused by indomethacin and other agents (Elliot and Wallace, 1998). GSH (reduced glutathione) is a major antioxidant that is found to be reduced in

gastric tissues treated with different ulcerating agents (Boyd *et al.*, 1981). Elevated lipid peroxide levels induced by these agents, in turn causes increased consumption of GSH thus leading to its depletion in the ulcerated tissues (Banerjee *et al.*, 1994, Krishnakumar *et al.*, 2014). In aerobic organisms, high doses of reactive oxygen species can result in oxidative stress, against which several enzymatic and non-enzymatic anti-oxidant defense mechanisms exist. These defenses include superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), ascorbic acid (vitamin C), α -tocopherol (vitamin E), glutathione (GSH), β -carotene, and vitamin A (Mates *et al.*, 1999). Previous reports have already established that NSAIDs, ethanol and pylorus ligation causes a decline in the levels of GSH, SOD and GPx in damaged tissues (Takeuchi *et al.*, 1997; El-Missiry *et al.*, 2001; Bafna *et al.*, 2004). Treatment with the CHE extract resulted in increased level of total tissue sulfhydryl (thiol group) compared to the untreated ulcerated rats. The antioxidant activity of the herbal extract would have prevented excessive peroxidation. Thus, it is evident that this extract can be attributed to have antioxidant nature which neutralizes the oxidative damage generated by the necrotizing agents (Sheeba and Asha, 2006).

From the pylorus ligation studies, it was observed that *C. halicacabum* ethanol extract produced a significant decrease in gastric acid secretion. Treatment with the CHE extract brought the gastric juice pH to near normal and caused a decline in total acidity. The exact mechanism by which the extract inhibits gastric acid secretion is unknown. Exposure to irritating agents (Robert *et al.*, 1983) increased PG production by the gastric mucosa as a consequence of a reduction in gastric lumen pH (Aly *et al.*, 1985).

The major classes of compounds in the extract were saponins, flavonoids, cardiac glycosides, alkaloids, phenols, tannins and these compounds were previously reported to have gastroprotective activity (Zayachkivska *et al.*, 2005). It is likely that some of these principles may be responsible for the reduction in acid secretion.

Gastric lesions arising due to administration of NSAIDs and infection with *H. pylori* can be associated with the inhibition of prostaglandin (PG) synthesis (Tegeeder *et al.*, 2000). This can be rooted to the milestone discovery in inflammatory research by Vane (1971) and coworkers that the analgesic, antipyretic and anti-inflammatory properties of acetylsalicylates were based on the inhibition of PG synthesis. NSAIDs inhibit the activity of cyclooxygenases (COX), the key enzyme in PG production (Herschman, 1996). Being found in the gastric mucosa at a high concentration, prostaglandins exert a variety of roles including stimulation of bicarbonate and mucus secretion, maintenance of mucosal blood flow, inhibition of acid secretion and regulation of mucosal cell turnover and repair (Hayllar and Jarnason, 1995). Endogenous prostaglandin E₂ plays an important role in the healing process of gastric ulcers (Wang *et al.*, 1989). The suppression of PG synthesis by NSAIDs such as indomethacin results in increased susceptibility to mucosal injury and gastroduodenal ulceration (Atay *et al.*, 2000). As inhibition of prostaglandin synthesis alone cannot be the sole mechanism for gastric ulceration by NSAIDs, neutrophils and neutrophil derived factors have also been shown to induce gastrointestinal damage (Wallace *et al.*, 1990). It is well known that treatment with conventional NSAIDs causes a delay in the healing of gastric ulcers; this delay is associated with the blockage of PG production in ulcerated tissues in rats and humans (Szelenyi *et al.*, 1982; Wang *et al.*, 1989; Levi *et al.*, 1990; Lancaster *et al.*, 1991).

H. pylori showed resistance against *C. halicacabum* ethanol extract under *in vitro* conditions. However, in the *in vivo* studies, the viable colonies of *H. pylori* in the gastric mucosa were reduced in

the extract treated groups. A significant and marked reduction in viable colonies was observed at the highest dose (600 mg/kg) of the extract treated animals. Thus, the number of viable bacteria was reduced by the extract under *in vivo* conditions. The exact mechanism for the same has to be explored further.

H. pylori induced gastritis is histologically characterized by infiltration of inflammatory cells including neutrophils into gastric mucosa. Gastric mucosal PGE₂ production is higher in *H. pylori* patients than in *H. pylori* negative patients (Jackson *et al.*, 2000; Hudson *et al.*, 1993). Increased expression of growth factors contributes to increased COX-2 expression in *H. pylori* infected mucosa, resulting in the increased PG synthesis. In this study, extract treatment did not show any significant alteration in the level of PGE₂.

In conclusion, to study the effectiveness of ethanol extract of *C. halicacabum* against gastric ulcer induced by various factors such as indomethacin, pyloric ligation and *H. pylori* infection, the animals were experimentally induced with ulcer by different necrotizing agents. Upon treatment with the plant extract, it could successfully reverse gastric ulcer induced by indomethacin and pylorus ligation. The CHE extract, the ALP activity while increasing GSH activity in a remarkable degree. It is of interest to note that unlike other anti-inflammatory and analgesic agents such as aspirin and indomethacin, *C. halicacabum* possesses gastroprotective activity also. This effect might be due to a relation between mucosal injuries, inhibition of acid secretion and previously observed antioxidant and anti-inflammatory effect. The same authors have previously established the antioxidant, anti-inflammatory and antisecretory activities of the plant. The extract also has inhibitory effect on NF- κ B activation (Sheeba and Asha, 2009). Therefore, further studies are required to unravel the effect of the extract on *H. pylori* infected rat gastric mucosa in the perspective of NF- κ B activation and NF- κ B dependent COX-2 expression.

Our studies have undoubtedly established the efficacy of *C. halicacabum* against gastric ulcers, arising from exposure to various necrotizing agents. Evaluation of the potential of *C. halicacabum* ethanol extract demonstrated that it has maximum antiulcerogenic activity against ulcers induced by indomethacin and pylorus ligation. More studies are in the pipeline to identify the specific chemical entities present in *C. halicacabum* that confers to it the gastroprotective capability.

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Conflict of interest

We declare that we have no conflict of interest.

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