Antioxidant and Antiulcer Potential of *Hydrolea Zeylanica* (L.) Vahl against Gastric Ulcers in Rats

**Abstract**

*Hydrolea zeylanica* (L.) Vahl (Hydroleaceae) an aquatic, edible medicinal plant is useful in healing ulcer. Objective was to establish antioxidant principles in antiulcer activities of *H. zeylanica* in ulcer induced in rats. In order to explain the role of antioxidant principles in antiulcerogenic activities, *in vitro* 1, 1-diphenyl-2-picryl hydrazyl (DPPH), nitric oxide (NO), superoxide anion (SOD) and hydroxyl (OH) free radical scavenging activities of successive solvent extracted fractions of leaves were performed. Antikeratolytic activities of ethyl acetate fractions of *H. zeylanica* (HZEA, 300 and 400 mg/kg) were evaluated by performing diclofenac sodium and pylorus ligation induced ulcer in rats. Gastric protective effects of HZEA were determined by assessing gastric volume, gastric pH, free acidity, total acidity, ulcer index and % protection. Oxidative stress markers as MDA, GSH, CAT of gastric mucosa were evaluated. Histological studies were performed on stomach tissues. Antioxidative activities of HZEA were better than other fractions with IC$_{50}$ of DPPH, SOD, NO and OH were 41.2±3.21, 59.71±1.45, 73.82±1.89, 48.91±0.89 µg/mL respectively. Total phenolic and flavonoid contents of HZEA were 28.6±3.9.16 mg GAeq/g and 208.2±4.55 mg Queeq/g. Gastric volume, free and total acidity, ulcerative index were significantly (p<0.01) reduced whereas gastric-pH and % protection were significant increased (p<0.01) in both models. There was a significant (p<0.01) reduction observed in MDA and increased in GSH, gastric-pH, free and total acidity, ulcerative index were significantly (p<0.01) reduced whereas gastric pH and % protection were significant increased (p<0.01) in both models. Those findings revealed that *H. zeylanica* can be used as a natural alternative therapy to treat gastric ulcer. Objective was to establish antioxidant principles in antiulcer activities of *H. zeylanica* in ulcer induced in rats. In order to explain the role of antioxidant principles in antiulcerogenic activities, *in vitro* 1, 1-diphenyl-2-picryl hydrazyl (DPPH), nitric oxide (NO), superoxide anion (SOD) and hydroxyl (OH) free radical scavenging activities of successive solvent extracted fractions of leaves were performed. Antikeratolytic activities of ethyl acetate fractions of *H. zeylanica* (HZEA, 300 and 400 mg/kg) were evaluated by performing diclofenac sodium and pylorus ligation induced ulcer in rats. Gastric protective effects of HZEA were determined by assessing gastric volume, gastric pH, free acidity, total acidity, ulcer index and % protection. Oxidative stress markers as MDA, GSH, CAT of gastric mucosa were evaluated. Histological studies were performed on stomach tissues. Antioxidative activities of HZEA were better than other fractions with IC$_{50}$ of DPPH, SOD, NO and OH were 41.2±3.21, 59.71±1.45, 73.82±1.89, 48.91±0.89 µg/mL respectively. Total phenolic and flavonoid contents of HZEA were 28.6±3.9.16 mg GAeq/g and 208.2±4.55 mg Queeq/g. Gastric volume, free and total acidity, ulcerative index were significantly (p<0.01) reduced whereas gastric-pH and % protection were significant increased (p<0.01) in both models. There was a significant (p<0.01) reduction observed in MDA and increased in GSH, gastric-pH, free and total acidity, ulcerative index were significantly (p<0.01) reduced whereas gastric pH and % protection were significant increased (p<0.01) in both models. Those findings revealed that *H. zeylanica* can be used as a natural alternative therapy to treat gastric ulcer.

**Keywords:** *Hydrolea zeylanica*; Antioxidant; Antiulcer; Diclofenac sodium; pyloric ligation

**Abbreviations:** DPPH: Diphenyl-2-Picryl Hydrazyl; NO: Nitric Oxide; SOD: Superoxide Anion; OH: Hydroxyl; NSAIDs: Non-Steroidal Anti-Inflammatory Drugs; ROS: Reactive Oxygen Species; TBA: Thiobarbituric Acid; PMS: Phenazine Methosulphate; TPC: Total Phenolic Contents; TFC: Total Flavonoid Contents; GAE: Gallic Acid Equivalents; TPC: Total Phenolic Contents; SOD: Superoxide Anion Scavenger; NIN: National Institute of Nutrition

**Introduction**

Gastric ulcer is a commonest form of gastro-intestinal disorder, posed a major threat to the world’s population with a high rate of morbidity and mortality [1]. The etiopathogenesis of this acid-driven disease is associated with self medication or excessive administration of non-steroidal anti-inflammatory drugs (NSAIDs) like diclofenac sodium and aspirin, smoking, excessive alcohol use, emotional stress and psychosocial factors etc [2]. The management of acid-driven disease remains a challenge in modern world because of change in life style and culture. The pathogenesis of this disease appears because of the system in imbalance stage between defensive factors (mucous-bicarbonate layer, prostaglandins, cellular regeneration, and mucosal blood flow) and aggravating factors (hydrochloic acid, pepsin, ethanol, bile salts, drugs) [3]. Most precipitating to this disorder is the drug abuse and chronic administration of NSAIDs plays a major role in the pathogenesis of gastric ulcer. It is reported that 35-60% of patients suffer gastric ulcer due to the chronic administration of diclofenac and other NSAIDs [4,5]. The prime cause of NSAIDs induced gastric ulcer is the generation of reactive oxygen species (ROS) which play an important role in the etiology and pathophysiology of gastric ulcer [6]. Generated ROS cause internal tissue damage and leads to cell destruction which may lead to chronic hyperacidity and severe ulceration [7]. For this reason, diclofenac sodium [Sodium (2-[(2,6-dichlorophenyl) amino]phenyln)acetate] has been widely used in gastric ulcer models as a referral inducer while evaluating the gastroprotective activities of other therapeutic agents.

More and more herbal and synthetic drugs are coming up offering newer dimension and better options for the treatment of ulcer. Based on the mechanism of action, synthetic drugs are classified as proton-pump inhibitors, prostaglandins analogs, histamine receptor antagonists and cytoprotective agents but on other side, these synthetic agents produce several side effects like arrhythmias, impotence, gynaecomastia, hyperplasia and haemopoietic changes etc [8]. For this reason, world is reversing back to use the traditional medicinal because natural products are the safe in use with better efficacy in treating ulcer. Other advantages of natural products are economical and widely available. For this reason, investigations continue with an objective to explore antioxidant principles of natural products as an alternative therapy in gastritis and based on antioxidant principles, natural remedies are now days have taken a special place in filling the research gap created by synthetic drugs [9,10]. Therefore, there is a need for *Hydrolea zeylanica* to be evaluated as a natural alternative therapy to treat gastric ulcer.
Hydrolea zeylanica (L.) Vahl (Hydroleaceae); commonly known as “Blue water leaf” or “Water olive” found in Indian subcontinent, China, Myanmar, Thailand, Malaysia, Indonesia, Philippines, and Australia. In some part of India, people are used aerial parts of leaves as vegetables. But ethnomedically these leaves are used in the treatments of diabetes wound healing, antiseptic and healing ulcers [11-17]. Since there is no scientific investigation to define the gastroprotective activity of H. zeylanica, the present study was aimed at investigating the antiulcer activity of the ethyl acetate fraction of the leaves of H. zeylanica against various experimental models, namely diclofenac sodium and pyroliglic induced ulcer by using ranitidine as a reference drug. In addition, the mechanism by which H. zeylanica exerts its efficacy is elucidated in terms of oxidative stress measures by performing in vitro and in vivo assays.

Materials and Methods

Chemicals and reagents

Diethyl-1-picyrylhydrazyl (DPPH), Folin-Ciocalteu reagent, thiobarbituric acid (TBA), ascorbic acid, nitro blue tetrazolium (NBT), trichloroacetic acid (TCA), NADH (DPNH), peroxidemethosulphate (PMS), sodium nitroprusside, EDTA, sodium carbonate, sodium nitrate, sodium hydrosyde, quercetin, trichloroacetic acid (TCA), gallic acid, sodium nitroprusside were purchased from Himedia, India. Phenolphthalein (Qualigen Chemicals, India), rutidine (Torrent pharmaceuticals, Ahmadabad, India), diclofenac sodium (Trolka Pharmaceuticals, Ahmadabad, India), Absolute alcohol (Merck, India), Topfer’s Reagent (Merck, India), Tween 80, Sodium hydrosyde, TBARS, GSH and CAT assay kits (Sigma Aldrich, USA) and all solvents used for extraction were of analytical grade and purchased from Sisco Research Laboratories, India.

Plant materials collection

Leaves of Hydrolea zeylanica (L.) Vahl (Hydroleaceae), were collected during the month of October and November, 2015 from the wetland of Pipilli and its adjacent areas of Puri District, Odisha, India (Latitude: 20°12’23.62”N; Longitude: 85°53’40.72”E; Altitude: 51 ft). The plant was identified and the voucher specimen was deposited at our Centre for future references (No. 7727/RPRC).

Extraction and phytochemical analysis

Leaves of H. zeylanica were shade dried and ground to powder. Before to follow the successive extraction, leaf materials were defatted and removal of gums by treating the materials with petroleum ether. Then successive extractions of leaf materials were performed by using hexane, chloroform, ethyl acetate and methanol solvents. Each fraction was evaporated using rotary evaporator (R100, Buchi, Switzerland). The yield of ethyl acetate fraction was 8.84 and 9.31 (w/w), respectively. Qualitative phytochemical test were performed for all fractions to assess the presence of various secondary metabolites [18].

Total phenolic contents (TPC)

Total phenolic contents (TPC) of different fractions were determined by using Folin-Ciocalteu reagent [19]. About 10µL of 1mg/mL different fractions of H. zeylanica, 450µL of distilled water and 2.5mL of 0.2 N Folin-Ciocalteu reagents were added. After 5min, 2mL of 10% sodium carbonate was added and incubated at 37 °C for 30 min. Then the absorbance was recorded at 765 nm by multimode micro plate reader (Synergy H1MF, BioTek, USA). Gallic acid was considered as internal standard and TPC was expressed as mg/g gallic acid equivalents (GAE) per gram of dried fraction of H. zeylanica (mg GAEq/g).

Total flavonoid contents (TFC)

Total flavonoid contents (TFC) of different fractions of H. zeylanica HGR, CGR, EGR and MGR were performed according to standard protocol [19]. About 500µL of 1mg/mL different fractions of H. zeylanica were mixed with 1.5mL ethanol (95%), 0.1mL of 10% aluminium chloride hexahydrate, 0.1mL of 1 M potassium acetate and 2.8mL of deionized water. The reaction was kept for incubation at room temperature for 30 min, and after that the absorbance of the reaction mixture was recorded at 415 nm by multimode micro plate reader (Synergy H1MF, BioTok, USA). Quercetin was used as reference drug and results obtained were expressed as mg/g Quercetin equivalents per gram of dried fraction of H. zeylanica (mg Eqv/g).

DPPH free radical scavenging activity

The ability of H. zeylanica fractions to scavenge 1, 1-diphenyl-1-picyrylhydrazyl (DPPH) radicals was determined by following a standard method with minor modification to it [20]. About 2mL of DPPH solution (5.9mg in 100mL methanol) was added to 1mL of different concentrations of HZH, HZC, HZEA and HZM. The absorbance of the solution was measured at 517 nm after a 30 min incubation time by using a multimode micro plate reader (Synergy H1MF, BioTok, USA). Ascorbic acid was considered as a reference drug. % inhibition was calculated by the following equation: Inhibition (%) = (A_control−A_sample/A_control) X 100

Superoxide anion radical scavenging assay

Superoxide anion scavenger (SOD) assay was carried out according to the standard procedure with a minor modification [21]. The reaction mixture consisting of 1mg/mL of 1mL sample, 1mL 60µm phenazine methosulphate (PMS) in phosphate buffer (0.1 M, pH 7.4) and 150µm 1mL nitroblue tetrazolium (NBT) in phosphate buffer. The reaction mixture was incubated at ambient temperature for 5 min and the resultant colour was read spectrophotometrically at 560 nm against a blank by using a multimode micro plate reader (Synergy H1MF, BioTok, USA). Quercetin was taken as a reference drug. % inhibition was calculated by the following equation: Inhibition (%) = (A_control−A_sample/A_control) X 100

Nitric oxide radical scavenging assay

Nitric oxide (NO) radical scavenging was performed with a slight modification to the protocol [22]. Different concentrations of HZH, HZC, HZEA and HZM (10-100µg/mL) were prepared and treated with 3mL of 10mM sodium nitroprusside in 0.2 M phosphate buffered saline (pH 7.4) and allowed for incubation at room temperature for 150 min. After the incubation time was over, 0.5mL of Griess reagent was added and the absorbance of the reaction mixture was read at 546 nm by using a multimode
micro plate reader (Synergy H1MF, BioTek, USA). % of radical scavenging activity was calculated as follows.

\[
\text{Inhibition (\%) } = \left( \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \right) \times 100
\]

**Hydroxyl radical scavenging activity**

Standard protocol was followed to carryout hydroxyl radical scavenging activity [23]. The prepared fraction/solution of different concentration from the stock (1mg/mL) were added with 1mL of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5mL of EDTA solution (0.018%), and 1mL of dimethyl sulfoxide (DMSO) (0.85% v/v, in 0.1 M phosphate buffer, pH 7.4). In order to initiate the reaction, 0.5mL of ascorbic acid (0.22%) was added and kept for incubation at 80-90 °C for 15 min. After the incubation time was over, 1mL of ice-cold TCA (17.5% w/v) was added so as to stop the reaction. Then, Nash reagent of about 3mL was added and left at room temperature for 15 min. The reaction mixture was read at 412 nm against reagent blank to find out the intensity of the colour formation by using a multimode micro plate reader (Synergy H1MF, BioTek, USA). % of hydroxyl radical scavenging activity was calculated as follows.

\[
\text{Inhibition (\%) } = \left( \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \right) \times 100
\]

**Acute toxicity study and dose fixation**

A safe dose of the fraction was determined by acute oral toxic method of Organization of Economic Co-Operation and Development (OECD) as per 423 guidelines [24]. Based on the higher antioxidant potential, HZEA was selected for the animal study. HZEA up to 4000 mg/kg p.o route was found to be safe. Based upon toxicity study, two doses of EAHZ at 300mg/kg and 400 mg/kg b.w. were selected for in vivo antiulcer studies.

**Animals**

54 healthy Wistar albino rats of either sex weighing 150-180 g were obtained from the National Institute of Nutrition (NIN), Hyderabad and were kept in the laboratory under complete light and dark cycle for about 10 days to acclimatize. Throughout the experiment animals were fed with standard laboratory chow diet and water ad libitum. All animals were placed individually in cages with wide mesh wire bottoms to prevent coprophagy. The rats were deprived of food but had ad libitum access to tap water for 24h before ulcer induction. The study was approved by Institutional Animal Ethical Committee of Regional Plant Resource Centre vide Regd. No.1807/GO/Re/S/2015/CPCSEA.

**Determination of pH**

An aliquot of 1mL gastric juice was diluted with 1mL of distilled water and pH of the solution was measured using pH meter [28].

**Determination of total acidity**

An aliquot of 1mL of gastric juice was diluted with 1mL of distilled water was taken into a 50mL conical flask and two drops of phenolphthalein indicator was added to it and titrated with 0.01 N NaOH until a permanent pink color was observed. The volume of NaOH consumed was noted [29]. Total acidity was expressed as mEq/L/100 gm by the following formula:

\[
\text{Acidity} = \frac{\text{Vol of NaOH} \times \text{Normality of NaOH} \times 100 \text{ mEq/L/100 gm}}{1000} \times 0.1^{-1}
\]

**Determination of free acidity**

An aliquot of 1mL of gastric juice was diluted with 1mL of distilled water was taken into a 50mL conical flask and two drops of Topfer’s reagent was added and was titrated with 0.01 N NaOH until a permanent pink color was observed. The volume of NaOH consumed was noted [29]. Total acidity was expressed as mEq/L/100 gm by the following formula:

\[
\text{Acidity} = \frac{\text{Vol of NaOH} \times \text{Normality of NaOH} \times 100 \text{ mEq/L/100 gm}}{1000} \times 0.1^{-1}
\]

**Diclofenac sodium induced ulcer**

Wistar rats were divided in to five groups (6 animals in each group). Group-I (Normal control): 0.1% tween in distilled water; Group-II (Ucerated control): Diclofenac sodium (20mg/kg); Group-III: Ranitidine (13.5mg/kg); Group-IV: HZEA (300mg/kg); Group-V: HZEA (400mg/kg). The oral feeding of ranitidine and HZEA were continued for 3 days after 1 h of diclofenac sodium administration. On 4th day animals were sacrificed, stomach was removed and cut along the greater curvature to measure the volume of gastric juice, pH, free acidity, total acidity and ulcer index. Gastric juice was collected into the centrifuge tube and was centrifuged at 1000rpm/min for 10 min and the volume was recorded [25]. From the supernatant, aliquots (1mL from each animal) were taken for the determination of pH, total and free acidity by titration against 0.01 N NaOH. Each stomach was examined for lesions in the mucosal layer of the stomach portion and ulcer index was calculated [26]. Oxidative parameters as MDA, GSH and CAT were performed according to the standard protocol as mentioned.

**Pyloric ligation method**

Wistar rats were divided into five groups, consisting of six rats each. Animals were fasted for 48h before the study, but had free access to water. Group-I (Normal control): 0.1% tween in distilled water; Group-II (Ulcerated control): Single dose of diclofenac sodium (20mg/kg); Group-III: Ranitidine (13.5mg/kg); Group-IV: HZEA (300mg/kg); Group-V: HZEA (400mg/kg). Pyloric ligation was done by ligating the pyloric end of the stomach of rats 1 h after diclofenac sodium administration. Pyloric portion of the stomach was slightly lifted out and ligated by avoiding traction to the pylorus or damage to the internal blood supply. 19h later the ligated rats were sacrificed by decapitation and the abdomen was opened, content in the stomach were drained into a centrifuge tube and centrifuged at 2000 rpm, 10 min for assessing parameters like gastric pH, total acidity and free acidity. The gastric ulcers were counted and ulcer index was determined. Each stomach was examined for lesions in the fore stomach portion and indexed according to severity [27].

**Scoring of ulcer**

The number of ulcers was noted and the ulcer index was recorded with the following scores [28].

- 0= no ulcer; 1= superficial ulcers; 2= lesions; 3= deep ulcers; 4= perforation.

\[
\% \text{ Inhibition of ulceration} = \frac{\text{Ulcer index}_{\text{Control}} - \text{Ulcer index}_{\text{Test}}}{\text{Ulcer index}_{\text{Control}}} \times 100
\]
Evaluation of biochemical parameters of stomach tissues

The section of the stomach was washed in ice-cold saline, dried with blotting paper and weighed. The mucosa (100 mg) was collected and homogenized in Tris HCl buffer (0.1 M, pH 7.4) at 4°C. Then it was centrifuged at 12000 g for 30 minutes. The supernatant obtained was used for the analysis of different biochemical parameters as MDA and GSH.

a. Measurement of malondialdehyde (MDA): HZEA (300 and 400 mg/kg, p.o.) activity on lipid peroxidation was carried out by measuring MDA using thiorbarbituric acid test [30]. After washing the tissues with 0.9% NaCl, 1g of wet tissue was homogenized in M/150 phosphate buffer (pH 7.0) at 1-4°C and centrifuged at 5000 rpm. The reaction mixture contained 0.1mL of the sample, 0.2mL of 0.1% sodium dodecyl sulfate, 1.5mL of 20% acetic acid solution, and 1.5mL of 0.8% aqueous solution of thiorbarbituric acid (pH of 20% acetic acid solution was adjusted with NaOH to be above 3). The mixtures were finally adjusted to 4mL with distilled water and heated at 95°C for 60 min. After cooling with tap water, 1mL of distilled water and 5mL of the mixture of n-butanol and pyridine (15:1, v/v) were added and the mixtures were vigorously shaken. After centrifugation at 3000 rpm for 15 min, the absorbance of the organic layer (upper layer) was measured at 532 nm. The values were expressed as µM MDA/g tissue.

b. Measurement of reduced glutathione (GSH): The levels of GSH in gastric mucosa were measured as described using diclofenac sodium-ulcer model [31]. 5mL aliquots of homogenates were mixed in 15mL test tubes with 4mL distilled water and 1mL of 50% TCA. Tubes were intermittently shaken for 10-15 min, and then centrifuged for 15 min at 3000 rpm. 2mL of the filtrate or supernatant was mixed with 4mL of 0.4 M Tris buffer (pH 8.9), 0.1mL 5,5'-Dithio-Bis-(2-nitrobenzoic-acid) (DTNB) was added, and the samples were shaken. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a blank reagent without a homogenate. The results were expressed in µg GSH/g tissue.

c. Measurement of catalase (CAT): Catalase (CAT) activity was measured by modified method [32]. 0.1mL of supernatant was added to 1.9mL of 50mM phosphate buffer (pH 7). Reaction was started by addition of 1mL of freshly prepared 30mM H₂O₂. The rate decomposition of H₂O₂ was measured spectrophotometrically at 240nm. Activity of catalase was expressed as U/mg of protein.

Statistical analysis

Results were indicated in terms of mean±SEM. Statistical significance of data were assessed by analysis of variance (one-way ANOVA), followed by comparison between different groups using Dunnett’s multiple comparison test. The differences were considered significant when p < 0.05.

Result

Phytochemical analysis and biochemical estimation of H. zeylanica

Phytochemical tests were performed for different fractions of H. zeylanica but, the results obtained could inferred that HZEA contains substantial amount of polyphenols, flavonoids and tannins. Biochemical estimation like total phenolic (TPC) and Total flavonoid contents (TFC) of all fractions were investigated but HZEA found to contain the higher level of TPC, TFC i.e. 286.33±9.16 mg GAeqv/g and 208.24±5.56 mg Queqv/g respectively than other fractions.

In vitro antioxidant activities of H. zeylanica

In vitro test results of different fractions of H. zeylanica for their antioxidant activities were obtained (Table 1). In detail analysis of test results, it concludes that the HZEA fraction was more bioactive fraction than other fractions. In DPPH assay of H. zeylanica, a direct linear relationship was found between the concentrations of HZEA and HZM in the DPPH radical scavenging in a dose dependent manner. HZEA has demonstrated 50% inhibition (IC₅₀) at 41.2±3.21μg/mL followed by HZM at 73.89±1.31μg/mL and HZC (289.91±2.1 μg/mL). Whereas IC₅₀ reference drug ascorbic acid was recorded at 23.76±1.21μg/mL (Table 1).

Superoxide anion (SOD) scavenger activity by H. zeylanica was evaluated and amongst all fractions, IC₅₀ of HZEA was of 59.71±1.45μg/mL followed by HZM at 176.32±2.98μg/mL. Whereas, the reference drug quercetin IC₅₀ was 28.89±0.67μg/mL (Table 1).

HZEA showed good nitric oxide (NO) scavenging activity and % of inhibitions were increased with increasing in concentration of this fraction. IC₅₀ of HZEA was reported at 73.82±1.89μg/mL while IC₅₀ of ascorbic acid was of 34.2±0.54μg/mL (Table 1). HZEA exhibited a better scavenging activity than other fractions (Table 1). In hydroxyl (OH) radical scavenging activity study, IC₅₀ of test results of HZEA and reference drug quercetin were recorded at 49.81±0.89μg/mL and 26.09±0.56μg/mL respectively and found to be a better scavenger than HZH, HZC and HZM (Table 1).

In vitro antioxidant test results of HZH found to be a biological insignificant or less active fraction as 50% inhibitory concentrations (IC₅₀) were reported above 1000μg/mL and hence it was not considered further in other experimental studies.

Effect of HZEA in diclofenac sodium induced gastric ulcer

In diclofenac sodium induced ulcerated groups, different degrees of ulceration were observed by following certain parameters as gastric volume, free acidity and total acidity which were increased significantly (p < 0.01) in comparison to the normal group. However HZEA showed a significant (p<0.01) dose dependent reduction in gastric volume at 300 mg/kg (2.88±0.23) and 400 mg/kg (2.08±0.15) in comparison to the ulcerated group (3.83±0.24). HZEA at 400 mg/kg was found comparable with the standard treated group (1.90±0.07).
In the case of free acidity, HZEA at 300 and 400 mg/kg treated groups were significant (p< 0.01) dose dependent reduction 62.83±0.79 and 53.16±0.60, respectively in comparison to the ulcerated group (118.66±0.76). The result of HZEA at higher dose was comparable with the standard treated group (41.83±0.9). Whereas the total acidity of HZEA treated groups at 300 and 400 mg/kg showed significant (p<0.01) dose dependent reduction 70.33±0.66 and 57.50±0.76 respectively in comparison to the ulcerated group (128.76±0.63). The results were found comparable with ranitidine treated group (Table 2).

Table 1: Antioxidant activities and biochemical estimations of different fractions of leaves of Hydrolea zeylanica.

| Assays | Reference Drug (µg/mL) | HZC (µg/mL) | HZEA (µg/mL) | HZM (µg/mL) |
|--------|------------------------|-------------|--------------|-------------|
| DPPH  | 23.76±1.21             | 289.9±2.1   | 41.20±3.21   | 73.89±1.31  |
| SOD    | 28.89±0.67             | 323.2±1.89  | 59.71±1.45   | 176.32±2.98 |
| NO     | 34.2±0.54              | 245.7±1.76  | 73.82±1.89   | 197.45±1.61 |
| OH     | 26.09±0.56             | 271.7±2.21  | 46.91±0.89   | 138.87±1.09 |

*Reference drug ascorbic acid was considered for DPPH, nitric oxide (NO); whereas for superoxide anion (SOD), hydroxyl (OH) assays quercetin was considered as a reference drug to perform all assays. Total phenolic contents (TPC) and total flavonoid contents (TFC) were estimated by performing gallic acid equivalent (GAE) in GAeqv/g and quercetin equivalent (QE) in Queqv/g of different fractions of leaves of H. zeylanica. The hexane fraction of leaves of H. zeylanica (HZH) showed the negligible free radical scavenging activities and hence it was not considered in the comparative studies. HZC is the chloroform, HZEA is the ethyl acetate and HZM is the methanol fraction of H. zeylanica. Table 2: Antiulcer activity of ethyl acetate fraction of Hydrolea zeylanica against diclofenac sodium induced gastric ulcer.

| Groups          | Gastric Volume (mL) | pH of Gastric Juice | Free Acidity (mEq/L) | Total Acidity (mEq/L) | Mean Ulcer Index | % of Protection |
|-----------------|---------------------|---------------------|----------------------|-----------------------|------------------|-----------------|
| Normal control  | 1.28±0.16           | 4.4±0.40            | 35.66±0.95           | 43.66±0.71            | 0.3±0.12         | --              |
| Ulcerated control | 3.83±0.24          | 2.05±0.22           | 118.66±0.76          | 128.76±0.63           | 5.41±0.75        | --              |
| Standard (Ranitidine) | 1.90±0.07       | 4.21±0.16           | 41.83±0.90           | 46.16±0.60           | 1.83±0.30        | 66.17           |
| HZEA(300 mg/kg)  | 2.88±0.23           | 3.20±0.08           | 62.83±0.79           | 70.33±0.66           | 3.15±0.45        | 33.82           |
| HZEA(400 mg/kg)  | 2.08±0.15           | 3.72±0.22           | 53.16±0.60           | 57.50±0.76           | 2.16±0.47        | 60.07           |

Values are expressed as Mean±SEM. *p< 0.01 as ulcerated control group compared with normal control group; **p< 0.01 as standard group compared with ulcerated control group and p< 0.05; ***p<0.01 all other groups compared with standard group using one way ANOVA followed by Dunnett test. ns-not significant, HZEA is the ethyl acetate fraction of H. zeylanica.

Further on support to HZEA activity, the macroscopic appearances of anti-ulcer effects of HZEA against diclofenac sodium induced ulcer in rats were shown in Figure 1. Diclofenac sodium induced ulcer control group (20 mg/kg) produced severe gastric lesions including hemorrhagic bands and spots in surface mucous cells with an ulcer index score of 5.41±0.75 (Figure 1B). The rats that were received HZEA of 300 and 400 mg/kg showed a significant (P < 0.01) reduction in ulcer index 3.15±0.45 and 2.16±0.47 respectively. HZEA 400 mg/kg treated group was found comparable with the ranitidine treated group (1.83±0.30). % of gastro protection was evaluated and found 33.82% gastro protection at 300 mg/kg and 60.07% gastro protection at 400 mg/kg of HZEA and were found comparable with the ranitidine treated group (66.17%) (Figures 1C-2E). The higher dose of HZEA was effective in protecting ulcers in rats than the lower dose.

The effect of gastric pH was studied and found that HZEA 300 and 400 mg/kg treated group showed a significant (p < 0.01) increase in gastric pH of 3.20±0.08 and 3.72±0.22 respectively against the ulcerated control group (2.05±0.22) which caused a significant (p<0.01) decrease in pH of gastric juice as compared to the normal group (4.40±0.40). Both the results were comparable with the reference drug treated group (Table 2).
Antioxidant and Antiulcer Potential of Hydrolea Zeylanica (L.) Vahl against Gastric Ulcers in Rats

Figure 1: The effect of Hydrolea zeylanica on the macroscopic appearance of the gastric mucosa in diclofenac sodium-induced gastric mucosal lesions in rats. (A) Normal control: intact gastric mucosa tissues; (B) Toxic control as diclofenac sodium (20mg/kg) induced ulcer: arrow mark shows severe lesions are seen with extensive visible hemorrhagic necrosis of gastric mucosa; (C) Standard drug ranitidine (11.5mg/kg) treated group as it recovered with a single spot lesions of gastric mucosa were observed compared to ulcer group. Whereas (D) HZEA 300mg/kg recovered with a mild lesions of gastric mucosa and (E) HZEA 400mg/kg as compared to the standard treated group recovered with a spotted lesions of gastric mucosa.

Effect of HZEA in pyloric ligated rats

The ulcerogenic effects of pyloric ligation in ulcerated control groups were produced severe ulcer by analyzing different parameters such as mean ulcer index, mean gastric volume, pH, free acidity and total acidity as recorded 3.91±0.27, 5.35±0.14, 1.88±0.28, 36.66±0.55 and 75.58±0.75 respectively. Pyloric ligation also produced ulcers in some of the HZEA treated animals. Diclofenac sodium (20mg/kg) treated control group produced severe gastric lesions including hemorrhagic bands and spots in surface mucous cells (Figure 2). Rats received HZEA (400 mg/kg) showed a significant reduction in ulcer index (1.95±0.36) which was nearly equal to that of the reference drug ranitidine treated group (1.66±0.33). Gastroprotection of HZEA at 300 and 400 mg/kg were of 27.62 and 50.12% gastroprotection, respectively. But the higher dose of HZEA was found to be an effective gastroprotector as compared to the ranitidine treated group (57.54%).

The gastric content volume was analyzed and found that HZEA showed a significant (p < 0.01) dose dependent reduction in gastric volume at 300 mg/kg (2.15±0.26) and 400mg/kg (1.90±0.15) in comparison to the ulcerated controlled group (Table 3).

In case of free acidity, HZEA treated group at 300 and 400 mg/kg were significant (p < 0.01) dose dependent reduction in free acidity 23.3±0.66 and 19.16±0.65, respectively. Whereas, total acidity significantly (p < 0.01) reduced in rats treated with HZEA at 300 mg/kg (69.66±0.77) and 400mg/kg (39.41±0.66). All the results of HZEA at 400 mg/kg were found comparable with the ranitidine treated group (Table 3).

The effect of gastric pH was studied and HZEA treated group at 300 and 400 mg/kg were of 2.61±0.08 and 3.10±0.17 respectively as a showed significant (p < 0.01) increase in gastric pH as compared to diclofenac sodium induced group (1.88±0.28). The result of higher dose HZEA treated group was found comparable with the reference drug treated group (Table 3).

In vivo antioxidant activities of H. zeylanica

The status of oxidative stress markers were affected by the diclofenac sodium induced ulcerated rats. There was a significant (p< 0.001) increase in MDA levels in tissue homogenates of ulcerated control group as compared to normal control group (Table 3). HZEA at 300 and 400mg/kg significantly (p < 0.01) reduced the MDA level 66.39±3.08 and 58.28±3.73nM/mg protein. However, MDA level of HZEA at 400 mg/kg was nearly comparable to the ranitidine treated group (56.24±2.05nM/mg protein). There was a significant (p<0.001) decrease in glutathione (GSH) levels in tissue homogenates of ulcerated control group as compared to normal control group. The level of GSH in diclofenac sodium induced ulcerated rats, it was found that both HZEA at 300 and 400 mg/kg significantly increased (p< 0.01) glutathione levels in tissue homogenates to 93.37±6.27 and 103.87±6.13nM/mg tissue respectively (Table 3). The results were found to comparable with the ranitidine treated group (110.65±8.54nM/mg; p< 0.01).
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Figure 2: The effect of Hydrolea zeylanica on the macroscopic appearance of the gastric mucosa in pyloric ligated gastric mucosal lesions in rats. (A) Normal control: intact gastric mucosa tissues; (B) Toxic control a single dose of diclofenac sodium (80mg/kg)-induced ulcer: arrow mark shows severe lesions are seen with extensive visible hemorrhagic necrosis of gastric mucosa; (C) standard drug ranitidine (50mg/kg) treated group as it recovered with a single spot lesions of gastric mucosa were observed compared to ulcer group. Whereas (D) HZEA 300 mg/kg recovered with a reduced lesions of gastric mucosa and (E) HZEA 400 mg/kg with a single mild lesions of gastric mucosa as compared to the standard treated group recovered with a spotted lesions of gastric mucosa.

Table 3: Antiulcer activity of ethyl acetate fraction of Hydrolea zeylanica (HZEA) against pylorus ligated ulcer.

| Groups               | Gastric Volume (mL) | pH    | Free Acidity | Total Acidity | Mean Ulcer Index | % Protection |
|----------------------|---------------------|-------|--------------|---------------|------------------|--------------|
| Normal control       | 1.55±0.32           | 3.96±0.07 | 9.16±0.60    | 27.16±0.70    | 0.30±0.13        | --           |
| Ulcerated control    | 5.35±0.14 a         | 1.88±0.28 a | 36.66±0.55 a | 75.58±0.75 a  | 3.91±0.27 a      | --           |
| Standard (Ranitidine 13.5 mg/kg) | 1.76±0.22 b         | 4.45±0.39 b | 14.16±0.47 b | 31.41±0.61 b  | 1.66±0.33 b     | 57.54        |
| HZEA (300 mg/kg)     | 2.15±0.26 ns        | 2.61±0.08 * | 23.33±0.66 ** | 69.66±0.77 ** | 2.83±0.30 ns     | 27.62        |
| HZEA (400 mg/kg)     | 1.90±0.15 **        | 3.10±0.17 ** | 19.16±0.65 ** | 39.41±0.66 ** | 1.95±0.36 **     | 50.12        |

Values are expressed as Mean±SEM. *p <0.01 as ulcerated control group compared with normal control group; †p < 0.01 as standard group compared with ulcerated control group and *p < 0.05, **p < 0.01 all other groups compared with standard group using one way ANNOVA followed by Dunnett test, ns-not significant, HZEA-Hydrolea zeylanica ethyl acetate fraction.

The activities of CAT in the gastric tissues were significantly lowered (p < 0.001) in the diclofenac group (17.4±1.21) as compared with the control group (43.3±1.81). Treated groups of rat with diclofenac sodium along with HZEA at 300 and 400 mg/kg increased significantly (p < 0.01) the activity of CAT in the gastric tissues by 24.4±1.14 and 28.5±1.17 in a dose-dependent way as compared with the diclofenac sodium-treated group. Both the results were comparable with the standard treated group (Table 3).

Citation: Sahoo AK, Kanhar S (2017) Antioxidant and Antiulcer Potential of Hydrolea Zeylanica (L.) Vahl against Gastric Ulcers in Rats. Int J Complement Alt Med 10(1): 00324. DOI: 10.15406/ijcam.2017.10.00324
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Discussion

Some of prime factors which are responsible for the development of gastric ulcers are bacterial infections, increase in acid secretion, generation of ROS, inhibition of endogenous PGs, and degradation of extracellular matrix [33,34]. Diclofenac sodium induced gastric ulcer mainly associated with the generation ROS which cause denaturation of mucosal glycoprotein [35]. Scavenging these free radicals can play an appreciable role in healing the ulcer and most of herbal medicines are good antiulcer agents because of free radical scavenging abilities. The scavenging activities of herbal medicines are based on presence of polyphenols [10]. Mostly these polyphenols are beneficiary to health and have demonstrate a number of pharmacological effects in the GIT area, acting as antisecretory by depleting the aggressive factors, cytoprotective by increasing the mucous secretion, and antioxidant activities by scavenging the free radicals [36,37]. Therefore, it would be important to quantify the amount of TPC and TFC which were present in leaves of H. zeylanica fractions.

Table 4: Effect of ethyl acetate fraction of Hydrolea zeylanica on diclofenac sodium induced oxidative stress parameters in rats.

| Groups               | Malondialdehyde Levels (MDA) (nM/mg of Protein) | Reduced Glutathione (GSH) (nM/mg Tissue) | Catalase (CAT) (U/mg of Protein) |
|----------------------|-----------------------------------------------|----------------------------------------|---------------------------------|
| Normal control       | 52.58±2.21                                    | 116.65±7.01                            | 43.3±1.81                       |
| Ulcerated control    | 81.65±4.21<sup>a</sup>                        | 86.54±5.21<sup>a</sup>                | 17.4±1.21<sup>a</sup>          |
| Standard (Ranitidine)| 56.24±2.05<sup>b</sup>                        | 110.65±8.54<sup>b</sup>               | 39.8±1.43<sup>b</sup>          |
| HZEA (300 mg/kg)     | 66.39±3.08<sup>b</sup>                        | 93.37±6.27<sup>b</sup>                | 24.4±1.14<sup>b</sup>          |
| HZEA (400 mg/kg)     | 58.28±3.73<sup>b</sup>                        | 103.87±6.13<sup>b</sup>               | 28.5±1.17<sup>b</sup>          |

HZEA is the ethyl acetate fraction of *Hydrolea zeylanica* at dose of 300 and 400 mg/kg. Values are expressed as Mean±SEM. (n=6) animals in each group. *p < 0.001; diclofenac sodium induced ulcerated control group compared with normal group. *p < 0.01; rest experimental groups compared with standard group. Data analyzed by one way ANOVA followed by Dunnett test.

Figure 3: Antioxidant activities of hexane, chloroform, ethyl acetate and methanol extract leaves of *Hydrolea zeylanica*. The standard drug ascorbic acid was considered for DPPH, nitric oxide assays; whereas for superoxide dismutase, hydroxyl assays quercetin was considered as a standard drug to perform the assays.
successive extraction process of leaves of *H. zeylanica* by considering non-polar to polar solvents. HZH, HZC, HZEA and HZM were estimated for quantifying TPC and TFC. Among them, HZEA contained higher level of TPC (286.3±3.916 mg GAE/g fraction) and TFC 208.2±5.56 mg Quev/g (Table 1).

The antioxidant and antiulcer activities of *H. zeylanica* leaves were performed which is the first report in this plant. From *in vitro* test results of different assays, HZEA demonstrated better activities in DPPH free radical scavenging assay with IC50 41.20±3.21µg/mL than, other fractions. The presence of higher amount of phenols and flavonoids in HZEA could be responsible for neutralizing DPPH radicals by donating hydrogen to a free radical generated by oxidants [38]. In case of superoxide anion (SOD) assay, powerful and dangerous hydroxyl radicals generated along with singlet oxygen, which are responsible for oxidative stress. But HZEA had effectively neutralized by scavenging SOD radicals with the IC50 59.71±1.45 µg/mL and the presence of polyphenols in this fraction were responsible for neutralizing the radicals generated by SOD [36]. At physiological pH, due to oxidative stress a spontaneously generation of nitric oxide may interact with oxygen and produce nitrite ions which were efficiently countered by HZEA as found in nitric oxide (NO) radical scavenging study. HZEA was a better scavenger of radicals with IC50 73.82±1.89 µg/mL due to the presence of phenolic and flavonoid contents (Figure 3) [38]. One of the dangerous radical generations in the form of hydroxyl (OH) radicals, which react with polyunsaturated fatty acid moieties of cell membrane phospholipids may cause a serious damage to cells. In the OH assay, amongst all, HZEA showed a good scavenger of OH radicals with IC50 48.91±0.89µg/mL (Figure 1).

Based on earlier reports, it may be inferred that phenolic and flavonoid contents were responsible for OH radical scavenging activities [10,38].

Oxidative stress induced generation of ROS and its overproduction is one of the prime aetiologic factors that cause gastric ulcer. As a result, some relevant biochemical marker status quo gets affected as peroxidation of lipids, proteins and nucleic acids, which may lead to cellular damage and cell death [39]. Some of the relevant *in vivo* antioxidant markers were studied. One of the important biochemical markers was the evaluation of malondialdehyde (MDA). In this study, the increase in MDA level in ulcerated rats was caused by diclofenac sodium and generated free radicals caused lipid peroxidation led to membrane fluidity and increased in influx of Ca2+ ions. As a result, membrane integrity was reduced of surface epithelial cells and led gastric ulcers [40]. Pretreatment with HZEA at 300 and 400mg/kg provided a significant (p<0.01) protective effect to the mucosal membrane by reducing the production of oxygen free radicals (Table 4). The antioxidant effect of HZEA could be attributed to the presence of phenols and flavonoid contents and this study corroborate with earlier reports where polyphenolic compounds were responsible for antiulcer activities [41].

Gluthathione is an important constituent of the intracellular protective mechanism against oxidative stress. Excessive generation of oxygen radicals in the extracellular space resulted in depletion of gluthathione by forming oxidized form of glutathione (GSSG) which are responsible for oxidative tissue damage of gastric mucosa leads to severe ulcer [42,43]. In this study, decreased glutathione concentrations were observed in ulcerated control group, whereas rats that were pretreated with HZEA at 300 and 400 mg/kg showed a significant (p<0.01) increase in the glutathione level, suggesting that GSH levels toward normal control have restored the steady state of GSH (Table 4). The results presented in this study corroborate with earlier reports where NSAID induced a significant depletion of GSH in gastric lesions [44].

Another antioxidant biochemical marker is catalase (CAT) and any disturbance in the level of this endogenous enzyme can result in generation of ROS. In this case, ROS caused a serious degradation to CAT by producing H2O2, which resulted into a molecule of oxygen and water. It was found that diclofenac sodium induced rats caused gastric damage and for which there was a decreased CAT levels in gastric induced rats. But administration of HZEA at 300 and 400 mg/kg showed a significant (p<0.01) increase in the CAT level by its antioxidant defense mechanism because of presence of phenols and flavonoids as reported in previous occasion [45].

The result of the antiulcer study demonstrated that HZEA exerted protective effects in both diclofenac sodium induced and pyridic ligation ulcer models. In both models, ulcer inductions were resulted in auto digestion of gastric mucosal barriers and this was probable due to the excess production and accumulation of HCl in stomach [46]. HZEA at a dose of 300 and 400 mg/kg significantly (p<0.01) reduced the volume of gastric juice, total acidity, free acidity and ulcer index and increased the gastric acid pH. The effects were comparable to ranitidine treated groups. Several NSAIDs like aspirin, indomethacin, diclofenac sodium are known to induce gastric ulcer by inhibiting the biosynthesis of cytoprotective prostaglandins [47]. In stomach, prostaglandins play an important role by stimulating the secretion of bicarbonate, maintaining the mucosal blood flow and regulating mucosal cell proliferation and repair [48]. In our study, the cytoprotective role of HZEA at 300 and 400mg/kg may be mediated through the endogenous prostaglandin because of the presence of phenols and flavonoids. *In vitro* test results confirmed that HZEA is a good scavenger of free radicals and also *in vivo* antioxidant mechanism of HZEA against gastric mucosal lesions was further supported by the restoration of oxidative stress marker levels in MDA, GSH and CAT. This proves that the presence the plant natural antioxidants as polyphenols were responsible for antiulcer properties of *H. zeylanica*.

**Conclusion**

In conclusion, this study provides evidences that *Hydrolea zeylanica* possess antioxidant and antiulcerogenic activity. Furthermore, our findings contribute to the validation of the popular claim that *H. zeylanica* as a natural antiulcer agent and this species may be a promising candidate for the development of a novel compounds to treat gastric ulcers. Further pharmacological studies are being undertaken with the ethyl acetate fraction of leaves of *H. zeylanica* (HZEA) and the bioassay guided isolation of HZEA is in progress in our laboratory in order to elucidate...
the precise mechanism by which HZEA acts in gastric mucosa protection.

Acknowledgement

Financial support for this work was provided by the Forest & Environment Department, Govt. of Odisha, Bhubaneswar, India (1580/RPRC; Dt.13-03-2015).

Conflict of Interest Statement

We declare that we have no conflict of interest.

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