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Original Article

# EFFECT OF AQUEOUS EXTRACT OF *AZOLLA FILICULOIDES* IN GASTRIC MUCOSA OF ULCERATED RATS

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**ABSTRACT**

**Objective:** To investigate the anti ulcer effect of aqueous extract of *Azolla filiculoides* (AF) in experimentally induced gastric ulceration in male Wistar rats.

**Methods:** Twenty four rats were divided into four groups of six rats in each group. The group I and III rats were fed with standard diet, whereas, group II and IV rats were pre-treated orally with aqueous extract of *Azolla filiculoides* (200 mg/kg body) twice a day for 15 d. Twenty four hours before sacrifice, group III and IV rats were induced with ulcer by pylorus-ligation plus oral administration of a single dose of HCl-ethanol mixture (1.5 ml). The gastric juice and mucosal scraps were collected from all the groups for biochemical analyses.

**Results:** In ulcer induced rats, there observed a significant ($p<0.05$) increase in the following parameters like ulcer index, gastric output, acid output, lipid peroxides and these levels were significantly ($p<0.05$) reduced to near normal values in AF pre-treated rats. A significant decrease was also observed in the levels of reduced glutathione, hexose, hexosamine, sialic acid and in the activities of antioxidant enzymes (glutathione-S-transferase and glutathione peroxidase) and antiperoxidative enzymes (catalase and superoxide dismutase) in ulcer induced rats. These values were restored back to near normalcy in AF pre-treated rats.

**Conclusion:** The results reinforce the antisecretory, acid neutralizing and the antioxidant potential of the whole plant extract of AF against experimentally induced gastric ulcer in rats. However, further studies are needed to identify the active principle involved in eliciting the antiulcer activity of the plant.

**Keywords**: *Azolla filiculoides*, Antisecretory, Acid neutralizing, Antioxidant.

## INTRODUCTION

The human population is more susceptible to gastrointestinal disorders owing to the changes in lifestyle and food habits. One of the gastrointestinal problems that commonly threaten the public is peptic ulcer. It happens mostly when the acids that helps to digest the food attacks the gastric mucosal lining of the stomach. The most common cause is the infection with a bacterium called *Helicobacter pylori* [1]. The other factor includes the long term consumption of nonsteroidal anti-inflammatory drugs (NSAIDs), increased stress, nutritional deficiencies, tobacco smoking, and imbalance in aggressive and defensive factors [2-3]. Lipid peroxidation-induced by the free radicals produced as a result of oxidative processes is the major rationale behind the aberration of gastric mucosa. The HCl-ethanol induced gastric mucosal lesions and other signs in the stomach appear more similar to the peptic ulcer caused by the long term consumption of NSAIDs. Allopathic drugs available in the market are capable of curing the ulcer symptoms, but are chosen diligently as it offers side effects like arrhythmia, nausea, abdominal pain, gynecomastia and impotence [4]. Due to the promising treatment results of herbal extracts, much interest is developing towards usage of the same in the treatment of Peptic ulcer [5-6].

Aquatic plants have become a potential source of beneficial substances like proteins, vitamins, fibers and medicinally active compounds. *Azolla* is an aquatic pteridophyte most commonly known for its agronomic usage due to its nitrogen-fixing ability. Recent studies in *Azolla* emphasized on the identification of its nutritional and pharmacological activities. From the previous research findings, it is evident that *Azolla* possesses antioxidant [7], antimicrobial [8], antifungal [9] and hepatoprotective activity [10], which can be attributed to the presence of secondary metabolites in significant quantities.

Based on these facts, an attempt has been made to investigate the effect of aqueous extract of *Azolla filiculoides* on gastric mucosa in experimentally induced ulcer in rats.

## MATERIALS AND METHODS

### Collection of plant and extract preparation

Whole plants of fresh *Azolla filiculoides* were collected from the Agricultural Microbiology Department of Tamil Nadu Agricultural University located at Coimbatore district, Tamil Nadu. The collected plant materials were washed thoroughly with tap water, rinsed with distilled water and air dried. The air dried plant materials were grounded to fine powder and the crude aqueous extract was obtained by extracting 20 g of the plant powder with 200 ml of distilled water kept in an orbital shaker maintained at 37 ˚C for 24 h. The extract thus obtained was filtered, evaporated to dryness and the dried extract was stored at 4 °C in an air-tight container until further use. The final concentration of the extract was made up to 100 mg/ml.

### Chemicals

Trichloroacetic acid, 5,5'-dithiobis-2-nitrobenzoic acid, hydrochloric acid, hydrogen peroxide, chloroform, tetra ethoxy propane, ethanol, epinephrine, 1-chloro-2,4-dinitrobenzene, sodium chloride, phenolphthalein, thiobarbituric acid, periodic acid, orcinol reagent and bovine serum albumin. All the chemicals and solvents used were of analytical grade and purchased from M/s Sigma-Aldrich Company located at Missouri, USA.

### Experimental animals

Male Wistar rats weighing in the range between 130-150 g were chosen for the experimental studies. They were placed in polypropylene cages at ambient conditions of temperature (24±2 ˚C), relative humidity (60-70 %) and light-dark schedule (12 h). The animals were fed with standard pelleted diets (M/s Hindustan Lever Foods, Bangalore, India) and water *ad libitum*. They were fasted for 24 h prior to the ulcer induction. The experiment using animals as model was carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals

(CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee (IAEC).

**Experimental design**

The animals were divided into four groups with each group constituting six rats. The treatment protocols are mentioned below:

Group I: Normal control rats with standard diet only.

Group II: Administered with 2 ml (200 mg/kg body weight p. o) of aqueous extract twice a day with an interval of 12 h for 15days.

Group III: Received standard diet but Ulcer induced 24 h prior sacrifice by the oral administration of 1.5 ml HCl-ethanol mixture containing 0.15 N HCl in 70 % v/v ethanol [11].

Group IV: Received *Azolla filiculoides* extract (200 mg/kg body weight p. o) pre-treatment for 15days and ulcer was induced by the similar manner as Group III rats.

**Excision of stomach and collection of gastric juice**

After the treatment period the rats were undergone surgery, according to the procedures mentioned by Takeuchi *et al.* [12] and 4 h post-surgery the rats were sacrificed using an overdose of Chloroform and the stomachs were removed after clamping the esophagus. Along the greater curvature the stomach was cut open and the gastric juice was collected in a graduated tube. It was spun at 2000 rpm/10 min and the volume was noted. The acid output was determined in the gastric juice by volumetric titration against 0.02 N sodium hydroxide using phenolphthalein as indicator.

**Lesions scoring**

Using a dissecting microscope (10x) the lesions were scored independently according to the severity of ulcers observed [13]. Latter, the Ulcer Index (UI) was calculated as the mean ulcer score for each rat. The percentage protection offered by the sample was calculated using the following formula: (UI control–UI test)/UI control *100.

**Preparation of tissue homogenate**

The mucosal scraps were gently collected using a thin glass slide and homogenized (600 rpm/5 min) with ice-cold 0.1 M Tris-HCl buffer (pH=7.4) using a Potter-Elvehjem homogenizer (Sigma-Aldrich). The filtrate obtained was used for the biochemical estimations.

**Biochemical estimations**

The total proteins were estimated using the method of Lowry *et al.* [14]. Bovine serum albumin was used as the internal standard for the determination of unknown concentration of proteins. The reduced glutathione content was determined based on the reaction of reduced glutathione with 5, 5'-dithiobis-2-nitrobenzoic acid to give a yellow compound, which was measured at a wavelength of 412 nm [15]. The reduced glutathione content was expressed as nmol/g of wet tissue. The extent of lipid peroxidation was assayed using the method of Ohkawa *et al.* [16], wherein the amount of malondialdehyde (MDA) liberated was estimated. 1, 1, 3, 3-tetraethoxypropane was used as a standard and the level of lipid peroxides formed was expressed as nmol of MDA formed/mg of protein. Sialic acids were analyzed by the addition of thio barbituric acid and periodate solution to the sample and the change in absorbance was noted at 540 nm [17]. Sialic acid levels were expressed in mg/g of tissue. The total hexose and hexosamine were determined using the method of Winzler *et al.* [18] and their corresponding concentrations were expressed as mg/g of wet tissue. The glutathione S-transferase activities were determined using the standard substrate 1-chloro-2,4-dinitrobenzene (CDNB) as mentioned by Habig *et al.* [19]. The activities of GST enzyme were expressed as µmol of CDNB conjugate formed/min/mg of protein. The glutathione peroxidase (GPx) activity was estimated by the method of Paglia and Valentine [20]. GPx catalyzes the reduction of hydroperoxides using reduced glutathione as the substrate. Thus, the enzyme activity of GPx was expressed as nmol of GSH oxidized/min/mg of protein. According to the method of Takahara *et al.* [21], the activity of enzyme catalase (CAT) was estimated based on the reaction with dichromate-acetic acid reagent in the ratio (1:3). The catalase enzyme activity was expressed as nmol of hydrogen peroxide ($H_2O_2$) decomposed/min/mg of protein. The activities of the enzyme superoxide dismutase (SOD) were estimated using the method of Misra and Fredovich [22]. One unit of SOD was calculated as, the amount of protein required to inhibit half the percentage of autoxidation of epinephrine.

**Statistical analysis**

The data were expressed as mean±SD. Statistical significance was assessed using one way ANOVA followed by Tukey's test and $p<0.05$ was considered significant. The analyses were performed using statistical program SPSS 10.0 for Windows.

**RESULTS**

The effects of aqueous extract of *Azolla filiculoides* on gastric mucosa in experimentally induced ulcer were studied. There observed a significant ($p<0.05$) increase in the values of an ulcer index, volume of gastric juice and the acid output in Group III rats as compared with that of Group I and Group II rats. In contrast, these values were significantly ($p<0.05$) reduced in the AF pre-treated Group IV rats. The results are shown in table 1.

**Table 1: Effect of aqueous extracts of *A. filiculoides* (whole plant) on the lesion index, volume of gastric output and total acidity in normal and experimental group of rats**

| Parameters | Group I (Normal Control) | Group II (AF pre-treated) | Group III (Ulcer induced) | Group IV (Group II+III) |
|---|---|---|---|---|
| Ulcer index | - | - | $9.58±1.15^{a}$ | $3.08±0.80^{a,b}$ |
| Volume of gastric juice (ml/4 h) | 2.11±0.14 | $2.08±0.12^{ns}$ | $4.32±0.12^{a}$ | $2.25±0.14^{a,b,c}$ |
| Acid output (µEq/4 h) | 139.33±5.13 | $134.17±4.88^{ns}$ | $256.5±6.66^{a}$ | $162.83±8.66^{a,b}$ |

Values are expressed as mean±SD for 6 rats.

**Table 2: Effect of aqueous extracts of *A. filiculoides* (whole plant) on the biochemical parameters of normal and experimental group of rats**

| Parameters | Group I (Normal Control) | Group II (AF pre-treated) | Group III (Ulcer induced) | Group IV (Group II+III) |
|---|---|---|---|---|
| Proteins[1] | 14.08±0.22 | $14.33±0.16^{ns}$ | $7.56±0.23^{a}$ | $13.55±0.13^{a,b}$ |
| GSH[2] | 4.79±0.14 | $4.68±0.12^{ns}$ | $2.08±0.12^{a}$ | $3.95±0.09^{a,b}$ |
| GST[3] | 5.20±0.07 | $5.13±0.06^{ns}$ | $2.97±0.04^{a}$ | $4.48±0.07^{a,b}$ |
| GPx[4] | 190.33±5.75 | $195.50±4.72^{ns}$ | $107.83±5.78^{a}$ | $172.67±2.80^{a,b}$ |
| CAT[5] | 3.39±0.13 | 3.75±0.06 | $1.93±0.12^{a}$ | $2.97±0.17^{a,b}$ |
| SOD[6] | 5.23±0.11 | 5.65±0.18 | $2.76±0.10^{a}$ | $4.92±0.13^{a,b}$ |
| LPO[7] | 2.86±0.13 | $2.62±0.40^{ns}$ | $7.91±0.19^{a}$ | $2.08±0.10^{a,b}$ |
| Hexose[1] | 13.57±0.21 | $13.20±0.36^{ns}$ | $7.14±0.16^{a}$ | $12.00±0.42^{a,b}$ |
| Hexosamine[1] | 8.09±0.15 | $8.18±0.12^{ns}$ | $4.41±0.27^{a}$ | $7.58±0.12^{a,b}$ |
| Sialic acid[1] | 1.26±0.09 | $1.33±0.12^{ns}$ | $0.68±0.06^{a}$ | $1.04±0.09^{a,b}$ |

Values are mean±SD for 6 rats.

**Group comparison**

[a]= significant ($p<0.05$) compared to group I & II rats

[a,b]= significant ($p<0.05$) compared to group I & III rats

[a,b,c]= significant ($p<0.05$) compared to group III rats

[ns]= No significant difference compared with group I rats

In HCl-ethanol plus pylorus-ligated Group III rats, a significant decrease in the concentration of total protein, reduced glutathione, hexose, hexosamine and sialic acid was observed. On the other hand, there observed a significant ($p<0.05$) elevation in the levels of lipid peroxides along with a concomitant reduction in the activities of glutathione dependent antioxidant enzymes (GST, GPx) and peroxidative enzymes (SOD, CAT). Oral pre-treatment with aqueous extract of AF markedly reduced the elevated levels of lipid peroxides, while the levels of reduced glutathione, hexose, hexosamine and sialic acid were augmented to near normalcy. The activities of antioxidant and antiperoxidative enzymes were also restored back to near normal values as shown in table 2.

**Group comparison**

[a]= significant ($p<0.05$) compared to group I & II rats

[a,b]= significant ($p<0.05$) compared to group I & III rats

[ns]= No significant difference compared with group I rats

**Units**

[1]=proteins, hexose, hexosamine, sialic acid= mg/g of tissue.

[2]=GSH nmol/g tissue.

[3]=1Unit of GST enzyme activity is the µmol of CDNB conjugate formed/min/mg of protein.

[4]=1Unit of GPx enzyme activity is the nmol of GSH oxidized/min/mg of protein.

[5]=1Unit of CAT enzyme activity is the nmol of $H_2O_2$ decomposed/min/mg of protein.

[6]=1Unit is the amount of enzyme required to inhibit 50 % autoxidation of epinephrine.

[7]=LPO nmol/mg of protein.

The percentage ulcer protection offered by the plant extract against HCl-ethanol induced ulcerogenesis was 67.85. The ulcer protective index along with other results suggests the cytoprotective, anti-secretory, and acid neutralizing potential of the plant extract in ulcer induced rats. The biochemical results of Group II rats did not show significant ($p<0.05$) differences when compared with that of Group I rats.

## DISCUSSION

In general, herbal medicines are considered to be less toxic, efficient and affordable to people of all age groups. Hence, this way of treatment will be most appropriate for the treatment of gastric ulcer and other gastrointestinal disorders. The oral pre-treatment with aqueous extract of AF (200 mg/kg body weight) ameliorated the deleterious effects caused by the administration of HCl-ethanol in the gastric mucosa of ulcer induced rats.

The ulcer is an outcome of an imbalance between the endogenous gastric mucosal defensive and offensive factors. In the present study, there observed a remarkable increase in the offensive factors like gastric juice and total acidity, whereas a decrease in defensive factors like mucopolysaccharides, reduced glutathione, antioxidant and peroxidative enzyme activities in ulcer induced rats. The ligation of pylorus results in the physiological accumulation of gastric juice, along with large amounts of pepsin that apparently leads to an auto-digestion of the mucosal barrier in the gastric mucosa [29]. An increase in gastric volume is encountered as a result of increased vascular permeability caused by the aberrations in the gastric mucosal barrier. The perturbation of this barrier is known to elicit excessive secretion of histamine that acts through the histamine receptors on the parietal cell and initiates increased acid secretion in the stomach. In general, the secretion of gastric acid plays a meticulous role in aggravating gastric mucosal injury and the antiulcer drugs accelerate the healing process by reducing this acid secretion. Oral administration of aqueous extract of AF significantly reduced the volume of gastric juice and total acidity in pre-treated rats. These results are in concordance with the reports of Oluwabunmi *et al.* [23], who reported that the methanolic extract of *Gomphrena celosioides* significantly reduced the volume of gastric juice and acidity in pre-treated rats. The anti-secretory effect observed in the present study, may be attributable to the presence of compounds having the ability to antagonize histamine receptors in the parietal cells. The reduction in the total acidity clearly indicates the acid neutralizing ability of the plant extracts.

The stomachs of ulcer induced rats in the present study were displayed with morphological aberrations, lesions and a reduction in total protein content. This loss of total gastric mucosal proteins may be due to its leakage into the gastric juice. In the present study, the pre-treatment of AF extracts not only reduced the ulcer indices, but also replenished the levels of total protein in group IV rats. These findings are in agreement with the results obtained by Mahendran *et al.* [24], who reported that the increased losses of protein are associated with HCl-ethanol induced damage to gastric mucosa in rats and their levels were returned to normalcy by the treatment with *Garcinia cambogia* extract. One of the major defensive factors in the gastric mucosa is the mucins that are mainly composed of glycoproteins and are responsible for the protection of gastric mucosa against physical, chemical and microbial disturbances. The ratio of carbohydrate to protein represents mucus activity in the gastric mucosa and this ratio was reduced in ulcer induced rats [25]. The presence of decreased level of glycoprotein component is a direct indication of disintegration and degradation of gastric mucosal glycoproteins caused by the HCl-ethanol administration. These effects were counteracted by the elevated concentration of total protein, hexose, hexosamine and sialic acid, subsequently replenishing the mucus contents in the gastric mucosa of AF pre-treated rats. This observation is in harmony with the results published by Jainu *et al.* [26].

Some physiologically unstable compounds like superoxide anion and hydroperoxy radicals are generated inside the cell as a result of the metabolism of ethanol, which makes the gastric mucosa more labile to chronic and acute ulceration through the induction of lipid peroxidation. This eventually leads to the destabilization of membrane proteins and inhibition of enzyme activities. The amount of gastric mucosal injury is aggravated by lipid peroxidation. Ganesan *et al.* [27] reported that the pylorus ligated rats subjected to oxidative stress were observed with an elevated LPO level along with a decline in the reduced-GSH content and SOD activities in the blood and these findings are in agreement with the present study. On the other hand, the activities of antioxidant enzymes (GST, GPx) and antiperoxidative enzymes (SOD, CAT) were reduced drastically in the ulcer induced rats, as observed by Sudheer *et al.* [28]. However, administration of aqueous extract of AF significantly reduced concentrations of LPO coupled with an elevated GSH content. The activities of enzymes like GST, GPx, CAT and SOD were also restored back to near normalcy, which is in concordance with the reports of Deoda *et al.* [29]. The foregoing observations indicate the *in vivo* antioxidant potential of the plant extract. Noor Nawaz *et al.* [7] investigated the *in vitro* antioxidant potential of two *Azolla* species and reported *Azolla* as a potential aquatic plant suitable for the development of pharmaceutical agent that counteracts physiological oxidative stress. A preliminary phytochemical study conducted in *Azolla microphylla* has indicated the presence of phenols, flavonoids, tannins, steroids [30] and in the same species Chowdhury *et al.* [31] isolated two bioactive flavonoids such as rutin and quercetin. In general, these compounds elicit antiulcer effect by virtue of its antioxidant potential [32]. The presence of such type of bioactive compounds in *Azolla filiculoides* might be responsible for producing the antiulcer effects.

## CONCLUSION

The results of the present study indicate the overall antiulcerogenic effect of aqueous extract of *Azolla filiculoides* in rats. The ameliorating effects elicited by the plant extract may be due to its

acid neutralizing, antioxidant potential and also by sustaining a balance between the concentrations of offensive and defensive factors. However, further studies must emphasis on the identification of the exact mechanism of action involved in ulcer healing and also the active principle involved in the plant.

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**CONFLICT OF INTERESTS**

The authors declare no conflict of interest.

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