Anti-ulcerogenic and *in vitro* antioxidant activities of *Lagenaria breviflora* (LB) whole fruit ethanolic extract in laboratory animals

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ABSTRACT

Background: The present study was undertaken to evaluate the anti-ulcer and antioxidant activities of the ethanol extract of *Lagenaria breviflora* (EELB) whole fruit in laboratory rats. **Methods:** The anti-ulcer property of the ethanolic extract of the whole fruit of *Lagenaria breviflora* (LB) was assessed using the cold-restraint stress-induced (CRU) gastric ulcer, pyloric ligation-induced (PL) gastric ulcer, aspirin-induced (ASP) gastric ulcer and alcohol-induced (AL) gastric ulcer models. The scavenging activity of the LB extract was examined with 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), Nitric oxide, Hydroxyl radical and Superoxide anion scavenging models. **Results:** EELB (50, 100, 150 and 200 mg/kg, b.w.) protected against the CRU gastric ulcer dose dependently. Similarly, 150 mg/kg b.w. of the LB extract protected against the PL gastric ulcer, ASP gastric ulcer and AL gastric ulcer and was comparable to omeprazole (10 mg/kg b.w.) or Suscralfate (500 mg/kg b.w.), respectively. The *in vitro* antioxidant activity of LB was demonstrated by its ability to quench free radicals generated by nitric oxide and superoxide anion with a concomitant scavenging potential against DPPH-induced radical formation. **Conclusion:** Taken together, the study showed that the whole fruit extract possess potent anti-ulcer and antioxidant activities.

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INTRODUCTION

Lagenaria breviflora (LB) belongs to the plant family Cucurbitaceae.[1,2] LB is a perennial climber ascending to the forest canopy, occurring from Senegal to the West Cameroons, and generally widespread in tropical Africa. The leaves are very scabrid and sandpapery. The stem when crushed has an unpleasant smell and a decoction from it is said to be used in Africa for headache and as a vermifuge.[3] Its seeds and fruits have been used in folk medicine since antiquity. Previous phytochemical screening of LB revealed the presence of triterpenoid saponins.[4] The alleged uses of the fruit are for the treatment of cold in man and coccidiosis in birds,[5] schistosomiaisis,[3] antifertility and hematinic effects.[6-8] A broad-spectrum antibacterial activity of the whole fruit of LB has also been reported.[9]

Reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, hydroxyl, nitric oxide and peroxynitrite radicals have been reported to play a significant role in oxidative stress related to the pathogenesis of various important diseases.[10,11] The antioxidant activities of the extracts of several plants, including their leaves, bark, roots,[12] fruits, seeds[13,14] and seedcake, have been extensively documented.[15,16] Lipid peroxidation is an important factor in the deteriorating reaction in food during storage and processing, and is believed to be associated with some diseases such as carcinogenesis, mutagenesis, ageing and arteriosclerosis.[17] The role of ROS and free radicals in tissue damage in such diseases is becoming increasingly recognized.[18] Generation of ROS has been linked to gastric ulceration.[19-21]

Peptic ulcer disease (PUD) encompassing gastric and

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duodenal ulcer is the most prevalent gastrointestinal disorder.[22] The pathophysiology of PUD involves an imbalance between offensive (acid, pepsin and *Helicobacter pylori)* and defensive factors (mucin, prostaglandin, bicarbonate, nitric oxide and growth factors).[23] There has been a rapid progress in the understanding of the pathogenesis of peptic ulcer and most of the studies have been focusing on newer and better drug therapy. Treatment of peptic ulcer has been made possible by the availability of the proton pump inhibitors, histamine receptor blockers, drugs affecting the mucosal barrier and prostaglandin analogue.[24] There has been no report on the antiulcer property of LB.

The aim of the present study was to investigate the anti-ulcer activity of LB using four experimental gastric acid methods (pyloric ligation, cold-restraint, alcohol and aspirin-induced gastric lesions) and its antioxidant activities, which was determined by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay, nitric oxide scavenging and superoxide anion radical scavenging activities.

MATERIALS AND METHODS

Lagenaria breviflora

Fresh fruits of LB were obtained from local markets in Ibadan, Oyo State, Nigeria. The fruits were washed, cut and weighed. They were tied up in small quantities in sieves and placed in plastic containers. Sufficient ethanol covering each portion was poured into each container and left for 4 days. The ethanol was decanted and stored and thereafter replaced with fresh ethanol. This procedure continued for an average of 1 week until the fruit was no longer extracting. The filtrate was kept in a refrigerator at 20°C. The filtrate was, concentrated in a rotatory evaporator at a reduced temperature of 40°C. The solvent was recovered in distillation *in vacuo* and the extract was stored in a desiccator and used for subsequent experiments. A semi-solid greenish-brown paste was obtained. A stock solution of the extract was prepared by dissolving 100 g of the extract in 100 ml of distilled water.

Animals

Sprague Dawley rats weighing 140–160 g and were obtained from the National Animal Laboratory Centre of Central Drug Research Institute, Lucknow, India. Animals were kept in raised mesh bottom cages to prevent coprophagy in an environmentally controlled rooms (25 ± 2°C, 12-h light and dark cycle), with free access to water. Animals were fed with standard Hind Lever diet pellets *ad libitum*. Animals were deprived of food for 24 h before subjecting them to ulcerogens and were randomly allocated to different experimental groups. Six rats were used in each group. Experimental protocols were approved by the institutional ethical committee following guidelines of the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), which complies with international norms of the INSA (Indian National Science Academy). The "Principle of Laboratory Animal Care" (NIH publication No. 85-23) guidelines and procedures were considered in this study (NIH publication revised, 1985).[25]

Drugs and Chemicals

All chemicals used were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO, USA) except otherwise stated.

Anti-ulcer Studies

Cold-restraint Stress-induced (CRU) Gastric Ulcer Model in Rats

The rats were subjected to cold-stress paradigm.[26] Animals of different experimental groups were subjected to cold and restraint stress after 45 min of treatment of the LB extract (50, 100 and 200 mg/kg, p.o.) and omeprazole (10 mg/kg, p.o.). All the animals were immobilized in restraint cages, kept at 4°C in an environmental chamber for 2 h and then sacrificed thereafter. The stomach was cut along the lesser curvature and the ulcers were scored with the help of a magnascope.

Pyloric ligation-induced (PL) Gastric Ulcer Model in Rats

This was carried out by ligating the pyloric end of the stomach of the rats under chloral hydrate anesthesia (300 mg/kg, i.p.). After 45 min of LB extract and omeprazole treatments, the abdomen was opened below the xiphoid process.[27] The pyloric portion of the stomach was slightly lifted and ligated, avoiding any damage to the adjacent blood vessels. The stomach was replaced carefully and the abdomen was stitched. The rats were allowed to recover and stabilize in individual cages during the post-operative period. After 4 h of surgery, the rats were sacrificed. The stomach was dissected out and the gastric juice was collected for the estimation of free and total acid, mucin and peptic activity.

Aspirin-induced (ASP) Gastric Ulcer Model in Rats

Aspirin at a dose of 150 mg/kg, p.o. was administered to induce ulcer after 45 min of treatment of the LB extract (150 mg/kg, p.o.) and omeprazole (10 mg/kg, p.o.). The animals were sacrificed 5 h after the aspirin treatment and the stomach was dissected out, incised along the lesser curvature and the lesion was scored.[28]

Alcohol-induced (AL) Gastric Ulcer Model in Rats

A gastric ulcer was induced in rats by administering absolute

alcohol at the dose of 1 ml/200 g, b.w.[29] The LB extract (150 mg/kg, p.o.) and Sucralfate (500 mg/kg, p.o.) were administered 45 min before alcohol treatment to the fasting rats. The animals were sacrificed after 1 h and the stomach was cut along the greater curvature to observe the gastric lesions appeared as hemorrhagic bands along the mucosal ridges of the stomach. The lesions were analyzed through a trinocular stereo zoom microscope and the lengths of the lesions were measured using the Biovis Image Analysis Software and summated to give a total lesion score.

Measurement of the Ulcer Index

The ulcers were scored with the help of a magnascope under 5X magnification using the ulcer scoring criteria.[30] The following scoring system was used to grade the incidence and severity of the lesions: (i) shedding of epithelium = 10, (ii) petechial and frank hemorrhages = 20, (iii) one or two ulcers = 30, (iv) more than two ulcers = 40, (v) perforated ulcers = 50. Length of the hemorrhagic band is measured in the AL model using Biovis Image Analysis Software (BIAS). Percentage protection index is calculated as follows:

% protection = $(U_c - U_t) \times 100/U_c$

Where, U_c = ulcer index in the control group; U_t = ulcer index in the treated group

Gastric Acid Secretion Analysis

The volume of gastric juice obtained in the pyloric ligation model was expressed in terms of milliliters/100 g of body weight. The free acidity, total acidity, peptic activity and dissolved mucous substances of the gastric juice were measured. Free and total acidity were measured by titrating the gastric juice with 0.01N NaOH using Topfer's reagent and phenolphthalein as indicators, respectively, and was expressed in terms of μeq/ml.[31] The peptic activity was determined by measuring the amount of liberated tyrosine by the action of pepsin on hemoglobin as a substrate, and was expressed in terms of U/ml.[32] The mucin level in gastric juice was quantified with a fluorometric assay as described by and expressed as micrograms of mucin/milliliter of gastric juice.[33]

Antioxidant Assays

Scavenging/Inhibitory Activity Coefficient

The scavenging or percentage inhibitory activity of the LB extract in each assay was calculated the equation:

% Inhibition = $(A_0 - A_1) \times 100 / A_0$

Where, A_0 = absorbance of the control (without extract) and A_1 = absorbance of the treated (with LB extract). The IC_{50} value of the LB extract was determined from the standard linear regression curve.

DPPH Photometric Assay

The free radical scavenging activity of the LB extract was estimated, *in vitro*, by the DPPH scavenging activity with slight modification.[34] Exactly 1 ml of 300 μM solution of DPPH in 100% ethanol was added to 3 ml of the LB extract dissolved in ethanol at different concentrations. The mixture was mixed and allowed to stand at room temperature for 15 min and the absorbance was measured at 517 nm with a spectrophotometer. The IC_{50} value of the LB extract was compared with that of ascorbic acid (standard).

Nitric Oxide Scavenging Assay

The nitric oxide radical scavenging activity was determined according to Garrat's method.[35] Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction. Two milliliters of 10 mM sodium nitroprusside in 0.5 ml phosphate-buffered saline (pH 7.4) was mixed with 0.5 ml of the LB extract at various concentrations and the mixture was incubated at 25°C for 150 min. 0.5 ml of the incubated mixture was taken and mixed with 1.0 ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min for total diazotization. Then, 1.0 ml Griess reagent was mixed and incubated at room temperature for 30 min. The absorbance at 540 nm was measured with a spectrophotometer. The IC_{50} value of the LB extract was compared with that of curcumin.

Hydroxyl Radical Scavenging Activity Assay

The hydroxyl radical scavenging activity of EELB was determined by studying the competition between deoxyribose and DMHBR for hydroxyl radical generated by Fenton's reaction.[36] The absorbance was read at 532 nm. The reference compound used was DMSO.

Superoxide Anion Scavenging Assay

The scavenging activity of the ethanol extract of *Lagenaria breviflora* (EELB) on superoxide anion radicals was measured by Liu's method.[37] Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments, the superoxide anion was generated in 3 ml of Tris-HCl buffer (100 mM, pH 7.4) containing 0.75 ml of NBT (300 μM), 0.75 ml of NADH (936 μM) and 0.3 ml of various concentrations of the EELB. The reaction was initiated by adding 0.75 ml of PMS (120 μM) to the mixture. The reaction mixture was incubated at 25C for 5 min, after which the absorbance was measured at 560 nm, spectrophotometrically. The IC_{50} value of EELB was compared with that of ascorbic acid.

Statistical Analysis

All values are expressed as mean ± SD. Data of the ulcer index were analyzed by non-parametric analysis of variance (ANOVA), while one-way ANOVA was used for statistical comparison of the other results, followed by Newman-Keul's multiple comparison test. Differences between means were considered significantly different when values of P <0.05 were obtained using the Graph-Pad Prism software.

RESULTS

Effect of the LB Extract on Gastric Ulcerations

The LB extract produced protection against CRU gastric ulcer in a dose-dependent manner at 50 mg/kg b.w. (19.5%), 100 mg/kg b.w. (20.8%), 150 mg/kg b.w. (68.1%) and 200 mg/kg b.w. (51.4%) comparably with standard anti-ulcer agent omeprazole (83.4%) as shown in Table 1. Similarly, 150 mg/kg b.w. of the LB also produced protection against gastric ulcerations in the range of 52.1%, 50% and 64.96%, respectively, comparable with omeprazole (69.0% and 80%) and sucralfate (77.25%) in PL, ASP and Al models respectively [Figures 1-4].

The LB extract at 150 mg/kg b.w. significantly reduced the free acidity (P < 0.05), total acidity (P < 0.05) and peptic activity (P < 0.05), with a concomitant increase (P < 0.001) in mucin secretion compared with control animals. Free acidity, total acidity and peptic activities in animals administered with omeprazole were also significantly lowered at P <0.001, P <0.001 and P <0.01 respectively compared with animals that received EELB and the control animals [Table 1].

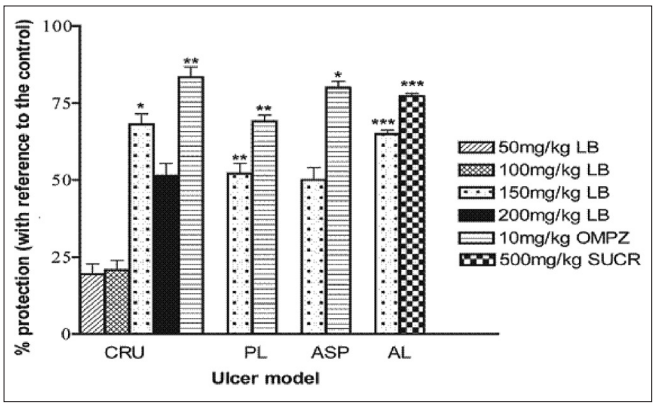

Figure 1: Anti-ulcer effects of the *Lagenaria breviflora* extract against acute gastric ulcer models in rats. Values are expressed as mean of % protection ± S.E.M. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with the control (ANOVA test). n = 6 in each group. Cold- stress-induced ulcer; alcohol-induced ulcer; aspirin-induced ulcer and pyloric ligation-induced ulcer.

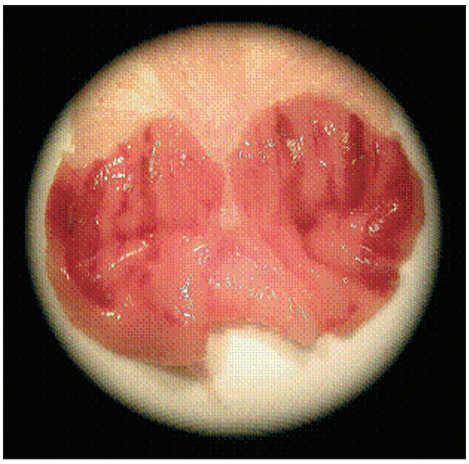

Figure 2: Alcohol-induced gastric ulcer in rats induced by administering absolute alcohol at a dose of 1 ml/200 g body weight

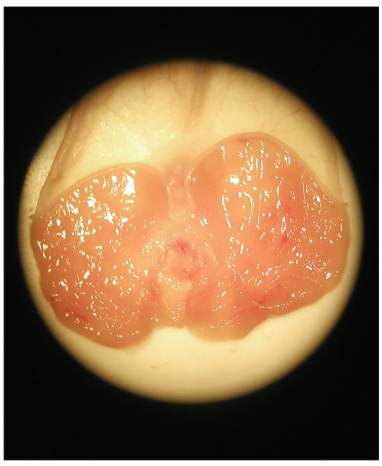

Figure 3: Alcohol-induced gastric ulcer in rats treated with 150 mg/kg of EELB

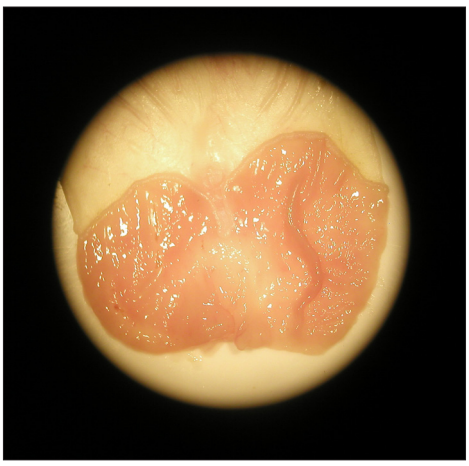

Figure 4: Alcohol-induced gastric ulcer in rats treated with 500 mg/kg of sucralfate

Table 1: Effect of the *Lagenaria breviflora* extract (150 mg/kg) and omeprazole (10 mg/kg) on free acidity, total acidity, peptic activity and mucus secretion of gastric juice in pylorus ligation-induced gastric ulcer model

Group (n = 6)	Free acidity (μeq/ml)	Total acidity (μeq/ml)	Peptic activity (U/ml)	Mucin secretion (μg/ml)
Control (10 ml/kg)	74.45 ± 4.56	141.4 ± 5.96	14.92 ± 1.82	102.6 ± 6.90
LB extract (150 mg/kg)	60.67 ± 4.58^{a*}, ^{b***}	70.93 ± 5.65^{a***}, ^{b***}	10.00 ± 1.06^{a*}, ^{b**}	275.4 ± 24.20^{a***}, ^{b***}
Omeprazole (10 mg/kg)	28.43 ± 1.94^{a***}	42.63 ± 6.17^{a***}	7.950 ± 1.10^{a**}	197.2 ± 15.75^{a**}

Values are expressed as mean ± SEM. *P < 0.05 and **P < 0.01, ***P < 0.001 compared with the control (ANOVA test) Superscript "a" represents comparison of the mean values of mice administered with the *LB* extract or omeprazole with control mice while "b" indicates the comparison between the mice

Effect of LB Extract on Free Radical Generation (*In Vitro*)

The extract exhibited antioxidant activity in the DPPH, nitric oxide, hydroxyl radical and Superoxide anion inhibition assays, as evidenced by the IC50 values in Table 2. The IC50 values obtained are 228.5 μg/ml, 364.3 μg/ml, 244.2 μg/ml and 253.7 μg/ml respectively, compared with the standard drug that had an IC50 of 42.5 μg/ml (ascorbic acid), 38.3 μg/ml (curcumin), 24 mM (DMSO) and 18.4μg/ml (ascorbic acid) respectively.

DISCUSSION AND CONCLUSION

Several plants and herbs have been reported for the treatment of gastrointestinal disorders including ulcers.[38] Antioxidants are known to play a significant role in repairing gastric damage.[39] Similarly, antioxidant parameters are reported to be reduced in the stomach tissue that is damaged by indomethacin.[40] The roles of oxygen radical have also been determined in the etiology and pathogenesis of indomethacin-induced gastric damage.[41]

The results of this study demonstrated that the LB extracts exerted protective effects on, cold restraint, and aspirin-induced ulcers. P and cold-restrained stress-induced ulcers have been shown to result in autodigestion of the gastric mucosal barrier due to excess production of hydrochloric acid in the stomach.[42] Histamine is a well-known powerful gastric secretagogue that evokes a copious secretion of acid from the parietal cells by acting on the H_2 receptors.[43] However, the pathophysiology of PUD with its attendant contributing factors have been documented.[23] The gastric mucus coat is thought to be important in both preventing damage and in facilitating the repair of the gastric epithelium.[44] The necrotizing agents produce ulceration in the gastric mucosa by depleting the gastric mucus and breaking the mucosal barrier.[45] The increase in gastric secretion has been considered as a pathogenic mechanism responsible for stress-induced gastric lesions.[46,47] Findings from this study show that the fruit extract of LB has an effective antiulcer activity against ethanol, aspirin, pylorus ligation and cold restrained induced ulcerogenesis and can therefore be used as therapy for ulcerogenesis and gastric mucosal injury. The ability of the LB extract to inhibit gastric mucus depletion, as observed in our study, might be responsible for increasing the mucosal resistance against noxious chemicals as described.[48] Also, the antiulcer activity in these models might be attributed to an antisecretory effect of the extract.

Table 2: *In vitro* antioxidant activities of the LB extract on some antioxidant models

Concentration	Scavenging activity of EELB (% inhibition)*			
(μg/ml)	DPPH	Nitric oxide	Hydroxyl radical	Superoxide anion
10	8.5 ± 4.32	17.3 ± 0.98	14.8 ± 6.77	-
100	14.9 ± 10.21	24.1 ± 3.45	41.9 ± 3.45	13.5 ± 2.01
200	27.7 ± 1.33	26.8 ± 0.76	49.5 ± 7.40	37.2 ± 2.14
300	38.1 ± 0.64	31.7 ± 1.37	57.9 ± 3.79	40.8 ± 2.87
400	52.4 ± 6.27	37.9 ± 3.83	66.2 ± 2.42	51.4 ± 3.81
500	66.3 ± 7.60	52.7 ± 3.71	74.5 ± 3.40	64.1 ± 1.76
IC50 (μg/ml)	228.5 ± 10.69	364.3 ± 5.77	244.2 ± 12.77	253.7 ± 13.94

*Values are expressed as percentage mean ± SEM of six replicates

According to our data, we observed that the LB plant extracts dose-dependently and significantly inhibited the free radical and superoxide anion. On the basis of the results of this study, it is clearly indicated that LB has a powerful antioxidant activity. LB can be used as a good source of natural antioxidants and as a possible food supplement. The mechanisms of the antioxidant activity of LB may be attributed to a metal-chelating ability, strong hydrogen-donating ability and their effectiveness as scavengers of hydrogen peroxide, superoxide anion and nitric oxide free radicals. In conclusion, LB has potent anti-ulcer and antioxidant properties. Further work is therefore recommended to elucidate the molecular mechanism(s) that might be responsible for this novel activity of LB.

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