Original Paper

Evaluation of the Antiulcer Activity of the Leaves of *Azadirachta indica*: An Experimental Study

Pranjit Santonu Bhajoni^{a, b} Girish Gulab Meshram^a Mangala Lahkar^b

^aDepartment of Pharmacology, Postgraduate Institute of Medical Education and Research and Dr. Ram Manohar Lohia Hospital, New Delhi, and ^bDepartment of Pharmacology, Gauhati Medical College and Hospital, Guwahati, India

Key Words

Azadirachta indica · Leaves · Aqueous extract · Antiulcer · Pyloric ligation · Aspirin-induced ulcer · Cold restraint stress-induced ulcer

Abstract

Background: *Azadirachta indica*, an evergreen tree, is used by several folkloric practitioners to treat peptic ulcers in India. The present study was carried out to evaluate the antiulcer activity of the aqueous extract (AE) of the leaves of *A. indica* in Wistar rats. **Methods:** Gastric ulcerations were induced by pyloric ligation, aspirin, and cold restraint stress. AE was used in doses of 150, 300, and 600 mg/kg body weight per os. Distilled water served as the control and ranitidine 20 mg/kg body weight intraperitoneal as the reference standard. The ulcer index (UI) and percentage inhibition (PI) values were determined in each model. The volume of gastric contents, free acidity, total acidity, and pH were measured in the pyloric ligation-induced ulcer model. **Results:** AE showed a dose-dependent and significant ($p < 0.05$) decrease in the UI and an increase in the PI in all models employed compared to the control group. AE caused a dose-dependent decline in the gastric content volume, free acidity, and total acidity. **Conclusion:** The leaves of *A. indica* possess significant antiulcer activity and act via multiple mechanisms.

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Introduction

Despite the decline in the incidence of peptic ulcer disease (PUD) in the recent years, the economic burden, morbidity, and mortality due to the disease are massive [1]. The most effective classes of drugs available to treat PUD include proton pump inhibitors, histamine-2 receptor blockers, and prostaglandin analogues [2]. The efficacy of these agents is marred by their numerous adverse effects which include gastrointestinal dysfunction, mental state changes, and an increased risk of respiratory/enteric infections. Furthermore, the various cytochrome enzyme interactions of these agents can also affect the therapeutic levels of other agents [3]. The limitations of the therapeutic agents available, their interactions, and their adverse effects are leading researchers to investigate various medical plants which could provide an excellent source for newer molecules that could be more safe, efficacious, and economical.

Azadirachta indica (A. Juss), called *Arishta* in Sanskrit, is an evergreen tree found in most parts of India. The Ayurvedic practitioners in India have been using the tree for curing illnesses such as peptic ulcers, leprosy, fever, asthma, epistaxis, intestinal worms, piles, diabetes, urinary tract infections, scabies, ringworm, and spermatorrhoea [4]. Modern-day research has led to the validation of most of these folkloric claims. The bark, leaves, and seeds of *A. indica* have emerged as valuable sources for new bioactive compounds due to their pleiotropic pharmacological activities. The leaves specifically have proven their value by their immunomodulatory, antiinflammatory, antihyperglycemic, antiulcer, antimalarial, antifungal, antibacterial, antiviral, antioxidant, antimutagenic, and anticarcinogenic properties [5]. High-performance thin layer chromatography studies of the leaves of *A. indica* have revealed the presence of several bioactive molecules such as azadirachtins, 6 de-acetylnimbin, azadiradione, nimonol, epoxyazadiradione, and cyclic sulfides [6, 7]. Since the reports about the antiulcer activity of the leaves of *A. indica* were sparsely documented, it was considered worthwhile to investigate the antiulcer activity of the aqueous extract (AE) of the leaves of *A. indica* and substantiate its ethnopharmacological claim of providing relief in PUD.

Materials and Methods

Plant Material

The leaves of *A. indica* were obtained from the campus of the Gauhati Medical College (GMC), Guwahati, and authenticated by a botanist from the Department of Botany, Gauhati University. A voucher (specimen number GMC-AI-24) was deposited at the Department of Botany for further reference. Five hundred gram of the powder prepared from shade-dried leaves was subjected to Soxhlet extraction for 16 h using 5 liter of distilled water. The greenish, semisolid AE obtained was dried under partial vacuum using a rotary evaporator. The percentage yield of the AE of the leaves was 20.34%, based on the starting quantity. The doses of 150, 300, and 600 mg/kg body weight (b.w.) were prepared by suspending the dried AE in distilled water and administering it to the rats by the per os (p.o.) route.

Animals

Male Wistar rats weighing 150–200 g were obtained from the Animal House, GMC, Guwahati. The animals were maintained in proper conditions, at temperatures of $25 \pm 1^\circ\text{C}$, a 12-hour light/dark cycle, and a relative humidity of 44–56%, and were fed with standard rodent diet and water ad libitum. Animals were fasted for 12–24 h but had free access to water up to 1 h before the commencement of the induction of ulcers.

Drugs

Ranitidine and aspirin were procured from Merck, Bangalore, India. NaOH, Toppfer's reagent, and phenolphthalein were obtained from Scientific OEM, Mumbai, India.

Groups

The rats were randomly divided into 5 groups (n = 6) for each of the 3 models employed in the study. Each rat in the respective group received distilled water (control), or ranitidine 20 mg/kg b.w intraperitoneally (i.p.) (reference standard), or AE 150 mg/kg b.w. p.o., or AE 300 mg/kg b.w. p.o., or AE 600 mg/kg b.w. p.o. for 7 days.

Pyloric Ligation-Induced Ulcer Model

The experiment was conducted according to the method described earlier [8]. After the 7th day of drug administration, the rats were fasted for 24 h. Light ether anesthesia was used to make a midline abdominal incision so as to ligate the pylorus without causing it any traction or damage to its blood supply. The stomach was replaced carefully and the abdominal wall was closed with sutures. The rats were deprived of water during the postligation period. Four hours after the procedure, the rats were sacrificed, and the stomachs were dissected out and cut open along the greater curvature so as to determine the ulcer index (UI) by the Ganguly and Bhatnagar method [9]. The volume of the gastric content was measured after centrifugation, while the acidity was determined by titration with 0.01 N NaOH using Toppfer's reagent and phenolphthalein as indicators [10]. The percentage inhibition (PI) of the ulcer production was also calculated [11].

$$PI = \frac{UI \text{ of the control group} - UI \text{ of the treatment group}}{UI \text{ of the control group}} \times 100.$$

Aspirin-Induced Gastric Ulcer Model

The experiment was carried out according to the method described earlier [12]. Following 1 h of the administration of the last dose of the test and control compounds, the rats were fasted for 24 h and were administered aspirin in a dose of 200 mg/kg b.w. p.o. Four hours after aspirin induction, the animals were sacrificed and their stomachs were dissected out. The UI and PI were determined as described in the previous model.

Cold Restraint Stress-Induced Ulcer Model

The experiment was conducted according to the method described earlier [13]. The test and control groups were deprived of food for 12 h after the administration of the last dose of the drugs. The rats were then immobilized in a steel cage and placed at a temperature of 3–5°C for 3 h, following which they were euthanized by cervical dislocation. The ulcers were then examined on the dissected stomachs so as to determine the UI and PI as described in the previous models.

Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by multiple Tukey's comparison test. A value of p < 0.05 was considered statistically significant.

Ethical Considerations

The experimental protocols and procedures used in the study were approved by the Institutional Animal Ethics Committee of the GMC and conformed to the *Guidelines for Care and Use of Animals in Scientific Research* (Indian National Science Academy, 1998, Revised 2000).

Fig. 1. Photographs showing aspirin-induced gastric ulceration in a rat's stomach subjected to normal saline (a), ranitidine 20 mg/kg b.w. i.p. (b), AE of *A. indica* leaves 150 mg/kg b.w. p.o. (c), AE of *A. indica* leaves 300 mg/kg b.w. p.o. (d), and AE of *A. indica* leaves 600 mg/kg b.w. p.o. (e).

Results

The AE in doses of 150, 300, and 600 mg/kg b.w. caused a significant ($p < 0.05$) and dose-dependent decrease in the UI and an increase in the PI compared to the control group in all 3 models employed in the study. The UI of AE 600 mg/kg b.w. was comparable to that of the standard reference group (ranitidine 20 mg/kg b.w.) in the pyloric ligation-induced ulcer model and the aspirin-induced ulcer model (fig. 1) but not in the cold restraint stress-induced ulcer model. The ranitidine 20 mg/kg b.w. i.p. group showed the highest PI values in all 3 models employed in the study (table 1). There was a significant rise in the pH with a reduction in the volume of gastric contents, free acidity, and total acidity in the AE-treated groups as compared to the control group (table 2).

Discussion

Acute toxicity studies of the leaves of *A. indica* determined its LD_{50} to be 4,800 mg/kg i.p., hence the doses of 150, 300, and 600 mg/kg b.w. p.o. were chosen for the study [14].

The pyloric ligation-induced ulcer model evaluates the antisecretory and gastroprotective effects of investigational agents. The ligation of the pyloric end of the stomach leads to accumulation of gastric acid which causes ulcers due to the autodigestion of the mucosa [15]. AE caused a significant decrease in the gastric volume, free acidity, and total acidity compared to the control group, indicating an antisecretory mechanism. The antisecretory

Table 1. UI and PI values obtained in the models examined

Models	Groups	UI	PI
Pyloric ligation-induced ulcer model	Control	1.63±0.04 [†]	
	Ranitidine 20 mg/kg b.w. i.p.	0.32±0.04*	80.37
	AE 150 mg/kg b.w. p.o.	0.65±0.05*, [†]	60.12
	AE 300 mg/kg b.w. p.o.	0.57±0.05*, [†]	65.03
	AE 600 mg/kg b.w. p.o.	0.45±0.14*	72.39
Aspirin-induced ulcer model	Control	3.66±0.32 [†]	
	Ranitidine 20 mg/kg b.w. i.p.	1.08±0.21*	70.49
	AE 150 mg/kg b.w. p.o.	1.53±0.13*	58.20
	AE 300 mg/kg b.w. p.o.	1.38±0.14*	62.30
	AE 600 mg/kg b.w. p.o.	1.27±0.13*	65.30
Cold restraint stress-induced ulcer model	Control	1.81±0.05 [†]	
	Ranitidine 20 mg/kg b.w. i.p.	0.29±0.03	83.98
	AE 150 mg/kg b.w. p.o.	0.75±0.05*, [†]	58.56
	AE 300 mg/kg b.w. p.o.	0.59±0.05*, [†]	67.40
	AE 600 mg/kg b.w. p.o.	0.51±0.07*, [†]	71.82

One-way ANOVA followed by multiple Tukey's comparison test. Values are mean ± SEM, n = 6 in each group. * p < 0.05 compared to the control group. [†] p < 0.05 compared to the ranitidine 20 mg/kg b.w. i.p. group.

Table 2. pH, volume of gastric contents, free acidity, and total acidity of the test and control groups in the pyloric ligation-induced ulcer model

Groups	pH	Volume of gastric contents, ml	Free acidity, mEq/l	Total acidity, mEq/l
Control	2.33±0.17 [†]	11.33±0.88 [†]	38.33±1.33 [†]	73.25±1.93 [†]
Ranitidine 20 mg/kg b.w. i.p.	4.92±0.11*	5.05±0.33	24.83±2.55	50.83±1.82*
AE 150 mg/kg b.w. p.o.	2.60±0.15 [†]	9.33±0.25*, [†]	36.50±1.52 [†]	67.33±2.25 [†]
AE 300 mg/kg b.w. p.o.	3.40±0.17*, [†]	8.23±0.39*, [†]	31.17±1.14*	59.83±1.30*, [†]
AE 600 mg/kg b.w. p.o.	4.51±0.14*	6.32±0.17*	27.17±0.70*	55.50±1.31*

One-way ANOVA followed by multiple Tukey's comparison test. Values are mean ± SEM, n = 6 in each group. * p < 0.05 compared to the control group. [†] p < 0.05 compared to the ranitidine 20 mg/kg b.w. i.p. group.

activity could be due to the inhibition of the H⁺-K⁺-ATPase enzyme [16, 17]. The UI and PI are parameters commonly used to determine the gastroprotective effect of investigational agents. AE in all doses caused a significant reduction in the UI and an improvement in the PI compared to the control group, indicating a gastroprotective effect.

Aspirin is a nonsteroidal antiinflammatory drug which induces ulcers by inhibiting prostaglandin synthesis in the stomach by blocking the cyclooxygenase enzymes [2]. Nonsteroidal antiinflammatory drugs also cause an inflammatory response increasing the reactive oxygen species in the gastric mucosa [18]. Previous studies have shown that the leaves of *A. indica* possess reactive oxygen species scavenging activity, suggesting the role of antioxidation as one of the mechanisms responsible for its gastroprotective action [19, 20]. In the present study, AE in all doses caused a significant reduction in the UI and an improvement in the PI,

indicating a possible involvement of the prostaglandin pathway. Mechanistic studies measuring levels of prostaglandin E₂, myeloperoxidase, and proinflammatory cytokines (interleukin 8, tumor necrosis factor- α) could elucidate this reasoning.

Cold restraint causes both psychological and physical stress to the rats. The induced stress releases histamine in the stomach, which leads to increased acid secretion and decreased mucus production, ultimately leading to ulcers [21]. AE caused a dose-dependent significant reduction in the UI in this model, suggesting the role of histamine in its mechanism, as suggested by a previous study [17]. An earlier study revealed that *A. indica* prevented stress-induced DNA fragmentation in the gastric mucosal cells, thus preventing their apoptosis [16]. The mast cell stabilization activity of *A. indica* inhibiting the release of histamine is also postulated as one of the mechanisms involved in its antisecretory action [22].

Phytochemical studies of *A. indica* have revealed that the leaves are a rich source of potentially bioactive alkaloids, flavonoids, tannins, and saponins [23]. High-performance thin layer chromatography and gas chromatography mass-spectrometry studies have determined that the leaves contain several compounds such as nimbic acid B, nimbolide B, azadirachtins, 6-deacetylnimbin, azadiradione, nimonol, epoxyazadiradione, quercetin-3-O- β -D-glucosamine, myricetin-3-O-rutinoside, quercetin-3-O-rutinoside, kaempferol-3-O-rutinoside, kaempferol-3-O- β -D-glucoside, quercetin-3-O- α -L-rhamnoside, hydroxypivalic acid, phytol, 4-cycloocten-1-ol, 8,8'-(iminodi-2,1-phenylene) bis-, 1,3-diphenyl-2-azafluorene, 3 β -lup-20(29)-en-3-ol, 3 β -lup-20(29)-en-3-yl acetate, germanicol, and cyclic sulfides [6, 7, 23–26]. Flavonoids and saponins are known to exhibit a myriad range of pharmacological activities, hence the anti-ulcer activity of the leaves of *A. indica* could be attributed to its flavonoids and saponins. However, the role of other secondary alkaloids cannot be eliminated [27, 28]. Future mechanistic studies could help determine the exact pharmacodynamics and mode of action of the bioactive alkaloids of *A. indica*.

Conclusion

In conclusion, the leaves of *A. indica* possess antiulcer activity and possibly act via multiple mechanisms including inhibition of the histamine-2 receptors/H⁺-K⁺-ATPase, prostaglandin modulation, or antioxidation. The present study confirms the folkloric claim of *A. indica* being effective in the treatment of PUD.

Disclosure Statement

The authors have no conflicts of interest to declare.

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