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Andrographaloide from *Andrographis pannicuolata* significantly inhibited the H^+ K^+-ATPase and increased the PGE2 level in rats

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ABSTRACT

Evidences have suggested that andrographolide (AP) attenuates gastric mucosal injury; however its mechanism has not yet been established. The aim of the present study was to evaluate the gastroprotective mechanism of andrographolide isolated from *Andrographis paniculata*. Andrographolide was evaluated against cold restraint (CRU), aspirin (AS), alcohol (AL) and pyloric ligation (PL) induced gastric ulcer models in rats. Potential anti-ulcer activity of andrographolide was observed against CRU (62.5%), AS (57.81%), AL (72.41%) and PL (60.00%) induced ulcer models. The standard drug omeprazole (10mg/kg, p.o.) showed 77.40% protection against CRU, 57.08% against AS and 69.42% against PL model. Sucralfate, another standard drug (500 mg/kg, p.o.) showed 62.72% protection in AL induced ulcer model. andrographolide significantly reduced free acidity (50.42%), total acidity (24.43%) and upregulated mucin secretion by 34.24% respectively. Further, andrographolide inhibited H^+ K^+-ATPase activity *in vitro* with IC_{50} of 71.435 µg/ml respectively as compared to the IC_{50} value of omeprazole (30.24 µg/ml) confirming their anti-secretory activity. Conclusively, the anti-secretory mechanism of andrographolide mediated apparently through inhibition of H^+ K^+-ATPase with corresponding decrease in plasma gastrin level, which is a novel property in our finding. Andrographolide was found to possess anti-ulcerogenic activity which might be due to its anti-secretory activity and subsequent strengthening of the defensive mechanism.

KEYWORDS

Gastric ulcer;
Proton pump;
Gastric acid secretion;
Andrographolide;
Gastrin level.

INTRODUCTION

Gastric ulcer disease (GUD) is one of the major gastrointestinal disorders which occur due to an imbalance between offensive (acid, pepsin and *Helicobacter pylori*) and defensive (mucin, prostaglandin and bicarbonate) factors. Consequently reduction of gastric acid production as well as reinforcement of gastric mucosal protection has been the major therapeutic approaches of gastric ulcer disease[1]. A number of anti-ulcer drugs including proton pump inhibitors (PPI) and H_2 receptor antagonists are available for the treatment of GUD, but

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clinical evaluation of these drugs has shown incidence of relapse, side effects and drug interactions. This has been the rationale for the development of new anti-ulcer drugs and thus the search for novel molecules has been extended to medicinal plants that can offer better protection and decrease relapses.

Several Indian medicinal plant species like *Allophylus serratus*[2], *Desmodium gangeticum*[3], *Ocimum sanctum*[4], *Xylocarpus granetum*[5] etc. have been reported to possess anti-ulcer activity. Studies on different biological activities of *A. paniculata* in general are also available. But there is less information available regarding its mechanism of action on gastrointestinal system. Keeping these facts in considerations, we have assessed the anti-ulcer activity of the andrgrapholide isolated from *Andrographis paniculata*.

Andrographis paniculata belongs to the Natural Order Acanthaceae. *A.panniculata* is a medicinal plant, commonly known as king of bitters. *A.panniculata* was reported to possess antimicrobial activity[6], antiviral properties[7] hepatoprotective and antioxidant[8], antidiabetic[9], anti hyperglycaemic activity (10), anti angiogenicactivity (11), anti inflammatory property[12], and treatment of upper respiratory tract infections[13]. The present study was undertaken to investigate the underlying mechanism of the anti-ulcer property of Andrographolide isolated from *A.panniculata* responsible for these gastroprotective effects by which mechanism of action.

MATERIALS AND METHODS

Plant material

A. paniculata plant grows naturally in tidal forests along the East and West coastal areas up to Maharastra and in Andaman Island. The *A. paniculata* leaves were purchased from Lucknow market and was authenticated by botany division of the Central Drug Research Institute (CDRI), Lucknow.

Extraction/fractionation procedure

The shade dried leaves (1.0 Kg) was powdered and extracted with 95% ethanol (4x2.0 lit). Combined extrac was filtered and concentrated under reduced pressure below 50^0C in a rotavapour To a green viscous mass.(31.2g). The ethanol extract thus obtained was macerated with chloroform and concentrated in a rotavapour to get chloroform soluble fraction (4.2 g). The chloroform soluble fraction was dissolved in 25 ml of methanol and left in a refrigerator. White deposit in the solution was filtered and identified as andrographolide mixture by physicochemical methods reported in the literature. This mixture of andrographolides was used for biological screening of antiulcer activity.

Experimental animals

Adult Sprague Dawley rats of either sex, weighing 180-200g were housed in raised bottom mesh cages to prevent coprophagy and were kept in environmentally controlled rooms (25 ± 2°C, 12 hours light and dark cycle). Animals were fed with standard laboratory food pellets and water was provided *ad libitum*. All experimental protocols were approved by our Institutional Ethical Committee following the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) which complies with International norms of INSA (Indian National Science Academy).

Materials

Sucralfate was obtained from Meranani Pharmaceuticals, India, whereas omeprazole and other chemicals were obtained from M/s. Sigma Chemicals, St Louis, MO, USA.

Treatment schedule

Andrographolide (20 mg/kg), standard drug omeprazole (Omz) (10 mg/kg) and sucralfate (SUC) (500 mg/kg) were prepared in 1% carboxymethyl cellulose (CMC) as suspension and administered orally 45 mins prior to exposure of ulcerogens to the animals at a volume of 1ml/200g of body weight. All animals were deprived of food for 16 h before ulcerogens exposure and were divided into three groups, (n=6).

1. Control group of animals were treated with vehicle 1% CMC.
2. Graded doses of Andrographolide (20 mg/kg p.o.) were tested against Cold restraint ulcer (CRU) model to identify the effective dose and selected for further studies in other ulcer models.
3. Experimental group was treated with standard anti-ulcer drugs such as Omz (10 mg/kg, in (CRU), aspi-

rin (AS), pyloric ligation (PL), and SUC (500 mg/kg, p.o.) in Alcohol (AL) induced ulcer model.

Anti-ulcer studies

(a) Cold restraint induced gastric ulcer (CRU)

Animals were subjected to cold restraint stress after 45 mins of treatment with andrographolide and Omz. All the animals were immobilized in restraint cage and kept at 4^0C in an environmental chamber[14]. Two hours later the animals were sacrificed and stomachs were observed and scored under Magnascope for ulcers.

(b) Aspirin induced gastric ulcer model (AS)

Aspirin at a dose of 150 mg/kg was administered to induce ulcer after 45mins of treatment of andrographolide and omeprazole. The animals were sacrificed 5 hours after aspirin treatment[15] and the stomach was dissected out, incised along the lesser curvature and the lesion was scored.

(c) Alcohol induced gastric ulcers in rats (AL)

Gastric ulcer was induced in rats by administering chilled absolute alcohol (1ml/200g, body weight of animals)[16]. The Andrographolide and sucralfate were administered 45 minutes before alcohol treatment. After 1 hour of alcohol administration, the animals were sacrificed and stomach was cut open along the greater curvature to observe the gastric lesions which appear as hemorrhagic bands along the mucosal ridges of the stomach. The lengths of the lesions were measured using Biovis image analyzer software and summated to give a total lesion score.

(d) Pyloric ligation induced ulcer model (PL)

After 45 mins of administration of Andrographolide and Omz, ulcer was induced in rats by pyloric ligation. Under Chloral hydrate anesthesia (300mg/kg, i.p.), the abdomen was opened and the pyloric end of the stomach was ligated avoiding any damage to the adjacent blood vessels[17]. Stomach was replaced carefully and the animals were allowed to recover with free access to water. After 4 hours the animals were sacrificed and the stomach was dissected out. Lesions were scored and gastric fluid was collected and centrifuged at 2000 rpm for 10 mins. The collected supernatant was used for the estimation of gastric secretion studies, mucin estimation and peptic activity.

Gastric secretion study

Free and total acidity was measured from the collected gastric juice by titrating against 0.01N NaOH, using phenolphthalein as an indicator and expressed in terms of μequiv./ml[18]. Mucus content was expressed in terms of mg%[19].

Measurement of ulcer index

Ulcer formed due to treatment with different ulcerogens were observed under Magnascope (5X magnification) and were scored according to the arbitrary scoring system as described by[20]. The severity and intensity of the lesions were graded as following: i) Shedding of epithelium = 10; (ii) Petechial and frank hemorrhages = 20; (iii) one or two ulcers = 30; (iv) more than two ulcers = 40; and (v) Perforated ulcers = 50.

Gastrin measurement

In order to determine the gastrin levels in plasma, blood was collected by cardiac puncture, centrifuged, and the plasma was analyzed for gastrin levels using rat gastrin I enzyme immunoassay kit (assay designs, Hines Drive Ann Arbor, USA) following the manufacturer's instructions. The results were expressed as pg/ml.

PGE_2 estimation

PGE_2 was determined in gastric tissue obtained from sham, control and treatment groups. Briefly, mucosa was scrapped and rapidly rinsed with ice-cold saline. The tissue was weighed and homogenized in 10 volumes of phosphate buffer (0.1 M, pH- 7.4) containing 1 mM EDTA and 10 μM indomethacin. The homogenate was centrifuged (10,000 rpm, 10 min, 4°C), and the supernatant was processed for PGE_2 estimation using the Biotrak enzyme immunosorbent assay kit (Cayman), following the manufacturer's instructions. Results were expressed as pg PGE_2/mg protein.

In vitro assay of H^+ K^+-ATPase activity

H^+ K^+-ATPase activity was assayed in gastric microsomes isolated from normal fasted rat stomach[21]. For the enzyme assay, gastric microsomes incubated with or without different concentrations of andrographolide as well as standard drug Omz for 10 min at 37°C, were added to an assay buffer containing (in mM) 150 KCl, 10 PIPES, 1 $MgSO_4$, 5 Mg ATP, 1

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EGTA and 0.1 ouabain, at pH 7.2 and 10µg/ml valinomycin, 2.5µg/ml oligomycin. The reaction was carried out at 37°C for 20 min and was stopped by adding 10% ice-cold trichloroacetic acid. After centrifugation (2000 g for 1 min), inorganic phosphate release was determined from the resulting supernatant spectrophotometrically at 310 nm wavelength[22] and expressed as µM/hr/mg protein.

Statistical analysis

All values shown in the figures and tables represent the means ± S.E.M. IC_{50} values with 95% confidence limits were estimated using Maximum Likelihord Iterative Procedure[23]. Statistical analysis was performed with Prism version 3.0 software using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. P<0.05 was considered to be statistically significant.

RESULTS

Anti-ulcer effect of *Andrographolide* against cold restraint induced ulcer in rats

Graded doses of andrographolide (10, 20 and 40mg/kg, p.o.) showed percentage protection of 37.5, 62.5 (P<0.01) and 68.7 (P<0.01) respectively whereas standard drug, omeprazole showed a percentage protection of 77.4 (P<0.01) in comparison to control against CRU model. From this observation 20mg/kg dose of Andrographolide was identified as the effective dose and selected for further studies. The results are graphically represented in Figure 2.

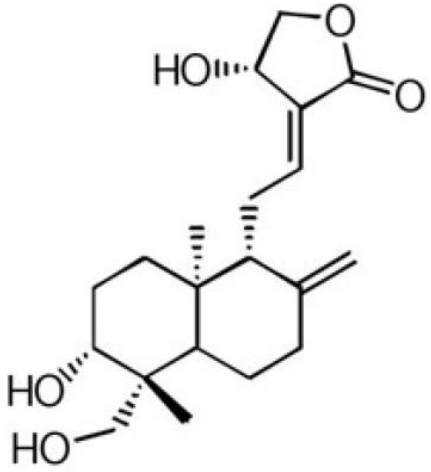

Figure 1 : Chemical structure of andrographolide.

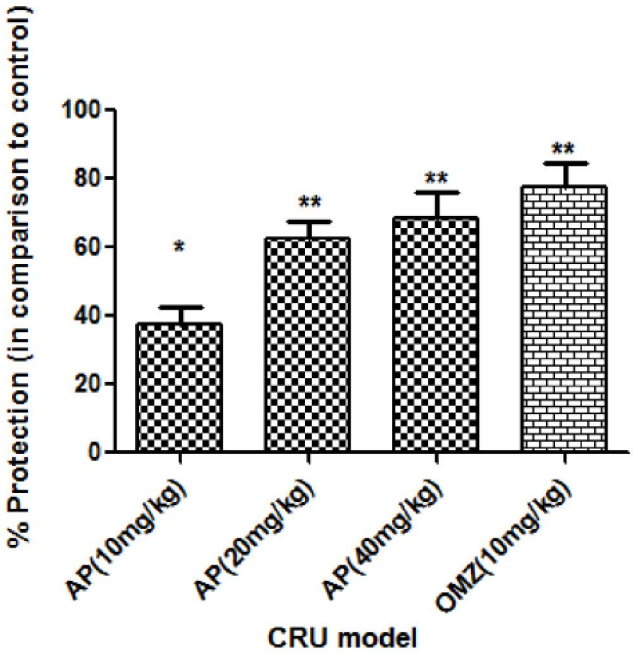

Figure 2 : Effect of graded dose of andrographolide (AP) and standard drugs (Omz) on percentage protection of ulcer against cold restraint induced gastric ulcer models in rats. Data expressed as mean % protection ± S.E.M. Statistical analysis was done by One Way ANOVA followed by dunnett's multiple comparison test. *Statistically significant at P<0.05 and **P< 0.01, in comparison to control. n = 6 in each group.

Effect of andrographolide against aspirin induced ulcer in rats

Potential anti-ulcer activity of andrographolide was observed when its efficacy was tested against aspirin induced ulcer model. 57.81% protection (P<0.01) was observed when andrographolide was administered whereas omeprazole showed 57.08% protection in comparison to control as shown in Figure 3.

Effect of andrographolide against alcohol induced ulcer

Andrographolide showed significant anti-ulcer activity against ethanol induced ulcer having 72.41% protection (P<0.01), whereas the standard drug, sucralfate, showed 62.72% protection (P<0.05) as depicted in Figure 3.

Anti-ulcer effect of andrographolide against pyloric ligation induced ulcer in rats

Anti-ulcer activity of andrographolide was also observed against pyloric ligation induced ulcer in rats where it showed protection of 60.00% (P<0.01) and omeprazole showed 69.42% (P<0.01) protection

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(Figure 3).

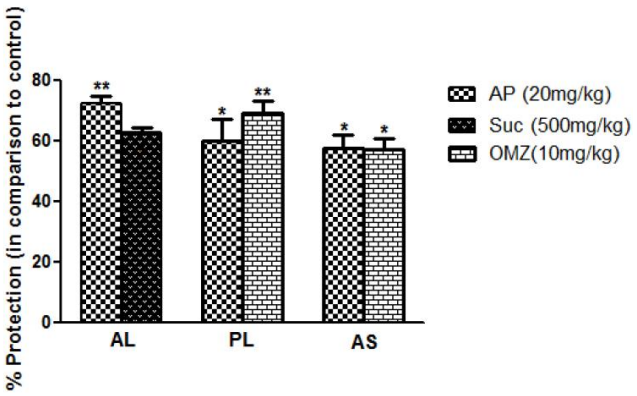

Figure 3 : Effect of andrographolide (AP) and standard drugs (Omz and SUC) on percentage protection of ulcer against cold restraint, aspirin, pyloric ligation and alcohol induced gastric ulcer models in rats and histamine induced duodenal ulcer in guineapigs. Data expressed as mean % protection ± S.E.M. Statistical analysis was done by one way ANOVA followed by dunnett's multiple comparison test. *Statistically significant at P<0.05 and **P< 0.01, in comparison to control. n = 6 in each group.

Effect of Andrographolide on gastric secretion

The antisecretory effect of andrographolide was evaluated by estimating free and total acidity of gastric juice and by estimating the activity of mucin as shown in TABLE 1. Andrographolide has reduced free acidity (50.42%, P<0.01), total acidity (24.43%) which was comparable with standard drug omeprazole (60.37%, P<0.01) and (30.50%, P<0.05) respectively. It significantly upregulated mucin secretion by 34.24% (P<0.01) whereas omeprazole increased mucin secretion by 17.53% (P<0.05) in comparison to control.

TABLE 1 : Effect of CA and Omz on free acidity, total acidity and mucin contents in pyloric ligation model (n= 6 in each group).

Treatment	Free acid μequiv./ml	Total acid μequiv./ml	Mucin μg/ml
Control	58.30±3.182	142.10±2.560	3829.027±44.16
Andrographolide	28.9±2.256*	107.38±3.950	5823.127±205.2**
Omz(10mg/kg)	23.10±3.760**	98.75±1.320*	4643.294±12.432*

Effect of *Andrographolide* on H^+ K^+-ATPase activity

The antisecretory mechanism of action of andrographolide (10-100μg/ml), has been confirmed through the inhibition of gastric H^+K^+-ATPase activity in comparison with control with an IC_{50} value of 71.435μg/ml. Omeprazole (10-50μg/ml) used as positive control reduced the enzyme activity with an IC_{50} value of 30.24μg/ml (Figure 4.)

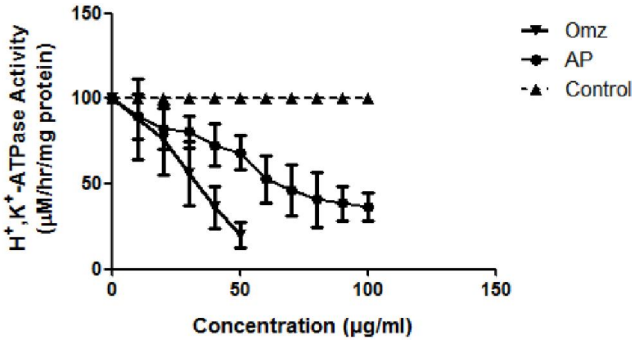

Figure 4 : Effect of AP and standard drug Omz on H+ K+-ATPase activity in the rat gastric microsomes. Dots and lines are mean±S.E.M. of experiments performed in triplicates (n=3). *Statistically significant at P<0.05 and **P< 0.01, in comparison to control. n = 6 in each group.

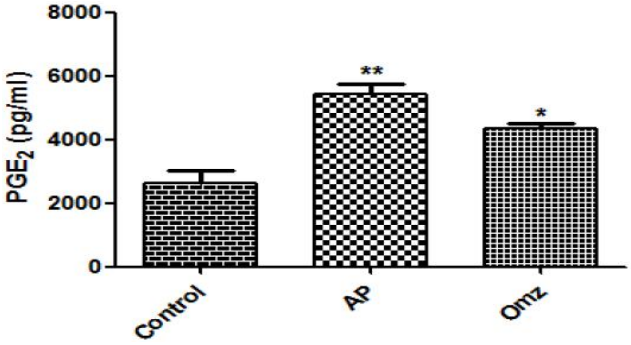

Figure 5 : Effect of AP and Omz on gastric PGE_2 level in comparison to ulcer control group. *Statistically significant at P<0.05 and **P< 0.01, in comparison to control. n = 6 in each group.

DISCUSSION

In our modern times, the use of medicinal plants and natural products has become universal. Natural products have gained powerful attention due to its effective roles in chemo-therapeutic agents. The discovery of new and novel pharmaceutical products from plants used in traditional system of medicine or folklore for the treatment or amelioration of the incidence of gastric ulcers. The anti-ulcer activity of andrographolide isolated from *A. paniculata* has been studied against various models of experimentally induced gastric ulcer in order to evaluate its mechanism of action involved in prevention of ulcer formation. The finding receives an impetus by considering the fact that Andrographolide isolated from *A. paniculata* showed anti-ulcerogenic activity in all the models, each of which induced ulcer

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through a different mechanism.

Gastric ulcer is postulated to develop when there is a disbalance of aggressive and defensive factors either because of increased secretion of acid or pepsin or because of impairment of mucosal resistance. So we select two models one of antisecretory and other of cytoprotective for the preliminary gastroprotective study of andrographolide.

We performed a dose dependent anti-ulcer study of andrographolide in CRU model. CRU is a well-accepted model for the induction of gastric ulcers, in which peripheral sympathetic activation and increased acid secretion play important roles. Andrographolide exhibited significant protection in a dose dependent manner in the CRU model, In addition, Andrographolide exerted a protective effect against ethanol-induced gastric lesions in contrast to standard drug, sucralfate. Since ethanol damages the superficial epithelial layers and inhibit the release of mucosal prostaglandins and depresses the gastric defensive mechanisms, these agents appear to augment the gastric mucosal defense indicating the cytoprotective potentials of andrographolide.

Graded doses of andrographolide exerted anti-ulcer effect in the CRU model, offering maximum protection at much lower dose 20mg/kg indicating active constituents isolated from *A. paniculata*. Hence, 20mg/kg dose was considered to be the optimal dose for evaluation in further studies. Andrographolide was highly effective in decreasing the hemorrhagic lesions induced by ethanol in contrast to standard drug, sucralfate, reflecting its cytoprotective activity.

Furthermore, gastric acid is an important factor for the genesis of ulceration in pyloric-ligated model. In this model, auto-digestion of mucosa by gastric acid and pepsin results in the development of ulcers. Andrographolide significantly reduced free and total acidity in this model, which suggests its anti-secretory potency.

In an attempt to clarify the mode of action of andrographolide, through the anti-secretory pathway, its influence on gastric secretion was studied using inhibition of H^+ K^+ATPase (Proton pump). This proton pump is a membrane bound enzyme that catalyses H^+ transport at the expense of ATP hydrolysis. Thus the inhibition or the blockade of H^+ K^+-ATPase may account for suppressed acid secretion observed in the *in vivo* studies. The results obtained with gastric microsomes isolated from rat stomach showed that andrographolide potently inhibited the H^+ K^+-ATPase activity comparable to the positive control, Omeprazole, thus suggesting that andrographolide might be imparting anti-ulcer activity through decrease in acid secretion via proton pump inhibition.

The cytoprotective ability of andrographolide was evident with increase in mucin content in pyloric ligation model and protection against ethanol induced ulcer model in comparison with the standard drugs. To further substantiate the cytoprotective potency of andrographolide, its effect against NSAIDs induced ulcer model was explored. Studies suggest that NSAIDs induces ulcers due to their effect on cyclooxygenase enzyme leading to reduced prostaglandin production and increase in acid secretion. Andrographolide significantly reduced ulcer incidence, which further supports cytoprotective effect of andrographolide, which may be mediated by prostaglandins. We also measure the level of prostaglandins we found that it also significantly incresaes the level of prostaglandins.

Phytochemical investigations of *A. paniculata* demonstrated the presence of pure compounds namely andrographolide. The gastroprotective activity of the compounds andrographolide is established but the exact mechanism of gastroprotection is not well established. Thus, we investigated the effect of andrographolide on H^+ K^+-ATPase inhibitory activity in isolated gastric microsomes from rat stomach. Andrographolide inhibited the proton pump activity with an IC_{50} 71.435µg/ml showing that anti-secretory activity of andrographolide. Though different biological activities of the plant *A. paniculata* has been reported earlier, anti-ulcer mechanism of this plant has not been reported till date. Our study is the first of its kind to show significant anti-secretory and cytoprotective effect of andrographolide isolated from the andrographolide.

Thus, the present study demonstrated that the andrographolide impart gastroprotective effects through the inhibition of H^+ K^+-ATPase (proton pump) activity and increseing the PGE2 level. Thus, *andrographolide* may emerge as a more potent therapeutic agent in treating gastric ulcer incidences since they possess both antisecretory and cytoprotective potentials.

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