



***Lagerstroemia speciosa* L. tannins reduces the gastric mucosal damage caused by ethanol and cold restraint stress**

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ABSTRACT

The present study investigated the possible protective effect of tannins isolated from *Lagerstroemia speciosa* L. leaves against ethanol and cold stress induced gastric damage. In ethanol induced model, gastric damage was induced by ethanol, one hour after tannin administration by a single dose whereas in cold restraint stress model tannins were administered for seven days and were subjected to cold stress for 4 hours and animals were sacrificed and the stomachs were taken for biochemical and histopathological analysis. Nitric oxide scavenging activity and FRAP assay proved the *in vitro* antioxidant activity possessed by the tannins. The tannins isolated from *L. speciosa* were found to be very effective that it protects the mucosa by 72.48 % in ethanol induced model and 55.1% in cold restraint stress model by 50mg/kg. The severity of gastric lesions was markedly decreased by the administration of tannins in both models. Administration of tannins significantly increased both gastric mucous and protein contents. Tannins increased the GSH level and decreased lipid peroxidation dose dependently. It can be concluded that the protective effects of tannins in gastric mucosa are mediated atleast partially by upregulation of antioxidant system.

Keywords: *L. speciosa*, gastric mucosa, stress, ethanol, tannins

INTRODUCTION

India is one of the richest floristic regions of the world and has been a source of plants and their products since antiquity and man uses them in different ways according to his needs, particularly as food and medicine. Among the entire flora, 35,000 to 70,000 species have been used for medicinal purpose and number of them is used for ulcer [1]. Considering the morbidity caused by peptic ulcer disease and dyspepsia over the world, cheap and easily available treatments will always be in demand especially for the people of non-industrialized countries [2].

Lagerstroemia speciosa, called as banaba, is a tropical plant found in many parts of Southeast Asia including the Philippines, Vietnam, Malaysia, India and southern China. The ellagitannin Lagerstroemin and gallotannin Penta-O-galloyl-glucopyranose (PGG) were identified as some of the effective components of the banaba extract responsible for its activity. *L. speciosa* has become relatively popular in the form of health-promoting tea products in Eastern Asia and the United States [3]. Tannins are a group of compounds belonging to the phenolic class of secondary metabolites in plants and have the ability to complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects and also through covalent binding [4] and were associated with antiulcerogenic

activity in plants [5]. The flower of *L. speciosa* reported to have antimicrobial and free radical scavenging activity [6].

Gastric hyperacidity and gastro-duodenal ulcers are illnesses that affect a considerable number of people in the world and are considered a very common global problem today. They are induced by several factors for example stress, smoking, nutritional deficiencies and ingestion of non-steroidal anti-inflammatory drugs [7]. The etiology of gastro-duodenal ulcers are influenced by various aggressive and defensive factors, such as acid-pepsin secretion, parietals cell, mucosal barrier, mucus secretion, blood flow, cellular regeneration, endogenous protective agents (prostaglandins and epidermic growth factor etc [8]. The modern approach to control gastric ulceration is to inhibit gastric acid secretion, to stimulate epithelial cell proliferation for effective healing. Antioxidant activity has also been studied as the role of the ROS in ulcers in definite [9]. Reactive oxygen species (ROS) have been shown to be involved in the etiology of many inflammatory disorders of the gastrointestinal system [10]. This is evidenced by the increased oxidative stress by pro-ulcerative factors in the gut such as *H. pylori*, [11] use of non-steroidal anti-inflammatory drugs [12], psychological stress etc [13]. The aim of the current study was to examine the effects of tannins in ethanol and cold stress induced ulcer models on rats, and to evaluate its effects on oxidant and antioxidant parameters in rat stomach tissue.

EXPERIMENTAL SECTION

2.1 Plant material

The leaves of *Lagerstromia speciosa* L. (*L. speciosa*) was collected from the premises of Amala Ayurvedic Hospital, Thrissur, Kerala, India. The plant material was identified by Dr. C.N. Sunil, Department of Botany, of S.N.M. College, Maliankara and was authenticated at Botanical Survey of India, Coimbatore. The voucher specimen, (*L. speciosa* BSI No. 62373) has been kept in Fr. Gabriel Herbarium, Amala Ayurvedic Hospital and Research Centre, Thrissur.

2.2 Animals

Male Wistar rats (180-200g) were purchased from Small Animals Breeding Station, (SABS) Kerala Agricultural University, Mannuthy, Thrissur, India and were kept for a week under environmentally controlled conditions (12h light-dark cycle at 22-24°C of temperature and humidity 70-75%) with free access to pelleted food (Sai Durga foods, Bangalore) and water.

2.3 Isolation and estimation of tannins

The crude tannins were isolated from the *L. speciosa* according to the method of Reddy *et al.*, [14] (2007) with slight modifications. The powdered plant material of the plants material were warmed with water for three hours and concentrated to dryness. This extract is successively partitioned with ethyl acetate and n-butanol. The ethyl acetate portion was subjected to column chromatography using silica gel and is eluted with methanol and water. The removal of solvent from methanol portion afforded tannins. Normal TLC was carried out on pre-coated silica gel 60 F₂₅₄ plates (Merck) with CHCl₃: MeOH: AcOH (7:2.5:0.5) and sprayed with 5% FeCl₃ which gave bluish black colour and confirmed as tannins.

The tannin content in *L. speciosa* was estimated spectrophotometrically by Folin-Denis method. The method is based on oxidation of the molecules containing a phenolic hydroxyl group. Phosphotungstomolybdic acid is reduced by tannins in alkaline solution to produce a highly coloured blue solution; the intensity of which is proportional to the amount of tannin and estimated against standard tannic acid solution at wavelength of 775 nm [15]. Calibration curve from standard solution of tannic acid was prepared and with the help of this curve the tannin contents of the extract was estimated. The linear correlation between these concentration (x-axis) and absorbance (y-axis) were graphically presented and the slope (b), intercept (a) and coefficient correlation (r) were calculated out for linear equation ($Y = bx + c$) by regression analysis using the least square method [16].

2.4 High performance thin layer chromatography of tannins

The High Performance Thin Layer Chromatography (HPTLC) studies of tannins were carried out on a pre-coated silica gel plate (0.2 mm, Merck 60 F 254, Germany) as the stationary phase, and chloroform/methanol/acetic acid (7:2.5:0.5) as the mobile phase. The extract was spotted as a band using a Camag Linomat IV applicator (CAMAG, Switzerland). The plates were observed in the visible region after derivatization using 5% ferric chloride reagent.

2.5 Nitric oxide scavenging and Ferric Reducing Antioxidant Power (FRAP) Assay

Nitric oxide scavenging activity of extract is determined using griess reagent [17]. The reaction mixture and different concentrations of tannins were incubated at 25°C for 150 min. The scavengers of nitric oxide compete with oxygen leading to the reduced production of nitric oxide. The capability to scavenge the nitric oxide radical was calculated by using the following equation.

$$\% \text{ Inhibition} = [1 - \text{Absorbance of sample} / \text{Absorbance of control}] \times 100$$

The ferric reducing ability was measured by Pulido *et al* [18]. An intense blue color complex was formed when ferric tripyridyltriazine (Fe^{3+} TPTZ) complex was reduced to the ferrous (Fe^{2+}) form. A calibration curve was plotted against FeSO_4 in the range 0-1 mM concentration with absorbance at 595 nm.

2.6 Anti ulcerogenic activity of tannins by ethanol induced gastric model

Male Wistar rats weighing between 200-225g were fasted for 18 hours and deprived of water for 12 hours prior to the experiments. They were divided into five groups of six animals each. Acute gastric ulceration was induced by absolute ethanol [19]. Group I rats were served as normal. Group II was kept as control (Absolute ethanol (96%) 5ml/kg intra-gastrically). Group III rats were given ranitidine (25mg/kg) which was used as standard drug in this experiment. Group IV and V were given different doses of tannins (10 and 25 mg/kg) respectively. Group IV and V were pretreated (orally) one hour before with isolated tannins as a single dose. The gastric anti-secretory drug ranitidine was given to group III of animals 30 minutes before the ulcerogenic agent as the single dose. The normal group received only the normal saline. After one hour of ethanol administration animals were killed, the stomach was excised, opened along the greater curvature, and washed gently in ice-cold saline. The stomach weight was noted and the extent of erosion of stomach mucosa from a scoring system. The severity of mucosal lesions was scored as ulcer index as follows. 0=no pathology, 1=a small ulcer (1-2 mm); 2=a medium ulcer (3-4mm); 4=a large ulcer (5-6mm); and 5=a larger ulcer (>6mm). The sum of the total scores divided by the number of animals was expressed as the mean ulcer index.

2.7 Histopathological evaluation

The total stomach was washed with normal saline for the lesions and measurement of ulcer index. Part of the stomach was taken and fixed in 10% formalin embedded in paraffin and stained with haematoxylin and eosin (H & E). The histological study was performed using a light microscope.

2.8 Anti ulcerogenic activity of tannins by cold restraint stress induced model

The rats were divided as per the above method. Tannins were administered for seven days and on the seventh day, the overnight fasted rats were given test drugs and after 30 minutes they were subjected to cold stress for 4 hours in a cold chamber at 4-5°C [20]. After the period of immobilization, the rats were sacrificed and the stomachs were removed for ulcer scoring. The severity of mucosal lesions was scored as ulcer index as above method.

2.9 Collection of gastric mucosa and biochemical estimations

Rats were killed by ether anesthesia and the stomach portions were removed rapidly, opened along the greater curvature, and rinsed with ice-cold normal saline thoroughly. A longitudinal section of gastric tissue was taken from the anterior part of the stomach and then fixed in 100mL/L buffered formalin for 24h. It was cut into sections of 5µm and then used in histological examination. Gastric mucosa was taken from the remaining part of the stomach by scraping with a glass slide or blunt knife on a glass dish on ice. They were wrapped by a piece of aluminum foil, immediately froze and stored at -70°C until assayed.

2.10 Determination of nitrite level in gastric mucosa

The gastric mucosa was cooled in ice-cold distilled water before homogenization. The crude homogenate was prepared using phosphate buffer (pH 7.2) and centrifuged at 21000 g for 20 min at 4°C. Aliquots of the supernatants were taken to determine nitrite levels. The amounts of nitrite were measured in the gastric mucosa by performing the Griess reaction. 100 µL of sample were incubated with 100 µL of Griess reagent (Sigma) at room temperature for 20 min. Nitrite level was determined by measuring the absorbance at 550 nm using a spectrophotometer (DU 640B, Beckman, Fullerton, California, USA) [18].

2.11 Determination of gastric wall mucus and total protein

Gastric wall mucus was determined according to the procedure of Corne *et al* [21]. The glandular segments from stomach, which had been opened along their greater curvature, were weighed. Each segment was transferred immediately to 10 mL of 0.1% w/v alcian blue solution (in 0.16 M sucrose solution, buffered with 0.05 M sodium acetate adjusted to pH 5.8 with HCl). After immersion for 2 h, excess dye was removed by two successive rinses with 10 mL of 0.25 M sucrose, first for 15 minutes and then for 45 minutes. Dye complexed with gastric wall mucus was extracted with 10 mL of 0.5M MgCl₂ by shaking intermittently for 1 minute at 30 minutes intervals for 2 hours. The resulting blue solution was shaken vigorously with an equal volume of diethyl ether and then the emulsion was centrifuged at 3000 rpm for 10 min and the absorbance of the aqueous layer against blank standard MgCl₂ solution was recorded at 580 nm. The quantity of alcian blue recovered from per gram of net glandular tissue was then calculated. The protein content was quantified by the method of Lowry *et al*, [22] with BSA as the standard.

2.12 Estimation of Glutathione, MDA and protein carbonyl content

Glutathione in the gastric mucosa was determined by Ellman's reaction using 5,5'- dithio-bis- 2-nitro benzoic acid (DTNB) [23]. The level of TBARS in the gastric mucosa was measured as malondialdehyde formed (MDA) according to the method described by Ohkawa *et al.*, [24] with minor modifications. The amount of protein carbonyl content was estimated by the following method [25].

2.13 Statistical analysis

Data are presented as mean \pm Standard Deviation using Graph pad Instat software. Bonferroni test was used to analyse the data significance and the results were subjected to one-way ANOVA.

RESULTS AND DISCUSSION

Tannins are a large group of polyphenolic compounds which have received attention in recent years due to their claimed ability to cure a variety of diseases [26]. *In vitro* antioxidant activity of tannins was carried out using nitric oxide scavenging activity. It was found that tannins from *L. speciosa* required only 19 μ g/mL for 50 % inhibition of radicals. FRAP assay showed that concentration of total tannins from *L. speciosa* required 0.45 mM equivalents of FeSO₄.

The calibration curve for tannic acid was found to be linear in the range of 5-45 μ g/ml. The correlation coefficient (r), was found to be 0.9916 indicates the good linearity between the concentration and the absorbance. Slope (b) was found to be 0.0016. The amount of tannins present in the extract was found to be 474 μ g/mL. HPTLC analysis was carried out using the extracted tannins from *L. speciosa* and four peaks were obtained. The fourth peak was identified as gallic acid using as standard. The percentage peak areas of the peaks are 6.32, 38.97, 47.23 and 7.48% respectively (Data not shown).

The reported medicinal attributes and antioxidant property of the plant triggered us to assess the protective effect of this against ulcer. In this study, the anti ulcerogenic effect of tannins was investigated in rats using ethanol and stress induced ulcer models. In addition, the effect of tannins on oxidant and antioxidant parameters in rat stomach tissue was evaluated. Ulcers are thought to be due to the imbalances in gastric offensive and defensive mucosal factors. To regain the balance, different therapeutic agents are used to inhibit the gastric acid secretion or to boost the mucosal defense mechanisms by increasing mucus production or stabilizing the surface epithelial cells [27]. The ethanol model has been used widely to produce gastric mucosal damage and produces necrotic lesions in the gastric mucosa by its direct toxic effect, reducing the secretion of bicarbonates and production of mucus [28]. It is well established that gastric acid secretion and the products of the 5-lipoxygenase pathways play a key role in the development of ulcers, induced by irritant agents such as ethanol; [29] and many anti-ulcerogenic drugs act by reducing the acid secretion.

In our study no ulcer or erosions were observed in rats of normal groups indicating that handling and surgical procedure had no interference with experimental outputs. The mean ulcer score in the control animals was 4.07 \pm 0.050. The severity of lesions was markedly increased in control group. The tannins isolated from *L. speciosa* were found to be more effective that it protects the mucosa by 72.48 % by 50mg/kg body weight. Higher dose of tannins showed better activity than ranitidine treatment (Table 1). The gastroprotective effect of *L. speciosa* might be due to the decrease in gastric motility and increased the gastric emptying time. It is reported that the changes in gastric motility may play a role in the development and prevention of experimental gastric lesions. Relaxation of circular

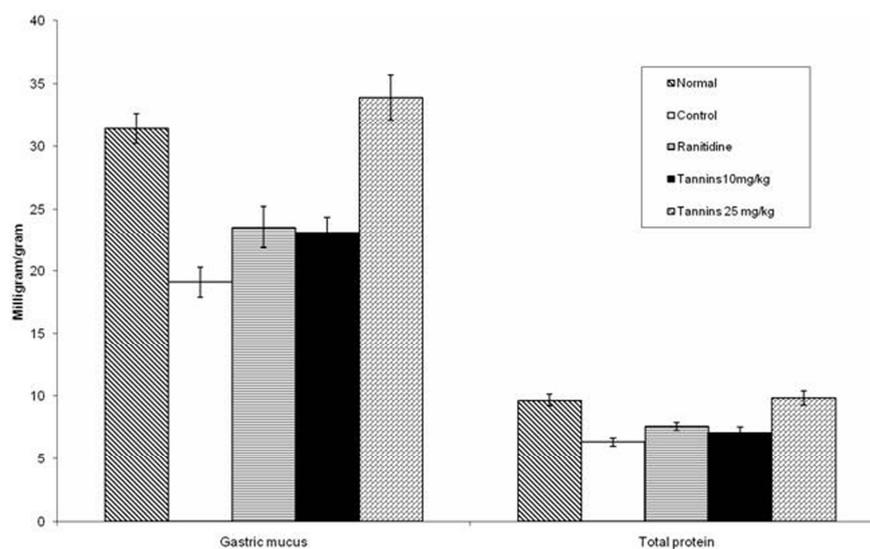
muscles may protect the gastric mucosa through flattening of the folds. This will increase the mucosal area exposed to necrotizing agents and reduce the volume of the gastric irritants on rugal crest. Ethanol produces a marked contraction of the circular muscles of rat fundic strip. This leads to ‘mucosal compression’ at the site of the greatest mechanical stress i.e. at the crests of mucosal folds leading to necrosis and ulceration [30]

Table 1. Effect of Tannins on ethanol induced gastric ulcers in rats

Treatment Groups	Dose (mg/kg)	Ulcer index	Inhibition (%)
Control	-----	4.07 ±0.050	-
Ranitidine	25 mg/kg	1.33 ±0.009	67.32
Total Tannins	10 mg/kg	2.08 ±0.031	48.89
Total Tannins	25 mg/kg	1.12 ±0.005	72.48

The figure 1 showed the result of the total protein and adherent mucous content of the glandular stomach. Administration of ethanol in control group significantly ($P < 0.001$) decreased both gastric mucous and protein contents, when compared with normal. Administration of ethanol significantly decreased both gastric mucous and protein contents, the inhibition being greatest in the control group. The protein carbonyl content was found to be higher in ulcerated control and reached to near normal level by the administration of tannins.

Figure 1. Effect of tannins on gastric mucus and protein content in ethanol induced gastric ulcer



In this study, ranitidine was used as reference drug to delineate in part the mechanism(s), accordance with previous report [31]. Also it is proved that flavonoids possess antiulcerogenic, antigastric; [32] as well as ability to inhibit acid secretion activities. The control gastric mucosal nitrite level in ethanol induced model was found to be 59.5 ± 2.9 nmol/g tissue (Figure 2). The control group showed depletion in the gastric nitrite level to 42.3 ± 1.69 nmol/g tissue. Tannins showed 46.2 ± 3.109 nmol/g tissue and 62.03 ± 3.209 nmol/g tissue. In cold stress model also efficacy of tannins was meaningful and comparable with ranitidine which was kept as standard. In our study, tannins reduced ethanol induced gastric damage and increased the mucus significantly. This may be explained with a correlation to strengthen the defense factors of gastric mucosa. The alcoholic solution gave blue colouration with ferric chloride

indicating the presence of tannins and phenolic compounds. It is likely that phenolic compounds may be responsible for gastroprotection [33].

Involvement of ROS in pathogenesis of gastric ulceration was first evident from the studies on ischemia-reoxygenation-induced gastric mucosal injury [10]. A growing body of experimental and clinical evidence suggests that the gastric mucosal damage caused by ethanol [35], non-steroidal anti-inflammatory drugs etc [36] is mediated through ROS. There is extensive experimental evidence that indicates certain substances, through scavenging of free radicals, protect the gastric mucosa [37]. The thiobarbituric acid reactive substance (TBARS) is used as an indicator of lipid peroxidation and free radical activity in biological samples [38].

Figure 2. Effect of tannins on gastric nitrite level in ethanol and cold stress induced ulcer model

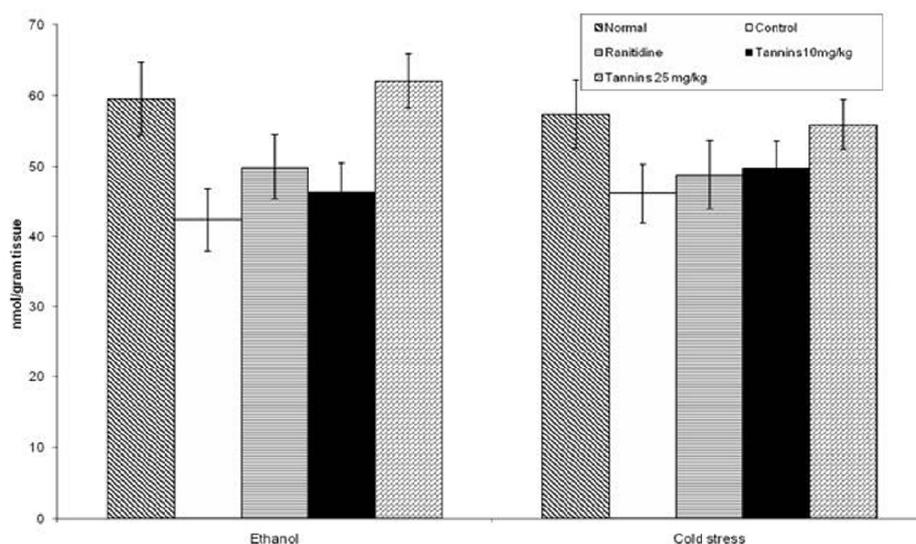


Table 2. Effect of Tannins on gastric mucosa in ethanol induced ulcers in rats

Treatment Groups	MDA (n mols/mg protein)	Protein Carbonyl ($\mu\text{g}/\text{mg}$ protein)	GSH (n mols/mg protein)
Normal	0.98 ± 0.13^a	0.502 ± 0.03^a	30.8 ± 2.61^a
Control (5ml/kg)	$2.31 \pm 0.27^{b*}$	$0.984 \pm 0.08^{b*}$	$16.7 \pm 1.12^{b*}$
Ranitidine (25 mg/kg)	$1.46 \pm 0.31^{c*}$	$0.592 \pm 0.06^{c*}$	$25.6 \pm 1.65^{c*}$
Tannins (10mg/kg)	$1.35 \pm 0.24^{d*}$	$0.690 \pm 0.04^{d*}$	$22.4 \pm 1.98^{d*}$
Tannins (25 mg/kg)	$1.09 \pm 0.12^{de*†}$	$0.569 \pm 0.08^{e*‡}$	$26.3 \pm 1.85^{de*}$

Values are mean \pm S.D, n=6 animals.

* $P < 0.001$, Comparison between normal group with other groups

† $P < 0.001$, Comparison between control group with other groups

‡ $P < 0.01$, Comparison between low dose level with higher dose levels of same extract

Alphabets a-e represents the experimental groups. Between the groups, same alphabets are not significant, but different alphabets are significant

In ethanol-induced model, in untreated control groups MDA level was found to be 2.31 ± 0.27 nmol/mg protein whereas in tannin treated groups it was observed that lipid peroxidation has decreased to 1.35 ± 0.24 nmol/mg protein and 1.09 ± 0.12 nmol/mg protein dose dependently. ROS also decreases the level of endogenous antioxidants such as GSH, α -tocopherol and ascorbate and make the mucosa more prone to oxidative damage [34]. In our study GSH levels were significantly decreased in ethanol alone-administered control group. Administration of tannins increased the GSH level dose dependently (Table. 2).

Figure 3 shows the histopathological changes in the rat gastric mucosa one hour after administration of ethanol. Figure 3B shows the cell necrosis after ethanol administration. The standard drug; Ranitidine (Fig. 3C) and tannins (25mg/kg) treatment (Fig. 3D) reduced the ethanol induced cell necrosis.

The cold stress increased the ulcer index and administration of ranitidine decreased the level significantly. The percentage protection in ranitidine treated group was only 36.4% and tannins show a much greater protection of 35.4 and 55.1% (Table. 3).

Table 3. Effect of Tannins on cold stress induced gastric ulcers in rats

Treatment Groups	Dose (mg/kg)	Ulcer index	Inhibition (%)
Control	-----	1.98 ± 0.21	----
Ranitidine	25 mg/kg	1.26 ± 0.23	36.4
Total Tannins	10 mg/kg	1.28 ± 0.40	35.4
Total Tannins	25 mg/kg	0.89 ± 0.45	55.1

Table 4. Effect of Tannins on gastric mucosa in rats with and without ulcers in and cold stress induced ulcers ethanol

Treatment groups	Dose (mg/kg)	LPO (n mols/mg protein)	GSH (nmols/mg protein)
Normal	-----	0.95 ± 0.19^a	32.5 ± 2.02^a
Control	-----	$2.42 \pm 0.20^{b*}$	$18.2 \pm 1.61^{b*}$
Ranitidine	25 mg/kg	$1.63 \pm 0.27^{c*}$	$24.7 \pm 1.72^{c*}$
Tannins	10mg/kg	$1.45 \pm 0.18^{cd*}$	$23.1 \pm 1.86^{bcd*}$
Tannins	25 mg/kg	$1.12 \pm 0.15^{ade\ddagger}$	$28.3 \pm 1.74^{a*\ddagger}$

Values are mean \pm S.D, n=6 animals

* $P < 0.001$, Comparison between normal group with other groups

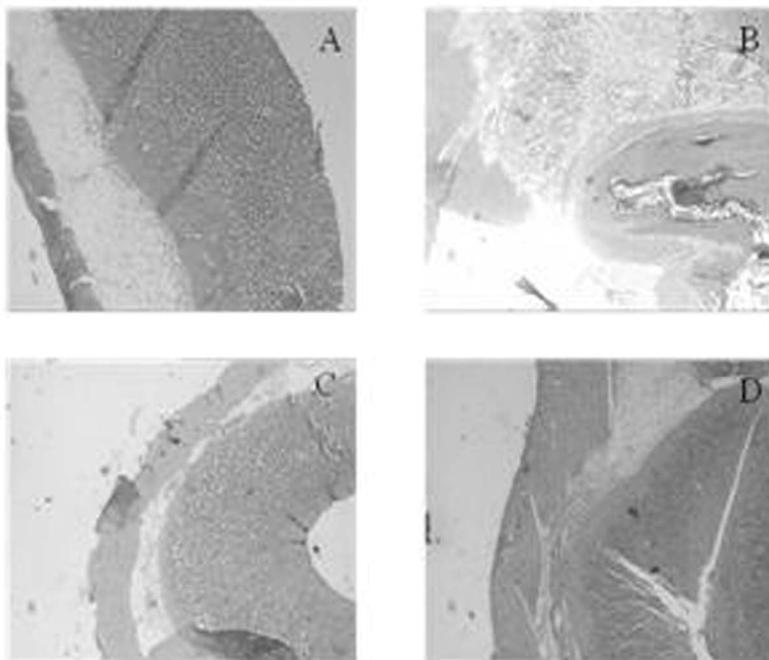
† $P < 0.001$, Comparison between control group with other groups

‡ $P < 0.01$, Comparison between low dose levels with higher dose levels of same extract

Alphabets a-e represents the experimental groups. Between the groups, same alphabets are not significant, but different alphabets are significant

In cold restraint stress model, ranitidine treated groups showed 1.63 ± 0.27 nmol/mg protein from the control value of 2.42 ± 0.20 nmol/mg protein. Tannins showed a dose dependent decrease in the formation of lipid peroxides. GSH levels was found to reduced significantly ($P < 0.001$) in cold stress induce ulcer model. Administration of tannins increased the GSH level significantly in dose dependent manner (Table.4).

Figure 4. Histological structure of gastric mucosa on experimental groups (Ethanol model) A; Normal, B; Control, C; Standard, D; Tannins



Our results revealed that the ethanol induced ulceration was accompanied with a severe oxidative stress in the gastric tissues causing damages to key biomolecules such as lipids, proteins etc. This was apparent from the stimulated lipid and protein oxidation leading to increased accumulation of MDA and protein carbonyls. GSH, an endogenous sulfhydryl compound, is an important substance in the cellular defense system [39]. In the gastric mucosa, NO also play dual roles in the regulation of gastric mucosal integrity. Tissue injury may result from a combination of NO with superoxide anion, yielding highly toxic species, which can degrade proteins, enzymes, and DNA [40, 41]. In both experimental models the tissue gastric nitrite level restored the normal level when compared with the untreated control. The increment in the gastric tissue nitrite in the tannins treated groups in the present study is therefore indicative for the protective capacity of endogenous NO.

The exact mechanism of pathogenesis in the ethanol and cold restraint stress ulcer model has not been fully known, but hypersecretion of gastric acid, deterioration of the mucosal resistance and promotion of gastric emptying are among the possible mechanisms [42]. Roughly 50% of new chemical entities introduced during the past two decades are from natural products. Recent technological advances have renewed interest in natural products in drug discovery. Therefore, efforts should be directed towards isolation and characterization of the active compounds and elucidation of the relationship between its structure and activity. Furthermore, detailed analysis of the active constituents of natural drugs should be directed towards clinical relevance.

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