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Effect of *Pithecellobium dulce* (Roxb.) Benth. fruit extract on cysteamine induced duodenal ulcer in rats

Jayaraman Megala[1] & Arumugam Geetha[2]*

[1]Department of Genetic Engineering, SRM University, Kattankulathur-603 203, Tamil Nadu, India
[2]Department of Biochemistry, Bharathi Women's College (Autonomous), Affiliated to University of Madras, Chennai-600 108, Tamil Nadu, India

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The edible fruits of *Pithecellobium dulce* (Roxb.) Benth. are traditionally used for various gastric complications in India. Here, we investigated the antiulcer activity of hydroalcoholic fruit extract of *P. dulce* (HAEPD) by applying cysteamine induced duodenal ulcer model in rats. Duodenal ulcer was induced in male albino Wistar rats by oral administration of cysteamine @ 420 mg/kg body wt. as a single dose. The rats were pre-administered orally with HAEPD @ 200 mg/kg body wt. for 30 days prior to ulcer induction. Rats pre-administered with ranitidine @ 30 mg/kg body wt. served as reference drug control. Ulcer score, thiobarbituric acid reactive substances (TBARS), glycoproteins, superoxide dismutase, catalase and glutathione peroxidase and reduced glutathione levels were measured in the duodenum. Rats pre-administered with the HAEPD showed significantly reduced ulcer score comparable to that of ranitidine pretreated rats. The co-administration of HAEPD lowered the TBARS level and also restored the levels of glycoproteins, enzymatic and non-enzymatic antioxidants. Histopathological observations confirmed the presence of inflammation, necrosis and hemorrhagic spots in the duodenum of ulcer control rats which were significantly reduced due to HAEPD treatment. No abnormal alterations were observed in normal rats treated with HAEPD at the dosage studied. The results demonstrated antioxidant and cytoprotective nature of *P. dulce*, and thereby its significant anti ulcer property.

Keywords: Antioxidant activity, Antiulcer property, Cytoprotective, Duodenum, Glycoproteins, Intestinal mucosa, Madras thorn, Peptic ulcer, Ranitidine

Duodenal ulcer is a chronic and recurrent peptic ulcer disease affecting 10% of population worldwide. Peptic ulcer disease is characterized by the shallow breaches in the lining of stomach and duodenum. Approximately, 95% of duodenal ulcer and 70% of gastric ulcer are associated with *Helicobacter pylori* infection[1,2]. Apart from this, imbalance between factors such as acidity, pepsin, active oxidants, NSAID's and the local defensive factors including prostaglandins, bicarbonate and mucosal glycoproteins leads to peptic ulcer disease[3,4]. The mucosal layer that covers the epithelial surface of intestinal tract constitutes the first line defense against a variety of exogenous or endogenous agents. This layer is an integral structural component of the intestine, acting as a medium for protection, lubrication and transport of ions[5].

Ulceration in the mucosa is caused either by breakdown of mucosa with the development of surface defects or by the failure of restitution of mucosal integrity which also delays the healing process. The defense mechanism of the gastrointestinal mucosa against aggressive factors mainly consists of functional, humoral and neuronal factors. Alkaline mucus secretion, mucosal microcirculation, cellular mucus, life span of mucosal cells and motility act as functional factors, while prostaglandins and nitric oxide act as humoral factors. Capsaicin sensitive sensory neurons act as neuronal factors. All the above factors contribute to mucosal protection in gastrointestinal system[6].

The duodenal mucosa is regularly exposed to intermittent pulses of gastric acid, with luminal pH varying rapidly between 2 and 7[7]. Biliary and pancreatic alkaline secretions act in concert with duodenal mucosal secretions to neutralize the luminal contents. Despite variable acidic conditions in the duodenal lumen, the surface epithelium is maintained at neutral pH. This is due to the bicarbonate ($HCO_3^{??}$) transport by the mucosa into a layer of mucus adherent to the surface of the duodenal epithelium[8,9]. Treatments available for peptic ulcer are usually aimed at reducing the production of gastric acid and re-enforcing gastric mucosal protection[2].

*Correspondence:
Phone: +91 9444902506
E-mail: drgeetha08@yahoo.in; geethav21@yahoo.co.in

The drugs used in the treatment of ulcer include receptor blockers and proton pump inhibitors[10]. Although a wide range of drugs are available for treatment of ulcers, many have side effects such as arrhythmia, headache, male hormone disturbances, and potential interference with drug metabolism[11,12]. In traditional system of medicine, several plants and herbs have been used to treat peptic ulcers[13]. Madras thorn [*Pithecellobium dulce* (Roxb.) Benth. (Fabaceae)] is one such plant being used in the treatment of leprosy, ear ache, peptic ulcer and tooth ache[14]. The plant parts have been found to possess anti-inflammatory and anti-venomous activity[15,16]. We have also demonstrated the antiulcer property of *P. dulce* using ethanol induced gastric ulcer model in rats[17]. Since peptic ulcer is comprised of both gastric and duodenal ulcers, here, we explored protective effect of *P. dulce* on duodenum against experimentally induced ulcer by cysteamine.

Materials and Methods

Drugs and chemicals—All the chemicals and reagents used for the study were of analytical grade purchased from the authorized dealers of Sigma-Aldrich and Merk Pvt Ltd. All the drugs and reagents were prepared freshly before use.

Preparation of hydroalcoholic fruit extract of Pithecellobium dulce—The fleshy fruits of *P. dulce* were washed thoroughly and air dried. The dried powder (300 g) was coarsely ground and mixed well with hydroalcoholic ethanol (70%) and left for 1 wk. The extract was obtained by filtration and evaporated to dryness. The yield was lyophilized (90 g). The residue was reconstituted with water.

Experimental design—Male albino Wistar rats weighing 150-200 g obtained from the Directorate of Centre for Animal Health studies, Madhavaram milk colony, Chennai were utilized for the study. Animals were housed in polypropylene cages, maintained under standard conditions of temperature (24±2°C) and relative humidity (30-70%) with 12 h light:dark cycle. Rats were fed normal commercial diet and given water *ad libitum*. The study protocol was approved by the animal ethics committee (290/CPCSEA/12/12/08-02). The animals were grouped as follows for experiment purpose and each group had 6 rats. Group I, without any treatment; Group II, cysteamine single oral dose @ 420 mg/kg body wt.[18]; Group III & IV pretreated with HAEP @ 200 mg/kg body wt. and ranitidine @ 30 mg/kg body wt. for 30 days, respectively prior to cysteamine administration at the same dose as given to Gr. II; Group V & VI, treated with HAEPD @ 200 mg/kg body wt. and ranitidine @ 30 mg/kg body wt. for 30 days.

Determination of optimum dose—Rats were treated with different doses of fruit extracts (100, 200 and 500 mg/kg body wt.) for 30 days before ulcer induction to find out the effective dose for ulcer prevention against duodenal ulcer. The fruit extract @ 200 mg/kg body wt. was selected based on the ulcer score. Similarly, different doses of ranitidine (15, 30 and 50 mg/kg body wt.) were tested to find out the optimum dose, and accordingly ranitidine @ 30 mg/kg body wt. was selected.

Induction of duodenal ulcer—The animals were administered with 200 mg/kg body wt. of HAEPD once daily orally for 30 days and cysteamine HCl (420 mg/kg body wt.) was administered orally 30 min after the last HAEPD dose and the animals were fasted for 24 h with water and *ad libitum*. At the end, the animals were sacrificed and the duodenum was excised carefully and opened along the anti mesentric side to determine ulcer score[19].

Determination of ulcer score—The duodenal lesions were measured in terms of ulcer score as done by Makovee *et al*.[20]. The intestine was excised and rinsed with 0.1 mol/L ice cold phosphate buffer saline. The mucosa was then examined with a 100X magnifier to determine the level of histological changes. Score was made based on the status of erosion. Ulcer score was expressed as mm^2 in duodenal mucosa.

Biochemical investigations

Duodenum was dissected out, washed in ice cold saline and 10% homogenate was prepared using 0.1 M Tris HCl buffer (pH 7.4) and used to assess the biochemical parameters.

Estimation of lipid peroxides—The level of lipid peroxidation products in duodenal tissues was estimated by the method of Nieahas &Samuelson[21] using 1,1,3,3-tetramethoxypropane as standard and TBARS was expressed as nM/mg protein.

Estimation of reduced glutathione (*GSH*) *and glutathione peroxidase* (*GPx*) *activity*—The total reduced glutathione content was determined by the method of Moron *et al*.[22] using GSH as reference standard and expressed as nM/g. The activity of GPx was assayed by the method of Folhe & Gunzler[23] and expressed as nM of GSH oxidized/min/mg.

Assay of superoxide dismutase (*SOD*) *and catalase* (*CAT*)— The enzyme superoxide dismutase was assayed by the method of Marklund & Marklund[24] using pyrogallol as reference standard. The degree of

inhibition of auto oxidation of pyrogallol at an alkaline pH by superoxide dismutase was used as a measure of enzyme activity. The activity of enzyme was expressed as U/mg protein. The catalase activity was assayed by the method of Sinha[25] using hydrogen peroxide as reference standard. Catalase catalyzes the decomposition of hydrogen peroxide and the enzyme activity was measured by the rate of decomposition and expressed as U/mg protein.

Estimation of glycoprotein—The membrane bound glycoproteins were extracted from the duodenal tissues by the method of Glossmann & Neville[26] and used for estimation of hexose (Niebes)[27], hexosamine (Wagner)[28], fucose (Winzler)[29] and sialic acid (Warren)[30]. The glycoprotein levels were expressed as mg/g tissue.

Histopathology

A portion of duodenum was excised from each rat and processed for histological observation. Formaldehyde (10% neutral) was used as a fixative. After 24 h, the tissues were washed, dehydrated with increased alcohol concentration and embedded. Micro sections (6 µm) were prepared and stained using hematoxylineosin. The stained tissue sections were fixed and microslides were viewed through light microscope and the pathological changes were recorded.

Statistical analysis—The values were expressed as mean±standard error of mean (SEM) for n = 6. The SPSS (Windows V.13) was used for analyzing the significance (ANOVA).

Results

Effect of HAEPD on ulcer score—The cysteamine induced duodenal ulcer was evidenced by lesions in the mucosal regions with hemorrhage and inflammatory changes. On gross examination, these spots and lesions were characterized by different sizes as shown in Fig. 1 (b-d in particular). Ulcerated rats pretreated with *Pithecellobium dulce* showed very mild lesions. Rats that received test drug alone showed the normal mucosa without any inflammation.

Ulcer score, a measure of lesion in duodenal mucosa observed in all the experimental animals is presented in Table 1. The lesions were characterized by different size of multiple red bands in duodenal mucosa as seen in fig. 1b-d. A significant reduction in ulcer score was observed in rats pretreated with the *P. dulce* fruit extracts and the effects were comparable to those of rats received the standard drug ranitidine.

Effect of HAEPD on lipid peroxidation—Table 2 shows the level of lipid peroxides in the duodenum of

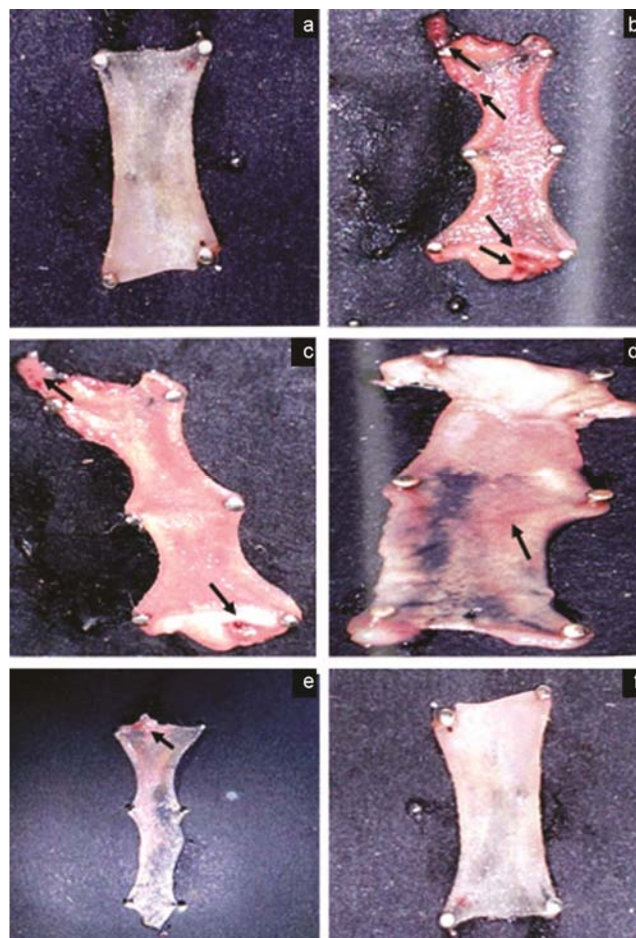

Fig. 1—Macroscopic examination of duodenal mucosa in control and experimental rats. Ulcer lesions are shown by arrows. (a) Control [Normal mucosal tissue]; (b) Cysteamine (420 mg/kg body wt.) [Duodenal lesions characterized by multiple hemorrhagic spots]; (c) HAEPD (200 mg/kg body wt.)+ Cysteamine [Very mild duodenal lesions with sparse hemorrhagic spots]; (d) Ranitidine (30 mg/kg body wt.)+ Cysteamine [Very mild duodenal lesions]; (e) HAEPD (200 mg/kg body wt.) [Duodenal mucosa without any abnormal changes]; and (f) Ranitidine (30 mg/kg body wt.) [Normal duodenal mucosa with no lesions]

Table 1—Effect of HAEPD and ranitidine on cysteamine induced duodenal ulcer in experimental animals

Particulars of treatment		Ulcer score (mm^2)	Ulcer protection (%)
Cysteamine		9.68 ± 0.06	Nil
HAEPD (mg/kg body wt) +	100	4.10 ± 0.22*	57.64
Cysteamine	200	3.83 ± 0.19*	60.43
	500	3.75 ± 0.15*	61.26
Ranitidine	15	3.15 ± 0.10*	67.45
(mg/kg body wt)+	30	2.86 ± 0.08*	70.45
Cysteamine	50	2.75 ± 0.06*	71.59

For statistical variation the comparison was made between control *vs*. fruit extract and ranitidine at different concentration. Values as mean ± SEM, n=6 in each group. *P <0.001.

experimental rats. It was found that the lipid peroxide level was significantly increased in ulcer control rats than in HAEPD pretreated rats (*P* <0.001) and the effects were comparable to those of rats received the standard drug ranitidine.

Effect of HAEPD on enzymatic and nonenzymatic antioxidants—The level of antioxidants such as GSH and GPx activity, and CAT and SOD levels are shown in Table 2. A significant reduction in GSH level and GPx activity (*P* <0.001) were observed in ulcer control rats. The levels of these antioxidants were found significantly restored in rats treated HAEPD prior to cysteamine administration and the effects were comparable to rats received the standard drug ranitidine. In tune with this, the levels of the antioxidant enzymes CAT and SOD were significantly decreased in ulcer control rats than in rats pretreated with HAEPD. The effects were comparable to those of rats received the standard drug ranitidine.

Effect of HAEPD on membrane bound glycoproteins—Table 3 illustrates the level of various glycoproteins in the duodenum of experimental rats. The ratio of protein bound carbohydrates to protein was significantly decreased (*P* <0.001) in ulcer control rats than in rats administered HAEPD prior to ulcer induction and the effects were comparable to the rats received the standard drug ranitidine.

Histological studies—Microscopic observation made on ultra-sections of duodenum extracted from control and experimental rats are presented in Fig. 2 (a-f).

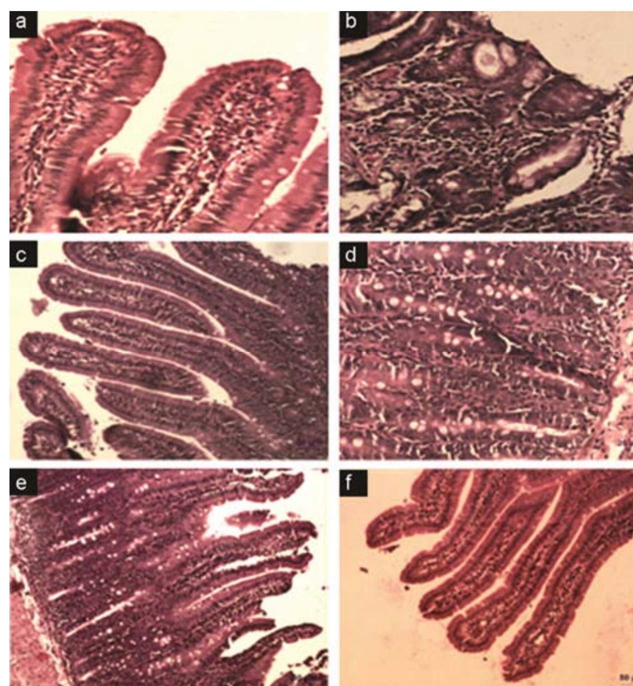

Fig. 2—Histological examinations of intestinal mucosa in control and experimental rats. Ultra section (HE 200X) of duodenum shows in (a) Control [Well defined basal epithelium and mucosal villi]; (b) Cysteamine [Mucosal degeneration, fragmentation of villi and mononuclear cell infiltration]; (c) HAEPD + Cysteamine [No changes in the structure of basal epithelium and mucosal villi]; (d) Ranitidine + Cysteamine [Very mild degeneration and necrosis of mucosal epithelium, degeneration of crypt epithelium and mild nucleophilic cell infiltration]; (e) HAEPD [No changes in the structure of basal epithelium and mucosal villi]; and (f) Ranitidine [Normal pattern of tissue architecture]

Table 2—Levels of duodenal TBARS, enzymatic and non enzymatic antioxidants in experimental rats

Groups	TBARS (nM/mg protein)	GSH (nM/g tissue)	GPx (nM of GSH oxidized/min/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)
I	0.71±0.01	8.14±0.22	262.41±0.58	61.29±0.51	5.49±0.006
II	2.23±0.15*	4.49±0.09*	109.18±0.43*	42.87±0.47***	4.42±0.02**
III	0.66±0.01NS	7.30±0.22*	238.03±0.31*	58.79±0.40*	4.91±0.03***
IV	0.63±0.006NS	7.61±0.02*	245.23±0.63*	60.46±0.99*	5.33±0.11**
V	0.71±0.005NS	7.99±0.16NS	258.02±0.57NS	60.15±0.39NS	5.47±0.04NS
VI	0.75±0.008NS	8.02±0.13NS	260.42±1.001NS	60.36±1.01NS	5.40±0.02NS

For statistical variation the comparison was made between groups as: Control *vs*. cysteamine/HAEPD/ranitidine; Cysteamine*vs*. pre-treated HAEPD (200 mg/kg body wt.)/ranitidine (30 mg/kg body wt.). Values as mean ± SEM, n=6 in each group. **P* <0.001, ***P* <0.01, ****P* <0.05 and NS, non significant

Table 3—Levels of proteins and total protein bound carbohydrates in experimental rats (mg/g tissue)

Groups	Hexose	Hexoseamine	Sialic acid	Fucose	Protein	TPBC	TPBC:P
I	13.60±0.03	8.62±0.006	2.55±0.04	3.37±0.06	21.36±0.24	30.11±0.41	1.40±0.005
II	8.61±0.03*	3.94±0.01*	0.73±0.01*	1.25±0.01*	14.91±0.01*	15.66±0.06*	1.05±0.0005*
III	12.65±0.02*	7.94±0.01*	2.31±0.06NS	3.59±0.03NS	18.91±0.01*	28.27±0.52*	1.49±0.005NS
IV	12.69±0.03*	8.34±0.01NS	2.54±0.01NS	3.51±0.06NS	18.28±0.09*	29.47±0.33*	1.61±0.005*
V	13.48±0.01NS	8.70±0.02NS	2.47±0.01NS	3.80±0.03NS	20.50±0.08NS	30.51±0.10NS	1.48±0.005NS
VI	13.50±0.005NS	8.95±0.02NS	2.85±0.01NS	3.63±0.10NS	20.68±0.04NS	30.84±0.17NS	1.49±0.005NS

For statistical variation the comparison was made between groups as: Control *vs*. cysteamine/HAEPD/ranitidine; Cysteamine *vs*. pretreated HAEPD (200 mg/kg body wt.)/ranitidine (30 mg/kg body wt.). Values as mean ± SEM, n=6 in each group. *P <0.001 and NS, non significant.

Control rats showed normal duodenal tissue architecture. Rats administered with cysteamine showed abnormal basal epithelium degeneration, necrosis of mucosal epithelium, mononuclear infiltration and inflammation. Rats pretreated with 200 mg /kg body wt. of HAEPD showed mild neutrophilic villi tubes with normal basal layer of epithelium and the rats pretreated with 30 mg/kg body wt. of ranitidine showed very mild degeneration. Rats which received only the test drug or the standard did not show any significant alterations in the duodenal tissue architecture.

Discussion

The gastrointestinal tract is regularly exposed to a large volume of acid produced by gastric parietal cells, ingested toxins, ischemia/reperfusion injuries or infections such as *H. pylori.* The gastric mucosal barrier consists of tight intercellular junctions between surface epithelial cells that secrete HCO_3 and mucus but has a limited ability to protect the mucosa from acid, pepsin and mechanical damage[31].

The duodenal mucosal blood flow is also important as it is involved in removal of acid as well as supply of HCO_3. Moreover, prostaglandins produced by the mucosa protect by influencing mucus and HCO_3 production. We have earlier reported that PGE_2, an eicosanoid product which provides protection to the gastric mucosa was significantly depleted in rats induced with gastric ulcer by ethanol administration but found maintained in HAEPD pretreated rats[17].

Ulcer score is a measure of duodenal mucosal lesion which can be characterized by multiple red spots and hemorrhagic bands of the intestine (Fig. 1). We found a significantly elevated ulcer score in cysteamine administered rats. The HAEPD and ranitidine pretreatments showed only mild lesions in ulcer induced rats demonstrating the ulcer reducing effect of HAEPD comparable to that of ranitidine.

Free radicals are produced in the mitochondria of cell during biological oxidation and if not quenched rapidly, they damage the lipid membrane by peroxidation[32]. Stress induced ulceration involves damage by reactive oxygen species (ROS) apart from acid and pepsin related factors[33]. The present study revealed that during stress, LPO and enzymatic antioxidants such as SOD and CAT were significantly decreased in the ulcer induced rats that did not receive any drug treatment. However, *Pithecellobium dulce* pretreatment prevented such decrease and it was comparable to those rats which received the standard drug ranitidine.

The SOD administration was found to augment alkaline secretion significantly in the duodenum, suggesting that the mucosal antioxidative system including SOD may play a role in the regulation of alkaline secretion and contribute to duodenal mucosal defensive ability[34]. In cysteamine administered rats, SOD activity was significantly decreased concomitantly with the increase in lipid peroxidation products[35]. These reports suggest that an increase of oxygen-derived free radicals and a decrease of SOD activity in the duodenal mucosa may be involved in the pathogenesis of cysteamine-induced duodenal ulcer.

Inactivation of gastric peroximes during stress may also aggravate the mucosal damage[36]. This evidently caused increased lipid peroxidation and mucosal damage and results in increased ulcer scores. Infection with *H. pylori* is associated with generation of ROS which leads to oxidative stress in the gastro duodenal mucosa[37]. They found that gastro duodenal mucosal GSH level was significantly lower and MDA level was higher in duodenal ulcer patients with or without *H. pylori* infection. Our findings are concordant with these observations. Ulcerogen induced rats showed increased level of lipid peroxidation products whereas the administration of *Pithecellobium dulce* maintained the level of lipid peroxidation products to near normal and prevented the damage by free radicals.

Reduced glutathione, one of the most abundant non-enzymatic antioxidants in stomach, plays a major role in the gastrointestinal production of mucus. It protects the underlying gastro duodenal mucosa against acid secretion, pepsin and exogenous necrotizing agents[38,39]. It serves as a co-factor for glutathione peroxidase and glutathione S-transferase and can react directly with hydrogen peroxide, super oxide anion, hydroxyl and alkoxyl radicals by its free sulphydryl groups[40]. The present study revealed that during stress, GSH level was significantly decreased in the ulcer induced rats that did not receive any drug treatment. *Pithecellobium dulce* pretreated rats maintained the normal level of glutathione in duodenal mucosa comparable to that of standard drug ranitidine.

Glutathione peroxidase, an enzyme with selenium plays a crucial role in the elimination of H_2O_2 and lipid hydroperoxides in the gastro duodenal mucosal cell. GPx reduces peroxides to alcohol using

glutathione and, thus prevent the formation of free radicals[41]. A significant depletion in GPx activity has been observed in cysteamine treated rats. The reversal of GPX activity in animals pretreated with HAEPD could be due to the replenishment of GSH level. These observations clearly indicate that *Pithecellobium dulce* could reverse the GSH level to a great extent and provide evidence for GSH involvement in the antiulcer activity of *P. dulce*. It is further strengthened by the observation that *P. dulce* normalizes the decreased gastro duodenal mucosal GPX activity in cysteamine treated animals.

Superoxide dismutase is an important defense enzyme that catalyzes the dismutation of superoxide anions into O_2 and H_2O_2[42]. Catalase captures hydrogen peroxide (H_2O_2) and converts into water[43]. Superoxide and hydroxyl radicals are important mediators of oxidative stress that play vital role in some clinical disorders. It is reported that superoxide anion is involved in ulcer formation[44]. The decrease in SOD activity in rats exposed to reactive oxygen metabolite has been associated with a number of diseases such as ischemia reperfusion injury and inflammation in some organs[45,46]. In the present study, SOD and CAT were significantly decreased in ulcer control rats. This is probably due to the utilization of enzymatic antioxidants in decomposition of superoxide anion generated by lipid peroxidation. Pretreatment with HAEPD increased the activities of SOD and CAT which may be due to reduction in lipid peroxidation. It demonstrated the antioxidant nature of *P. dulce* that prevented the formation of free radicals. Similar results have been reported by Somani *et al*.[47] who studied the protective effect of *Dillenia indica* on acetic acid induced colitis in mice. Presence of flavonoids such as quercitrin, rutin, kaempferol, naringin and daidzein in *P. dulce* may possibly have some role in the antioxidant role of its fruit extract and can be claimed for the ulcer preventive effect[48].

The mucus content in the gastrointestinal tract plays a pivotal role in cytoprotection and repair of the gastro duodenal mucosa. Goel *et al*. observed the increased levels of adherent mucus content of gastric tissue pretreated with *Thespesia populnea* indicating its cytoprotective action on experimentally induced gastric ulcer[49]. Further, the *Aegle marmelos* fruit extract has been reported to decrease the colonic mucosal damage and inflammation in acetic acid induced colitis[50]. The increase in total carbohydrate: protein (TC:P) ratio is the direct reflection of mucin activity, which is indicated by the enhanced level of individual glycoproteins like hexose, hexosamine, fucose and sialic acid[51]. These glycoproteins are essential for the maintenance of membrane integrity of mucosal cells of duodenum. The membrane glycoprotein preserving effect of antiulcer drug from *Pongamia pinnata* has been reported[52]. Our study revealed that HAEPD pretreated rats significantly increased mucus secretion as observed from the increase in TPBC:P ratio which is taken as reliable marker for mucin secretion.

Brunner's glands are the duodenal glands located throughout the duodenum. The gland provides an alkaline condition for the intestinal enzymes to be active, thus enabling absorption to take place and lubricating the intestinal walls. The histopathological observation (Fig. 2) shows significant alterations in the duodenal mucosa of rats administered with cysteamine. The HAEPD pretreated rats showed normal villi tubes with normal basal layer of epithelium and did not show any significant alterations in the duodenal tissue architecture as that of the rats administered with ranitidine.

Conclusion

It can be concluded that HAEPD prevented ulcer induction as well as the related damage in the duodenal mucosa, by reducing the ulcer score, the extent of inflammation and free radical formation. Thus, the antioxidant role of *Pithecellobium dulce* may be claimed for the above mentioned therapeutic action as a duodenal ulcer preventing agent. The study suggests that consumption of Madras thorn (*Pithecellobium dulce*) fruits may be beneficial in preventing ulcers of gastrointestinal origin.

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