Section A-Research paper

Evaluation of antiulcer activity of p-Coumaric acid using 6 hours pylorus ligation induced peptic ulcer in rat.

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Abstract

The present study was aimed at investigating the possible antiulcer activities of p-Coumaric acid (p-CA) in a pylorus ligation induced ulcer model. The pylorus ligation induction was done in pretreated male wistar rats. The antiulcer activities of p-CA were estimated at different dose levels (50 and 100 mg/kg) using omeprazole as a standard drug (20 mg/kg).

After 6hrs of pylorus ligation rats resulted in gastric ulcer which was indicated by the accumulation of gastric secretion, increased free acidity & total acidity and decreased pH. The pretreatment of rats with p-CA significantly inhibited the ulcers induced by pylorus ligation. These results were attributed to significant reductions in ulcer index, gastric volume, total and free acidity, and pepsin while there is a marked increase in gastric pH (the antisecretory) as well as mucosal strengthening properties of this polyphenol compound. Significant reductions in level of IL- 1 beta and TNF- alpha while Antioxidant parameter such as SOD, GSH, CAT and MDA level is significantly restored. In histopathological observation of stomach revealed reduction in submucosal edema with infiltration of leukocytes and minimal congestion are noted with administration of p-CA. These results may further suggest that p-CA was found to possess antiulcer properties.

Keywords: p-Coumaric acid, pylorus ligation, gastric ulcer, wistar rat.

Introduction

An acid-induced digestive tract lesion known as peptic ulcer, also known as peptic ulcer disease (PUD), is typically found in the stomach or proximal duodenum and is defined by the defect extending into the submucosa or muscularis propria [1]. The aggressive (acid, pepsin, and Helicobacter pylori) and defensive (gastric mucus and bicarbonate secretion, prostaglandins, and inherent resistance of the mucosal cells) elements that contribute to peptic ulcer are out of balance [2].

In addition to having a greater prevalence of morbidity and mortality, the peptic ulcer is regarded as a chronic condition that affects roughly 4 million individuals globally [3-4]. Lifetime prospects of encountering peptic ulcer is about 10% in males and 4% in females [5].

Currently drug therapy is main clinical treatment for gastric ulcer. Synthetic antiulcer medications including H, anti-histaminics, proton pump inhibitors, ulcer protecting, and ulcer healing medications are used to treat ulcers, but these medications have certain common adverse effects include headache, nausea, vomiting, lethargy, constipation, giddiness, and dizziness as well as includes poor ulcer healing and ulcer recurrence, resulting in a huge economic burden on patients and public health systems. As alternative system, Herbal medications are effective in treating ulcers with no or minimal adverse effects [6-7].

Exploring efficient and secure gastroprotective drugs made from natural resources is thus essential. Currently, several edible resources have been found to help improve gastric ulcers [8]. In this aspects, on the basis of literature survey, we selected polyphenolic compound i.e. p-Coumaric (p-CA) acid for antiulcer activity.

p-Coumaric (p-CA) acid is phenolic acid which belong to family hydroxycinnamic acid and it is most commonly occure isomer in nature [9]. p-Coumaric acid is a phytochemical and nutraceutical that is present in a number of Vegetables (beans, potato, carrots, onions), fruits (apples, pears, grapes, orange, tomato, strawberries), cereals, and Mushroom species. [10-14]

Previous studies have indicated the P-Coumaric acid has very low toxicity profile with wide range of pharmacological effects includes: antioxidant effect[13], Antimelanogenic effect[15], Antimicrobial activity[16], Anti-inflammatory[17], Antidiabetic[18], Anticancer[19], Hepatoprotective, Nephronprotective[20], Immunomodulator[21], and Antiplatelet [22].

Based on previous research, this study aimed to evaluate the gastroprotective effect of p-Coumaric acid on ligation-induced gastric ulcers in rat, and to investigate the underlying mechanisms that involved.

Material and Methods

Drugs and chemicals

Omeprazole (Dr. Reddy's Laboratories Ltd.), phenolphthalein (Molychem, Mumbai), alcian blue, pyrogallol, anilinium sulphate, diethyl ether (Loba Chemie pvt. Ltd.), thiobarbituric acid & topfers reagent (Research lab Fine Chemical industries), mucus dye, folin reagent, NaOH, bovin serum albumin, trichloroacetic acid, tris-HCL buffer, EDTA, Dithiobisnitrobenzoate, Pyridine, ammonium molybdate, $NaH_2PO_4.2H_2O$, and KH_2PO_4. (Analab Fine chemicals, Mumbai)

Animals

Male wistar rats (180-220 gm) were procured from National Institute of Biosciences, Pune, and housed in polypropylene cages at 25 ± 2 °C temperature with 60% relative humidity and kept under 12:12 h light-dark cycles. They were fed with standard pellet diet (Nutrivet Life Sciences, Pune) and water ad libitum. The rats were allowed to acclimatize to laboratory conditions prior to experimentation. All procedures were carried out in the daylight period. The experiment protocol was approved by the Institutional Animal Ethics Committee (IAEC) (DYPCOP/IAEC/2022/03), and care of animals was taken as per guidelines of the Committee for Control and Supervision of Experimentation on Animals (CPCSEA). The animal were fasted for 24 for pylorus ligation.

Design of experiment: 30 male wistar rats randomly divided into 5 groups of six animals each as follows:

Group 1: (Control group): will receive Distilled water.

Group 2: (Ulcerated control): Pylorus ligation.

Group 3: (Pyloric ligation + Omeprazole): Omeprazole (20 mg/kg per oral) from day 1 to day 14 and pylorus ligation done at 15th day.

Group 4: (Pyloric ligation + PCA-I): Will receive PCA (50 mg/kg, p.o.) from day 1 to day 14 and pylorus ligation done at 15th day.

Group 5: (Pyloric ligation + PCA-II): Will receive PCA (100 mg/kg, p.o.) from day 1 to day 14 and pylorus ligation done at 15th day.

Acute oral toxicity study and dose selection of the drug

Acute toxicity study for the p-Coumaric acid was carried out on mice according to OECD guideline 423. The three mice were allowed free access to water and were starved overnight. Each animal received single dose of p-Coumaric acid (2000mg/kg p.o).

After the administration of test compound, animal were observed individually and continuously for 30 min, 2 hour and 24 hour to detect the changes in the autonomic or behavioral responses and also for tremors, convulsions, salvations, diarrhea, lethargy, sleep and coma and then monitored for any mortality for the following 14 days.

Pyloric ligation-induced gastric ulcer

The ulcer was induced by the pyloric ligation method described by Shay et al (1945) [23] with some modification. Rats were fasted for 48 h before operation, in individual cages. Following a 6-hour drugs therapy, they received anesthesia with diethyl ether, and the abdomen was opened through a short midline incision just below the xiphoid process. In order to prevent traction on the pylorus or harm to its blood supply, the pyloric section of the stomach was slightly pushed out and ligated. Carefully, the stomach was restored, and the abdominal wall was stitched up using interrupted sutures. After six hours after pyloric ligation, rats were killed by an overdose of anesthetic ether. The stomach's cardiac end was removed when the abdomen was opened, and the contents were then drained into a glass tube.

Ulcer index and % ulcer protection were calculated by using the methods described Kore Kakasaheb J. et al [24].

Percentage of ulcer protection = ulcer index of treated group / ulcer index of control group × 100

Estimation gastric secretion parameters

The effect of p- Coumaric acid on Gastric secretion parameters such as gastric volume, pH, free acidity, total acidity, pepsin and mucus was studied in pylorus ligation model.

After pyloric ligation, the gastric juice was collected and centrifuged at 2000rpm for 10 min to obtain the gastric supernatant. Measurements were made of the pH levels and gastric juice volume. Pepsin activity was determined according to wang et al.[25].Methyl orange and phenolphthalein were used as indicators in a sodium hydroxide titration to measure free acid and total acidity. Acidity was calculated using formula:

$$\text{Acidity} = \frac{\text{Volume of NaOH} \times \text{Normality of NaOH} \times 100}{0.1/100 \text{ gm}} \text{meq / Lit}$$

Total acid output ([H+] mEq/mL/h) was calculated according to Formula given below,

Total acid output = (volume of gastric juice × total acidity)/6 h (with some modification)[26-28].

Mucus content of gastric juice was detected according to the procedures reported by Berté et al. [29] with slight modifications. 4 μL of gastric juice supernatant was diluted with distilled water to 40 μL, and mixed with 1% (w/v) alcian blue GX solution (4 μL), citric acid phosphate buffer (pH 5.8, 132 μL), and distilled water (92 μL). After 24 h of incubation at 20 °C, the mixture was centrifuged (2236 × g, 10 min). The obtained supernatant's absorbance was measured at 615 nm.. Distilled water was used as blank control, and the D-value between the absorbance of distilled water and that of supernatant represented the amount of alcian blue binding (unit of mg/ mL), regarding as the free mucus content.

Antioxidant Parameters

The rats were sacrificed on day 15 and stomach of rats were isolated and washed in ice cold saline. Tissue homogenate were prepared with 0.1 M tris-HCL buffer (pH 7.4). The supernant obtained was used to estimate superoxide dismutase (SOD), Reduced Glutathione (GSH) estimation, Malondialdehyde (MDA) Value, Catalase activity (CAT) assay was performed as per method described by Mccord and Fridovich (1972); Slater and swayer 1971; Morgaon et. al. 1979 and Halim et. al. 2017 respectively. [30-33]

Estimation of IL-1 beta and TNF- alpha

Blood of rat was centrifuged to collect the serum. Levels of TNF-α and IL-1β in serum were detected by ELISA kits.

Macroscopic examination

The stomachs were opened along the larger curvature, rinsed with saline to remove any gastric contents or blood clots, and then the ulcers were examined under a 10x microscope. Analysis was based on mucosal colour, haemorrhages, and superficial and deep ulcers in addition to perforation, and these variables were ranked as follows: One is mild or focused, two is mild to moderate, and three is severe[34]

Histopathology

The stomach were identified and and dissected. The stomach tissue were washed with saline solution and instantly kept at 4^0C in 10% Neutral buffered formalin fixative solution.
The ulcerated gastric tissue was stained by using Haematoxylin and eosin stain and examined under the microscope for histopathological changes such as infiltration, inflammation and erosion.

Statistics

The data were expressed as the mean ± SEM. The experimental groups were statistically compared using one-way ANOVA followed by "Dunnet test". Statistical analysis was performed using the software GraphPad Prism version 8.00. Results were considered statistically significant when $^{**}p < 0.01$ & $^{***}p < 0.001$ when compared to ulcerated control group, $^{###}p<0.001$ when compared to normal control group.

Result

Acute toxicity test

In oral toxicity study administration of the p-CA did not exhibit any toxic symptoms and mortality when given orally at dose of 2000 mg/kg. Therefore two different doses of p-Coumaric acid i.e. 50 and 100 mg/kg were selected for antiulcer investigation.

Effect of p-CA on ulcer index and percentage protection

In the pyloric ligation-induced ulcer model, Oral administration of p-CA in two different doses (50 mg/kg and 100 mg/kg) showed a significant reduction in ulcer index, gastric volume, free acidity, total acidity as compared to the ulcerated control group. P-CA was showing a protection index of 44.79% and 62.57% at the dose of 50 mg/kg and 100 mg/kg respectively in comparison to ulcerated control whereas Omeprazole as a reference standard drug a protection percentage of70.10% has been observed. Since the ulcer protective percentage of p-CA at 50 mg/kg is 44.79% it can be considered to be less significant in the context of the study. (Table 1)

Table 1: Effect of p-CA on ulcer index and percentage protection in pylorus ligation induced ulcer model.

Groups	Ulcer Index	% Protection
Group 1 - Normal Control	-	100%
Group 2 - Ulcerated group	12.88±0.31	-
Group 3 - Reference standard group (omeprazole) 20 mg/kg per oral	3.85±0.13	70.10%
Group 4 – PCA 50 mg/kg	7.11±0.09	44.79%
Group 5 - PCA 100mg/kg	4.82±0.27	62.57%

Effect of p-Coumaric acid on acid secretory parameters

The results of various acid secretory parameters such as Gastric volume, pH, free acidity and Total acidity of p-Coumaric acid on pylorus ligation induced gastric ulcer in rats are summarized in Table 2.

Estimation of acid secretory parameters (except PH) was increased significantly in the ulcerated control group. Administration of p-CA exhibited a significant ($p < 0.001$) reduction in gastric volume, free acidity and total acidity and the results were comparable with the normal control group. While In case of gastric PH it is significantly low in the ulcerated control group. Administration of p-CA exhibited a significant ($p < 0.001$) increase in gastric PH.

Table 2: Effect of p-CA on gastric volume, pH, free acidity & total acidity in pylorus ligation induced ulcer model.

Groups	Gastric volume	pH	Free acidity	Total acidity
Group 1 -Normal Control	3.43±0.162	2.70±0.186	28.48±0.528	68.31±1.892
Group 2-Ulcerated group	8.01±0.257$^{###}$	1.73±0.158$^{##}$	43.71±0.462$^{###}$	85.63±1.380$^{###}$

Group 3 – Reference standard group (omeprazole)	1.41±0.122***	5.11±0.130***	24.27±1.363***	34.57±1.262***
Group 4 – PCA 50 mg/kg	2.25±0.143***	4.20±0.177***	36.89±1.272**	53.69±1.805***
Group 5 - PCA 100mg/kg	1.41±0.142***	5.05±0.147***	27.02±1.387***	38.47±1.423***

Effect of p-Coumaric acid on Mucin and Pepsin.

The effects p-Coumaric acid on Mucin and Pepsin in the experimental animals is shown table. 3
Significant (***p < 0.001) reduction in Pepsin, whereas significant increase in mucus content was noted with p-CA-II (High conc.) and p-CA-I (Low conc.) at 100 mg/kg and 50 mg/kg respectively when compared to induction control group.

Table 3: Effect of p-CA on Mucin and Pepsin in pylorus ligation induced ulcer model.

Groups	Mucus (mg/ml)	Pepsin (U/mL)
Group 1 - Normal Control	36.18±0.140	08.80±0.189
Group 2 - Ulcerated group	37.61±0.192$^{###}$	15.46±0.206$^{###}$
Group 3 - Reference standard group (omeprazole 20 mg/kg)	33.3±0.198***	10.43±0.264***
Group 4 - PCA 50 mg/kg	31.3±0.091***	12.26±0.168***
Group 5 - PCA 100mg/kg	33.3±0.346***	10.71±0.288***

Effect of p-Coumaric acid on antioxidant parameter

The effects p-Coumaric acid on antioxidant parameter in the experimental animals is shown table. 4

The induction of pylorus ligation caused a significant (###p < 0.001) decrease in the level of SOD, GSH, and CAT whereas significant increase in the level of MDA were noted in induction control group when compared with normal control group. Administration of PCA exhibited a significant (***p < 0.001) restoration in the levels of SOD, GSH, CAT and MDA when compared to induction control group.

The reference standard omeprazole (20mg / kg p. o.) was showed more potent and significant (*** p < 0.001) results in all above parameters.

Table 4: Effect of p-CA on antioxidant parameters in pylorus ligation induced ulcer model.

Groups	SOD(U/mg protein)	GSH (ug GSH /mg protein)	CAT (nmol/min/mL)	MDA (nmol of MDA/ mg protein)
Group 1 - Normal	17.43±0.189	13.16±0.302	6.36±0.120	4.42±1.101

Control				
Group 2 - Ulcerated group	08.25±0.190###	05.93±0.105###	4.46±0.166###	9.62±0.150###
Group 3- Reference Standard group (omeprazole 20 mg/kg)	15.89±0.406***	12.30±0.167***	7.40±0.169***	5.14±0.297***
Group 4- PCA 50 mg/kg	13.05±0.114***	10.43±0.223***	5.86±0.135***	6.54±0.078***
Group 5- PCA 100mg/kg	16.40±0.553***	11.73±0.255***	6.73±0.138***	4.47±0.118***

Effect of p-Coumaric acid on IL- 1 beta and TNF- alpha

The effect of p-Coumaric acid on IL- 1 beta and TNF- alpha in the experimental animals is shown in Figure 1 respectively.

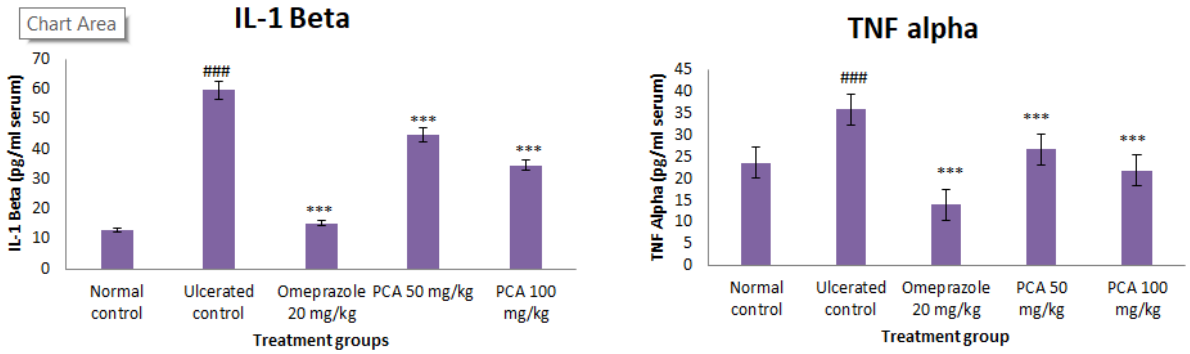


Figure 1: Effect of p-Coumaric acid on IL- 1 beta and TNF- alpha

IL- 1 beta and TNF- alpha significantly (###p<0.001) high in induction control group compared to normal control group whereas significant (p<0.001) and equipotent decrease in IL- 1 beta and TNF- alpha was noted with p-CA-II (High conc.) and p-CA-I (Low conc.) at 100 mg/kg and 50 mg/kg respectively when compared to induction control group. Reference standard Omeprazole was more potent and significant in this regard.

Macroscopic Examination

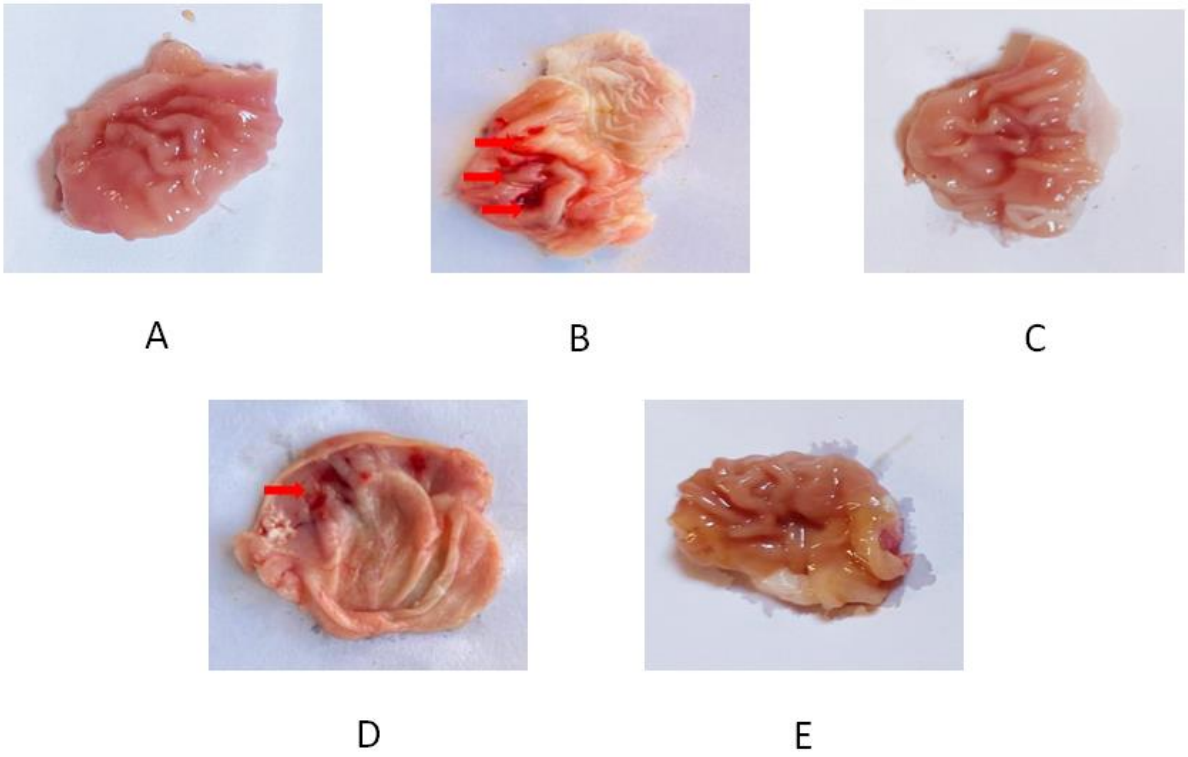
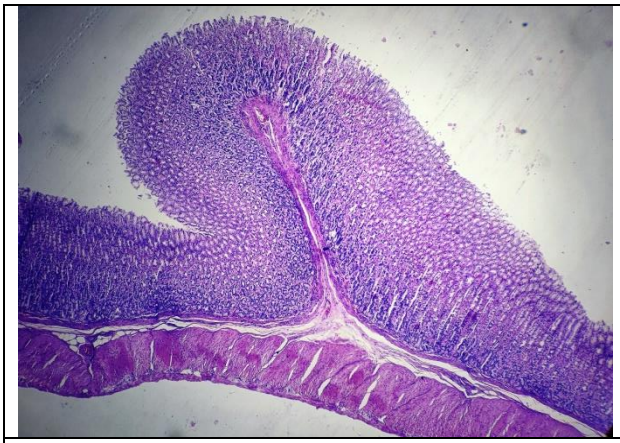
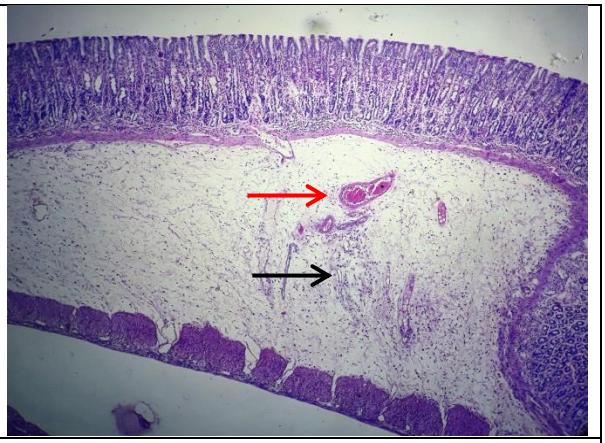
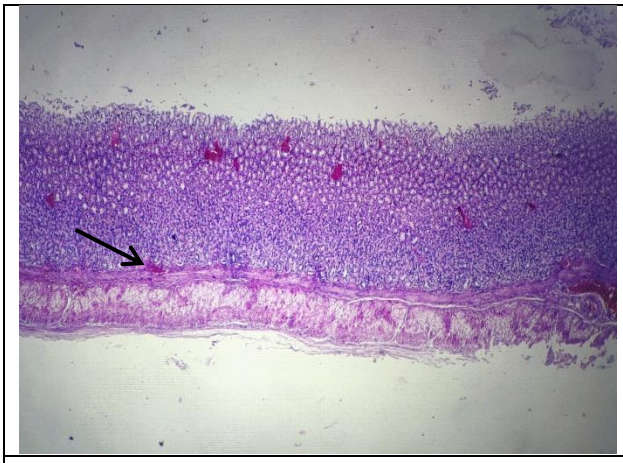


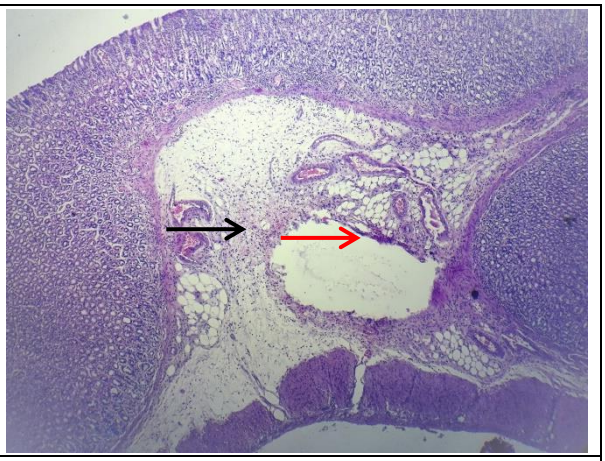
Figure 2: Macroscopic/Gross appearances of the internal aspect of gastric tissues of experimental animals (Group A-E) Arrows point to varying degrees of gastric mucosal erosion due to exposure to the acidic gastric secretions during pyloric-ligation method. Group A (Normal control) represents normal gastric mucosa; Group B (Ulcerated control) showing intense mucosal erosion; Groups C (omeprazole treated) showing null mucosal erosion; Groups D(PCA 50 mg/kg) showed mild mucosal erosion; Groups E (PCA 100 mg/kg) showed very mild/null mucosal erosion.

Histopathological examination

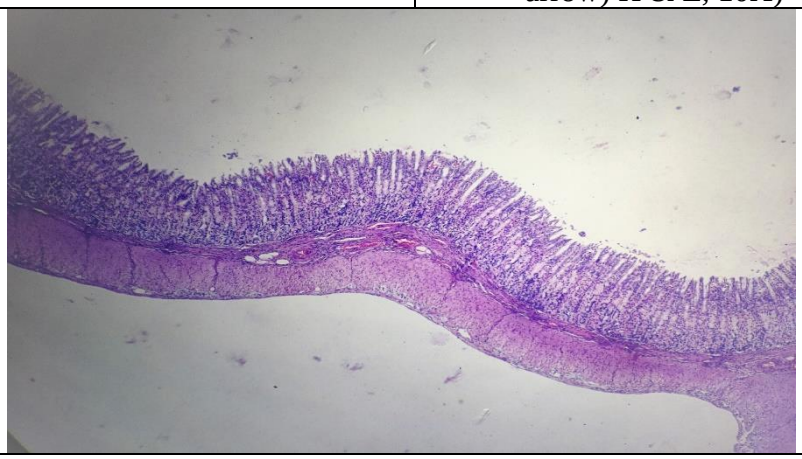
	
A. Control (No abnormality detected, H & E, 10X)	B. Ulcerated control: (Submucosal Edema with infiltration of leukocytes (Black arrow), Mild congestion (red arrow) H & E, 10X)



C. Reference standard (Minimal congestion in mucosa and submucosa (black arrow), (H & E, 10X)



D. p-CA-I (Submucosal edema with infiltration of leukocytes (Black arrow), Minimal congestion (red arrow) H & E, 10X)



E. p-CA-II (No abnormality detected, H & E, 10X)

Figure 3: Histopathological examination of pylorus ligation induced ulcer model

In pylorus ligation induction histopathological observation of stomach revealed mild submucosal edema with infiltration of leukocytes and mild congestion of blood vessels in pylorus ligation group when compared with control. There was significant reversal of gastric damage in group treated with PCA (100 mg/kg) in comparison with control, omeprazole (20 mg/kg) (standard drug) and PCA (50 mg/kg).

Discussion

This study revealed a significant antiulcer effect of p-Coumaric acid in experimental models of gastric ulcers induced by pylorus ligation in rats. 50mg/kg and 100 mg/kg as a low dose and high dose of test drug were chosen from the result of acute oral toxicity.

Although in most of the cases, the etiology of ulcer is unknown, it is generally accepted that it results from an imbalance between aggressive factors and the maintenance of the mucosal integrity through the endogenous defense mechanism. To regain the balance, different therapeutic agents including polyphenol compound are used to inhibit the gastric acid secretion or to boost the mucosal defense mechanism by increasing mucus production[35].

In pylorus ligated rats, gastric acid is associated with sever ulceration of the rat gastric mucosa. The activation of vagus–vagal-reflux by stimulation of pressure receptors in the antral gastric mucosa is believed to increase gastric acid secretion[24].

The present study demonstrated the potential p-CA at 100mg/kg and 50 mg/kg significantly reduces gastric ulceration based on using the pylorus ligation assay, the p-CA was suggested to shows its antiulcer effects by reducing the volume of gastric juice secreted, gastric free acidity, total acidity, total protein, pepsin and increasing the pH[36-38]. Thus, the possible mechanism of gastric mucosal protection by p-CA may be partly due to the reinforcement of resistance of the mucosal barrier by a protective coating. p-CA has shown increased pH and decreased total acidity of gastric fluid[39].

Mucus-bicarbonate barrier is the primary defense of gastric mucosa. Mucus is a gel adhering to the mucosa, preventing gastric acid penetrating into the mucosa and whittling some mechanical abrasion[40]. The mucus content in gastric juice was higher in p-CA at 100 mg/kg and 50mg/kg group than the UC group, indicating supplement of polyphenol compound could protect the integrity of gastric mucosa. Accumulation of pepsin in induction group leads to auto-digestion of gastric mucosa[41-42]. On treatment with the standard as well as the two doses of p-CA there was significant reduction in content of pepsin and significant increase in mucus content.

In response to the oxidative stress brought on by pylorus ligation the gastric tissue homogenate generated from the pre-treated groups with p-CA shown considerable antioxidant activity, with lower levels of MDA and higher levels of SOD, GSH & CAT. Superoxide is changed into hydrogen peroxide (H2O2) by SOD, and then either catalase in the lysosomes or glutathione peroxidase in the mitochondria transforms H2O2 into water [43] Lipid peroxidation's final product, MDA, is used to assess the amount of lipid peroxidation [44] Lipid peroxidation impairs ion membrane integrity, and fluidity of the membrane, which leads to a loss of cellular function.

Once gastric mucosa was damaged, inflammatory process was activated [45], thereby increasing inflammatory mediators, including TNF-α, & IL-1β [45]. TNF-α stimulates neutrophil infiltration and epithelial cell apoptosis, reduces gastric microcirculation around the ulcer region and delays gastric ulcer healing [46]. The extensive expression of IL-1β greatly contributes to ulcer formation [47]. Significant declines in expressions of TNF-α and IL-1β in serum of rats generated by two doses of PCA implying that the polyphenols was capable of reducing inflammatory response.

Acknowledgement

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Conflict of interest

This declaration is not applicable.

Financial support

This declaration is not applicable.

Ethics statement

The research protocol was approved by Institutional Animal Ethical Committee (IAEC) constituted as per the directions of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) under protocol number DYPCOP/IAEC/2022/03.

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