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Evaluation of antiulcer activity of protocatechuic acid ethyl ester in rats

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Abstract

To study the antiulcer activity of Protocatechuic acid ethyl ester using different models of gastric ulceration in rats. Antiulcer activity of Protocatechuic acid ethyl ester was studied in rats in which gastric ulcers were induced by oral administration of ethanol or aspirin or by pyloric ligation. Protocatechuic acid ethyl ester was administered in the dose of 30 mg/kg and 60 mg/kg i.p. 30 min prior to ulcer induction. The antiulcer activity was assessed by determining and comparing the ulcer index in the test drug group with that of the vehicle control group. Gastric total acid output and pepsin activity were estimated in the pylorus ligated rats. Ranitidine and Sucralfate were used as a reference drug. The ulcer index in the Protocatechuic acid ethyl ester treated animals was found to be significantly less in all the models compared to vehicle control animals. This antiulcer property was more prominent in animals in whom ulcers were induced by ethanol, aspirin and pyloric ligation. Ranitidine (30 mg/kg) produced a significant gastric ulcer protection when compared with the control group. The anti-ulcer activity of Protocatechuic acid ethyl ester was however, less than that of ranitidine. Our results suggest that Protocatechuic acid ethyl ester possesses significant antiulcer property which could be either due to cytoprotective action of the drug or by strengthening of gastric mucosa and thus enhancing mucosal defence.

Key-Words: Cyto protection, Mucosal defence, Ulcer Protection, Protocatechuic acid ethyl ester

Introduction

Peptic ulcer disease broadly refers to a group of disorders characterized by the presence of ulcers in any portions of g.i. tract, exposed to acid in sufficient duration and concentration although these ulceration most commonly occurs in the small intestine (duodenal ulcer) or stomach (gastric ulcer). The most common forms of peptic ulcers are gastric ulcer, duodenal ulcer, esophageal ulcer, meckel's diverticulum ulcer. The majority of gastric ulcer can be attributed to either *H. pylori* or NSAIDs induced mucosal damage. Gastric ulcer that occurs in the pyloric area or those in the body associated with a duodenal ulcer. Gastric acid output tends to be normal or decrease in gastric acid animals. When gastric acid develops in the presence of minimal acid level, impairment of mucosal defense factor may be present .Pathogenesis of gastric ulcer usually normal to low acid levels, hyper acidity if present is due to high serum gastrin, damage to mucus barrier.

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The present study nominated modulatory role of protocatechuic acid ethyl ester in gastric ulcer treatment. Protocatechuic acid ethyl ester is white or pale brownish yellow, crystalline powder; odourless or has faint phenol-like odour Chemical name of Protocatechuic acid ethyl ester is Ethyl 3,4-dihydroxybenzoate. Its molecular formula is $C_9H_{10}O_4$ and molecular weight is 182.2. Antioxidant[1], Cardioprotective[2], Iron deficiency[3], Collagen production inhibitor[4], Hypoxia tolerance[5], Neuroprotective[6], Antimicrobial & Antioxidant[7], Myoprotective activity[8] of protocatechuic acid ethyl ester have already been reported. All the new alkyl protocatechuate antioxidants studied possessed better radical-scavenging capacity than the natural antioxidant protocatechuic acid. The antioxidant activity indicates that protocatechuic acid ethyl ester may posess antiulcer activity, so we designed the protocol to evaluate the antiulcer activity protocatechuic acid ethyl ester.

Material and Methods

Experimental animals

The study was conducted on Albino rats (Wistar) of 200-250 g and maintained under standard conditions (room temperature 24- 27°C and humidity 60-65%) with 12 h light and dark cycle. The food in the form of dry pellets (Amrut Lab., Pune) and water were available ad libitum. The animal experiments were approved by the ethics committee of the institute.

Chemicals and drugs

Protocatechuic acid ethyl ester, ethanol (Yash Chem.,Pune),aspirin (Research lab, Mumbai), ranitidine (Cipla, Mumbai) sucralfate (Dr. Reddys lab, Mumbai), carboxy methyl cellulose (CMC), trichloroacetic acid (Research lab, Mumbai), phenol reagent (Research lab, Pune), bovine albumin serum (Research lab. Fine chem. industries, Mumbai), std. phenol solution (Research lab, Pune), conc. HCl (Research lab, Fine chem industries, Mumbai), NaOH (Research lab Fine chem industries, Mumbai) were used in the study.

Experiment design

Acute Toxicity Studies (OECD 423)[9]

The acute toxicity study was done as per the OECD guidelines (423). The compounds were administered i.p. in different doses, where 24 h toxicity was recorded to identify the toxic dose. No mortality and no signs of toxicity were found at the dose of 300 mg/kg body weight of protocatechuic acid ethyl ester. Therefore, it might be considered that protocatechuic acid ethyl ester have an LD50 value above 300 mg/kg. Two doses 30 mg/kg and 60 mg/kg were selected for present study.

Pyloric ligation method[10-12]

In this method, albino rats were fasted in individual cages for 24 h. Care was being taken to avoid coprophagy. Rats of either sex, were randomly divided in to 5 groups of 6 animals in each group. **Group-I:** Normal control (Normal saline), **Group-II :** Ulcerated control(1% CMC, 5 ml/kg, p.o.)., **Group-III:** Ranitidine(30 mg/kg,i.p.)., **Group-IV:** Protocatechuic acid ethyl ester (30 mg/kg, i.p.)., **Group-V:** Protocatechuic acid ethyl ester (60 mg/kg, i.p.).

Protocatechuic acid ethyl ester or reference drug or control vehicle was administered 30 min prior to pyloric ligation. Under light ether anesthesia, the abdomen was opened and the pylorus was ligated. The abdomen was then sutured. At the end of 4 h after ligation, the animals were sacrificed with excess of anesthetic ether, and the stomach was dissected out. Gastric juice was collected and its volume was measured. The glandular portion was then exposed and examined for ulceration. Ulcer index was determined.

Aspirin-induced gastric mucosal damage[10,13]

Albino rats (Wistar) of 200-250 g were selected, fasted for 36 h. Rats of either sex were randomly divided in to 5 groups of 6 animals in each group.**Group-I :** Normal control (Normal saline), **Group-II :** Ulcerated control (30 mg/kg, p.o.), **Group-III:** Ranitidine (50 mg/kg,i.p.), **Group-IV :**Protocatechuic acid ethyl ester (30 mg/kg, i.p.), **Group-V :** Protocatechuic acid ethyl ester (60 mg/kg, i.p.).

After 30 min., aspirin suspended in 1% CMC in water (20 mg/ml) at a dose of 30 mg/kg was administered orally to all the animals and 4 h later, the animals were sacrificed. The stomach was removed and opens along the greater curvature. The number of ulcer spots in the glandular portion of the stomach were counted in both control and drug treated animals and the ulcer index was calculated.

Gastric cytoprotection methods (Ethanol induced ulcers)[14-15]

Albino rats (Wistar) of 200-250 g are maintained under standard conditions (room temperature 24- 27oC and humidity 60-65%) with 12 h light and dark cycle. The food in the form of dry pellets (Amrut Lab., Pune) and water were available ad libitum. Rats of either sex, were randomly divided in to 5 groups of 6 animals in each group.**Group-I:** Normal control (Normal saline), **Group-II:** Ulcerated control (1 ml ethanol, p.o.), **Group-III:** Sucralfate (400 mg/kg,p.o.), **Group-IV:** Protocatechuic acid ethyl ester (30 mg/kg, i.p.), **Group-V:** Protocatechuic acid ethyl ester (60 mg/kg, i.p.).

Thirty minutes after the test or reference drug or the control vehicle treatment, 1 ml of ethanol was orally administered to each rat. After 1 h the rats were euthanized with excess of anesthetic ether and stomach was cut open along the greater curvature, cleared of residual matter with saline and the inner surface was examined for ulceration. Ulcer index and % ulcer protection were calculated by using the methods described earlier.

Determination of various parameters

Determination of ulcer index (UI)[16-17]

The ulcerative index was calculated by severity of gastric mucosal lesions and graded as follows;

Erosions	Score
1 mm or less	1
1-2 mm	2
More than 2 mm	3

Then the UI was calculated by using the formula:

UI = 1 (no. of lesions of grade 1) + 2 (no. of lesions of grade 2) + 3 (no. of lesions of grade 3). Then the overall score was divided by a factor 10, which was

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designed as ulcer index. % gastro protection was calculated according to

% gastro protection = (UIC-UIT)/UIC*100

Where, UIC-ulcer index of control, UIT-ulcer index of test.

Collection of gastric juice

Gastric juice was collected from pylorus ligated rats. The gastric juice collected was centrifuged at 60 rpm for 10 min. and the volume of gastric juice was measured. The gastric juice was used for biochemical estimation.

Determination of free acidity and total acidity[11]

Gastric juice (1 ml) was taken in to a 100 ml conical flask, to this 2-3 drops of Topfer's reagent was added and titrated with 0.01 N NaOH until all traces of red colour disappears and the colour of the solution turns yellowish orange (end point). The volume of alkali added was noted. This volume corresponds to free acidity. Then 2-3 drops of phenolphthalein solution was added and titration was continued until a definite red tinge reappears. The volume of alkali added was noted which corresponds to total acidity. Acidity was calculated by using the formula;

Acidity (mEq/litre) = Volume of NaOH* Normality of NaOH * 100/0.1

Estimation of pepsin[12,18-19]

For estimation pepsin, placed 4 test tubes (1) and (2) containing 5 ml of 1% bovine albumin in 0.01 M HCL, (3) and (4) containing 10 ml of 0.35 M trichloroacetic acid. The gastric juice was mixed with an equal volume of 0.01 M HCL, warmed to 370 c. 1 ml of this mixture was added to each of test tubes of (1) and (4). Incubated for 15 min. At the end mixed content of tube (1) with (3). Allow to stand for about 4 min. (1)+(3) gives test and (2)+ (4) gives blank.The mixture was filtered. To 2 ml of the filtrate, 10 ml of NaOH was added. Then 1 ml of phenol reagent was added and mixed by gentle rotation. After 30 min.,the absorbance was measured at 680 nm. The difference between test and blank gives the measures of peptic activity. As standard, mixed 2 ml of freshly prepared phenol solution containing 50 mcg/ml with 10 ml NaOH and 1 ml of phenol reagent was addd and was measured at 680 nm after 5 to 10 min.

Statistical analysis

The Statistical analysis was performed by using One Way ANOVA followed by Dunnet's comparision test and student t-test (unpaired).The values are expressed as mean ± SEM and the $P<0.05$ was taken as significant.

Results and Conclusion

Effect of Protocatechuic acid ethyl ester in pylorus ligated rats

Pylorus ligation in ulcerated control group had produced ulcer in all animals and the mean ulcer index was 4.20±0.19 indicating the ulcerogenic effect. Another ulcerogenic effect as compared to normal control was measured as follows; mean gastric content volume as 6.18±0.17, pH as 1.67±0.05, free acidity as 36.23 ± 1.60, total acidity as 75.23 ± 1.50, pepsin content as 5.16 ± 0.59, indicating the ulcer production in animals. Pylorus ligation also produced ulcers in all the protocatechuic acid ethyl ester pretreated animals. However, the ulcer index showed significant dose dependent reduction in the animal pretreated with protocatechuic acid ethyl ester 30 mg/kg (UI; 3.02 ± 0.10) and 60 mg/kg (UI; 2.15 ± 0.08).It indicated 28.09% gastroprotection at 30 mg/kg and 48.80% gastroprotection at 60 mg/kg as compared with ulcerated control. The results indicate that the higher dose of protocatechuic acid ethyl ester i.e. 60 mg/kg was effective in protecting ulcers in pylorus ligated rats. Pylorus ligation had produce ulcers in all animals pretreated with Ranitidine 30 mg/kg. However, ulcer index (1.58 ± 0.09) showed significant reduction as compared with ulcerated control and showed 62.38 % gastroprotection.

Effect of Protocatechuic acid ethyl ester in Aspirin induced gastric ulcer

Aspirin induced ulcerated control group had produced ulcer in all animals and the mean ulcer index was 2.15 ± 0.11 indicating the ulcerogenic effect. Pylorus ligation also produced ulcers in all the protocatechuic acid ethyl ester pretreated animals. However, the ulcer index showed significant dose dependent reduction in the animal pretreated with protocatechuic acid ethyl ester 30 mg/kg (UI; 1.15 ± 0.05) and 60 mg/kg (UI; 0.75 ± 0.12).It indicated 46.51% gastro protection at 30 mg/kg and 65.11% gastro protection at 60 mg/kg as compared with ulcerated control. The results indicate that the higher dose of protocatechuic acid ethyl ester i.e. 60 mg/kg was effective in protecting ulcers in pylorus ligated rats. Pylorus ligation had produce ulcers in all animals pretreated with Ranitidine 30 mg/kg. However; ulcer index (0.25 ± 0.02) showed significant reduction as compared with ulcerated control and showed 88.37 % gastro protection.

Effect of Protocatechuic acid ethyl ester in ethanol induced gastric ulcer

Ethanol induced ulcerated control group had produced ulcer in all animals and the mean ulcer index was 4.42 ± 0.05 indicating the ulcerogenic effect. Pylorus ligation also produced ulcers in all the protocatechuic

acid ethyl ester pretreated animals. However, the ulcer index showed significant dose dependent reduction in the animal pretreated with protocatechuic acid ethyl ester 30 mg/kg (UI; 2.28 ± 0.17) and 60 mg/kg (UI; 2.10 ± 0.09).It indicated 44.41% gastro protection at 30 mg/kg and 52.48% gastro protection at 60 mg/kg as compared with ulcerated control. The results indicate that the higher dose of protocatechuic acid ethyl ester i.e. 60 mg/kg was effective in protecting ulcers in pylorus ligated rats. Pylorus ligation had produce ulcers in all animals pretreated with Sucralfate 400 mg/kg. However, ulcer index (1.05 ± 0.19) showed significant reduction as compared with ulcerated control and showed 76.24 % gastro protection.

The finding of the present study demonstrated that protocatechuic acid ethyl ester possess antiulcer activity against the ulceration caused by pylorus ligation , aspirin and ethanol. 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. The digestive site of accumulated gastric juice and interference of gastric blood circulation responsible for ulceration. It is evident from the present result that protocatechuic acid ethyl ester has potent ulcer protective activity at a dose 30 mg/kg and 60 mg/kg, but at the dose 60 mg/kg was more potent. The ulcer index of protocatechuic acid ethyl ester treated rat was comparable to those of ulcerated control rats. There was significant decrease in ulcer index in protocatechuic acid ethyl ester treated rats and ranitidine treated rats.

There was significant decrease in the volume of gastric content, free acidity, total acidity, peptic activity and increase in pH in protocatechuic acid ethyl ester treated rats in dose dependent manner as compared with ulcerated control rats. The activity of protocatechuic acid ethyl ester was less than ranitidine (30 mg/kg).

Several non-steroidal anti-inflammatory drugs like; aspirin are known to induce gastric damage by suppression of prostaglandins. In the stomach, prostaglandins play a vital protective role, stimulating the secretion of bicarbonate and mucus maintaining mucosal blood flow and regulating mucosal cell turn over and repair. Oxy radicals may play important role in the aspirin induced erosive gastritis. After an initial hydrophobic intermolecular interaction, the free carboxyl group present in all NSAIDs forms a strong electrostatic bond with the positively charged head group of zwitterionic phospholipids of mucus layer and, in doing so, increase the phospholipids solubility, neutralize its surface activity. Thus, NSAIDs topically act on tissue to disrupt the hydrophobic protective lining of the mucus gel layer[20-21].

It is evident from the present result that protocatechuic acid ethyl ester has potent ulcer protective activity at a dose 30 mg/kg and 60 mg/kg, but at the dose 60 mg/kg was more potent. The ulcer index of protocatechuic acid ethyl ester treated rat was comparable to those of ulcerated control rats. There was significant decrease in ulcer index in protocatechuic acid ethyl ester treated rats and ranitidine treated rats.

Ethanol produce necrotic lesions in the gastric mucosa by its direct toxic effect reducing the secretion of bicarbonate and production of mucus, increase vascular permeability and decreases non-protein sulf-hydryl groups (NP-SH) of gastric mucosa. Also, increase xanthine oxidase activity and malonyl-dialdehyde level. The ethanol also depresses tissue level of DNA, RNA and proteins, leading to flow stasis and injured area.

In the present study, protocatechuic acid ethyl ester has potent ulcer protective activity at a dose 30 mg/kg and 60 mg/kg, but at the dose 60 mg/kg was more potent. The ulcer index of protocatechuic acid ethyl ester treated rat was comparable to those of ulcerated control rats. There was significant decrease in ulcer index in protocatechuic acid ethyl ester treated rats and ranitidine treated rats.

In conclusion, it appears that protocatechuic acid ethyl ester possess anti-ulcerogenic property against gastric mucosal damage induced by pylorus ligation, aspirin and ethanol, through inhibition of gastric acid, pepsin, histamine and free radical and stimulation of mucus secretion.

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Table 1: Effect of protocatechuic acid ethyl ester on volume and pH of gastric content in pylorus ligated rats

S/No.	Treatment (mg/kg)	Volume of gastric juice (ml)	pH of gastric juice
1.	Normal control	1.06±0.07	3.77±0.03
2.	Ulcerated control	6.18±0.17	1.67±0.05
3.	Ranitidine (30)	3.00±0.12**	3.50±0.06**
4.	Protocatechuic acid ethyl ester (30 mg/kg)	4.10±0.09*	2.38±0.07*
5.	Protocatechuic acid ethyl ester (60 mg/kg)	3.22±0.08**	3.34±0.10**

Values are expressed as mean ± S.E.M., n=6, Ranitidine 30 mg/kg, Protocatechuic acid ethyl ester 30,60 mg/kg, *p<0.05, **p<0.01, as compared with ulcerated control using one way ANNOVA followed by Dunnet test.

Table 2: Effect of protocatechuic acid ethyl ester on free acidity and total acidity in pylorus ligated rats

S/ No.	Treatment (mg/kg)	Free acidity (meq/L/100gm)	Total acidity(meq/L/100gm)
1.	Normal control	9.54 ± 0.40	24.6 ± 0.65
2.	Ulcerated control	36.23 ± 1.60	75.23 ± 1.50
3.	Ranitidine (30 mg/kg)	14.30 ± 0.36**	31.5 ± 0.66**
4.	Protocatechuic acid ethyl ester (30 mg/kg)	33.88 ± 0.55	70.05 ± 0.50
5.	Protocatechuic acid ethyl ester (60 mg/kg)	22.1 ± 0.95*	45.7 ± 1.21**

Values are expressed as mean ± S.E.M., n=6, Ranitidine 30 mg/kg, Protocatechuic acid ethyl ester 30,60 mg/kg, *p<0.05, **p<0.01, as compared with ulcerated control using one way ANNOVA followed by Dunnet test.

Table 3: Effect of protocatechuic acid ethyl ester on pepsin content in pylorus ligated rats

S/ No.	Treatment (mg/kg)	Pepsin content (mcg/ml)
1.	Normal control	0.77 ± 0.03
2.	Ulcerated control	5.16 ± 0.59
3.	Ranitidine (30 mg/kg)	2.29 ± 0.63**
4.	Protocatechuic acid ethyl ester (30 mg/kg)	4.27 ± 0.09*
5.	Protocatechuic acid ethyl ester (60 mg/kg)	2.98 ± 0.12**

Values are expressed as mean ± S.E.M., n=6, Ranitidine 30 mg/kg, Protocatechuic acid ethyl ester 30,60 mg/kg, *p<0.05, **p<0.01, as compared with ulcerated control using one way ANNOVA followed by Dunnet test.

Table 4: Effect of protocatechuic acid ethyl ester on ulcer index and % gastro protection in pylorus ligated rats

S/ No.	Treatment (mg/kg)	Ulcer index (Mean± SEM)	% Gastro protection
1.	Normal control	-	100
2.	Ulcerated control	4.2 ± 0.19	-
3.	Ranitidine (30 mg/kg)	1.58 ± 0.09	62.38**
4.	Protocatechuic acid ethyl ester (30 mg/kg)	3.02 ± 0.10*	28.09*
5.	Protocatechuic acid ethyl ester (60 mg/kg)	2.15 ± 0.08**	48.80**

Values are expressed as mean ± S.E.M., n=6, Ranitidine 30 mg/kg, Protocatechuic acid ethyl ester 30,60 mg/kg, *p<0.05, **p<0.01, as compared with ulcerated control using one way ANNOVA followed by Dunnet test.

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Table 5: Effect of protocatechuic acid ethyl ester on ulcer index and % gastro protection in aspirin induced gastric lesion in rats

S/ No.	Treatment (mg/kg)	Ulcer index (Mean± SEM)	% Gastro protection
1.	Normal control	-	100
2.	Ulcerated control	2.15 ± 0.11	-
3.	Ranitidine (30)	0.25 ± 0.02**	88.37**
4.	Protocatechuic acid ethyl ester (30 mg/kg)	1.15 ± 0.05*	46.51*
5.	Protocatechuic acid ethyl ester (60 mg/kg)	0.75 ± 0.12**	65.11**

Values are expressed as mean ± S.E.M., n=6, Aspirin 30 mg/kg, Ranitidine 30 mg/kg, Protocatechuic acid ethyl ester 30,60 mg/kg, *p<0.05, **p<0.01, as compared with ulcerated control using one way ANNOVA followed by Dunnet test.

Table 6: Effect of protocatechuic acid ethyl ester on ulcer index and % gastro protection in ethanol induced gastric ulcer in rats.

S/No.	Treatment (mg/kg)	Ulcer index (Mean± SEM)	% Gastro protection
1.	Normal control	-	100
2.	Ulcerated control	4.42 ± 0.05	-
3.	Sucralfate (400 mg/kg)	1.05 ± 0.19	76.24
4.	Protocatechuic acid ethyl ester (30 mg/kg)	2.28 ± 0.17*	44.41*
5.	Protocatechuic acid ethyl ester (60 mg/kg)	2.10 ± 0.09**	52.48**

Values are expressed as mean ± S.E.M., n=6, Sucralfate 400 mg/kg, Protocatechuic acid ethyl ester 30,60 mg/kg, *p<0.05, **p<0.01, as compared with ulcerated control using one way ANNOVA followed by Dunnet test.