



Evaluation of Protective Effect of Hydro-alcoholic Extract of Fruit Peels of *Punica granatum* Linn against Ulcerative Colitis in Rats

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ABSTRACT

The study was designed to evaluate the protective effect of hydro-alcoholic extract of fruits peels of *Punica granatum* Linn. against acetic acid and trinitrobenzene sulfonic acid (TNBS) induced ulcerative colitis in rats. Acute oral toxicity study was performed to find out the test dose according to OECD guidelines 425 and hydro-alcoholic extract of *P. granatum* fruit peel (PGPE) was found to be safe at a dose of 2000 mg/kg body weight. The animals pretreated with hydro-alcoholic extract of *P. granatum* fruit peel (200 mg/kg and 400 mg/kg respectively) significantly restored the altered hematological, biochemical parameters to normal levels when compared with control. The protective effect of *P. granatum* peel extract was comparable to standard sulfasalazine. The findings of the present study revealed that the hydro-alcoholic extract of *P. granatum* fruit peel possessed a dose dependent significant inhibitory activity against ulcerative colitis. The results obtained established the efficacy of the *Punica granatum* fruit peel against inflammatory bowel diseases possibly by its anti-inflammatory and antioxidant properties.

Keywords: Acetic acid, *Punica granatum*, Sulfasalazine, TNBS, Ulcerative colitis.

INTRODUCTION

Inflammatory bowel diseases (IBD) including ulcerative colitis (UC) and Crohn's disease (CD) are the most affecting human illness are chronic inflammatory bowel disease. [1-2]

Inflammatory bowel disease is an immune-mediated disease of the gastrointestinal tract caused by ulceration and inflammation. [3] The pathogenesis of IBD is a multi factorial process. [4] In addition, the progress of an unusual immune and inflammatory reaction occurs, which is mediated by activated neutrophils, monocytes and characterized by an increased development of reactive oxygen and nitrogen species. [5]

Ulcerative colitis, moreover known as chronic ulceration of the intestines, is a universal disease estimated to have an incidence of 1 in 1000 persons in western countries. [6] Effective treatment relies on correct and timely diagnosis. Various factors contribute to the disease's clinical physical components, but the etiology of the disease remains poorly understood. [7]

The majority of the current treatments for inflammatory

bowel disease include treatment with glucocorticosteroids and 5-aminosalicylic acid. Immunosuppressive drugs have also been used to heal chronic illness, regardless of the more dangerous complications and hazardous side effect associated with them. [8] In allopathic system of treatment, corticosteroids and aminosalicylates such as sulfasalazine, mesalamine are mainly used. But these drugs have adverse effects such as head ache, nausea, temporary male infertility in males, etc.

The use of many traditional remedies mainly plant products are also common throughout India. Some of these plants as well as plant formulations used in traditional medicines have been pharmacologically evaluated for their efficacy to alleviate ulcerative colitis. However, still more numbers of plants are needed to be screened for their ulcerative colitis protective property. One such plant is *Punica granatum* Linn locally available has been used in traditional system of medicine. It has good antioxidant property and has been proved for the treatment of parasitic, viral and bacterial infection. However, there is no scientific claim has been made on the ulcerative colitis protective activity of the plant of *Punica granatum* Linn fruit peels. In view of this, an attempt has been made to investigate the ulcerative colitis protective role of *Punica granatum* Linn fruit peels.

MATERIALS AND METHODS

Plant material

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The fresh peels of *Punica Granatum* used for the present studies were collected from Kondotty, Kerala in May 2013. It was authenticated by Mr. Prabhu Kumar, Scientist, Aryavaidyasala, Kotakkal, Kerala.

Preparation of plant extracts

The powdered material of *P. granatum* fruit peels was subjected to hydro-alcoholic extraction in Soxhlet apparatus by using water and ethanol as solvents. The powdered material (150 gm) was packed in Soxhlet extractor and extracted using solvents ethanol-water (1:1). The temperature was maintained on an electric heating mantle with thermostat control. Appearance of colorless solvent in the siphon tube was taken as the termination of extraction. The extract was concentrated by using rotary flash evaporator. The concentrated extract was then air dried at room temperature and stored in air tight container until used.

Phytochemical screening^[9-10]

Freshly prepared hydroalcoholic extract of the fruit peels of *Punica granatum* (PGPE) was subjected to preliminary phytochemical screening for detection of major chemical constituents.

Experimental animals

Healthy Wistar albino rats of either sex weighing 150-200 g were used. Animals used in the study were procured from registered breeder. The animal care and handling was carried out according to CPCSEA guidelines. Animals were acclimatized to the animal quarantine for one week prior to the experiment under controlled conditions of temperature ($27 \pm 2^\circ\text{C}$) and were housed in sterile polypropylene cages containing paddy husk as bedding material with maximum of six animals in each cage. The rats were fed on standard food pellets and water *ad libitum*. The studies conducted were approved by the Institutional Animal Ethical Committee, Srinivas College of Pharmacy, Mangalore, Karnataka (Approval No.: SCP/CPCSEA/P11/F150/2012).

Acute toxicity study

Acute toxicity study of hydroalcoholic extract of the fruit peels of *P. granatum* (PGPE) was determined in Wistar albino rats (150-200 g) according to OECD guidelines No. 425.^[11] The animals were fasted overnight and the extract was administered orally with a starting dose of 2000 mg/kg to different groups of animals. Animals were observed continuously for first 3 h and monitored for 14 days for mortality and any general behaviour of animals, signs of discomfort and nervous manifestations.

Acetic acid induced ulcerative colitis in rats^[12]

Healthy albino rats of either sex weighing 150–200 g were used in the study and divided into five groups with six animals each (n=6) as follow

- | | |
|---------------------------|---|
| Group I (Vehicle control) | : 1 ml distilled water |
| Group II (Toxic control) | : 2 ml acetic acid |
| Group III (Standard) | : Sulfasalazine (100 mg/kg, p.o.) + Acetic acid |
| Group IV (Test Low Dose) | : PGPE (200 mg/kg, p.o.) + Acetic acid |
| Group V (Test High Dose) | : PGPE (400 mg/kg, p.o.) + Acetic acid |

Trinitrobenzene sulfonic acid induced colitis^[13]

Healthy albino rats of either sex weighing 150–200 g were used in the study and divided into five groups with six animals each (n=6) as follow

- | | |
|---------------------------|------------------------|
| Group I (Vehicle control) | : 1 ml distilled water |
| Group II (Toxic control) | : 1 ml TNBS |

- | | |
|--------------------------|--|
| Group III (Standard) | : Sulfasalazine (100 mg/kg, p.o.) + TNBS |
| Group IV (Test Low Dose) | : PGPE (200 mg/kg, p.o.) + TNBS |
| Group V (Test High Dose) | : PGPE (400 mg/kg, p.o.) + TNBS |

In both acetic acid/TNBS induced colitis the animals were pre-treated with the respective drugs for 8 days along with the normal diet. On the 4th day of the treatment, the animals were fasted overnight with access to glucose containing water *ad libitum*. On the 5th day after 1 h of the aforementioned treatments, the animals (Groups II to V) were anesthetized by ether inhalation and a polypropylene tube with 2 mm diameter was inserted through the rectum into the colon to a distance of 8 cm. Initially, each rat was received a 1 ml saline (0.9%) flush followed by manual palpation of the abdomen to remove any feces. Then, the solution of 2 ml of acetic acid (3%, v/v) in 0.9% saline/ a solution of 1 ml of TNBS (dissolved in 1.0 ml 50% ethanol) was instilled into the lumen of the colon and maintained in a supine Trendelenburg position for 30 s to prevent the leakage of the intracolonic instillate.

Assessment of colon function

After 72 h of single dose administration of TNBS (on 8th day), clinical activity scores were measured and the animals were anaesthetized with ether and blood was collected by retro orbital puncture for biochemical estimation. The animals were sacrificed by cervical dislocation and colon was dissected out. The colon was flushed gently with saline and weighed. One portion of the colon was subjected to histopathological examination and the other portion was subsequently subjected to estimation of endogenous antioxidant parameters like LPO^[14], SOD^[15], GSH^[14] and CAT.^[16]

Statistical analysis

All data were expressed as Mean $\pm$ SEM. The statistical significance between groups was compared using One way ANOVA, followed by Dunnett's 't' test. The $p < 0.05$ was considered as statistically significant.

RESULTS

Phytochemical screening

Preliminary phytochemical investigation of the PGPE reveals the presence of flavonoids, carbohydrates, steroids, alkaloids, glycosides and tannins.

Acute toxicity study

Acute toxicity studies were carried out according to OECD guidelines. No mortality was observed at 2000 mg/kg body weight. Therefore 1/10th and 1/5th doses were selected as low and high effective dose for this study.

Effect on MPO, LPO, CAT and GSH in acetic acid induced colitis

It was observed that animals treated with acetic acid developed a colonic damage, observed as increase in LPO, MPO and decrease in GSH, CAT level when compared to normal control. Animals treated with standard (Sulfasalazine) showed extremely significant ($p < 0.001$) increase in GSH, CAT and decrease in LPO and MPO level. Treatment with PGPE (200 and 400 mg/kg, p.o.) significantly ($p < 0.05$) prevented the increase in LPO, MPO levels and brought them near to normal level, whereas GSH, and CAT levels were significantly raised, thus providing protection against acetic acid induced toxicity (Table 1).

Table 1: Effect of Sulfasalazine and PGPE on MPO, LPO, CAT, and GSH in acetic acid induced colitis

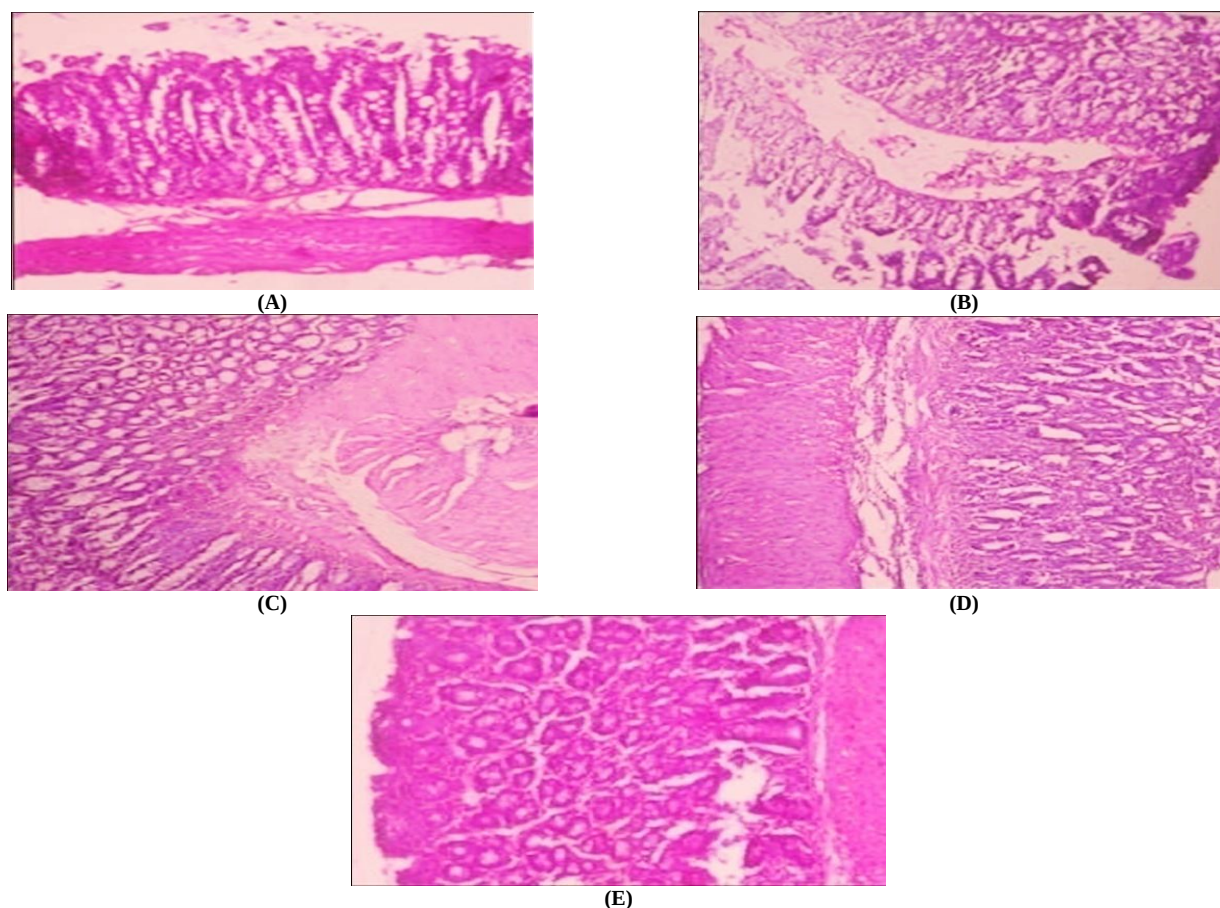
Treatment	MPO (Abs at 460 nm)	LPO (Abs at 535 nm)	CAT (Abs at 560 nm)	GSH (Abs at 412 nm)
Normal	0.08±0.01	0.08±0.03	0.48±0.05	0.46±0.05
Acetic acid control	0.46±0.08	0.36±0.01	0.17±0.02	0.13±0.08
Standard (Sulfasalazine 100 mg/kg)	0.12±0.04***	0.17±0.02***	0.40±0.08***	0.34±0.02***
PGPE (200 mg/kg)	0.38±0.02**	0.30±0.01**	0.22±0.07**	0.24±0.02*
PGPE (400 mg/kg)	0.22±0.04***	0.24±0.08***	0.29±0.01***	0.28±0.06**

All the values are in absorbance mean ±SEM, n= 6. Data were analyzed by One way ANOVA followed by Dunnett's 't' test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ when compared with acetic acid control group

Table 2: Effect of Sulfasalazine and PGPE on MPO, LPO, CAT, and GSH in TNBS induced colitis

Treatment	MPO (Abs at 460 nm)	LPO (Abs at 535 nm)	CAT (Abs at 560 nm)	GSH (Abs at 412 nm)
Normal	0.17±0.008	0.21±0.03	0.62±0.02	0.70±0.01
TNBS control	0.56±0.017	0.65±0.01	0.21±0.01	0.47±0.02
Standard (Sulfasalazine 100 mg/kg)	0.30±0.05***	0.28±0.34***	0.52±0.04***	0.66±0.01***
PGPE (200 mg/kg)	0.42±0.04*	0.55±0.01*	0.36±0.07**	0.49±0.02*
PGPE (400 mg/kg)	0.35±0.02**	0.51±0.08**	0.44±0.02***	0.60±0.08**

All the values are in absorbance mean ±SEM, n= 6. Data were analyzed by One way ANOVA followed by Dennett's 't' test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ when compared with TNBS control group


Fig. 1: Effect of Sulfasalazine and PGPE on colon histology in acetic acid induced colitis

(A) Normal control: normal texture of colon tissue, (B) Positive control (Acetic acid treated): Severe tissue damage and neutrophil infiltration, (C) Low dose (PGPE-200): Moderate tissue damage and neutrophil infiltration, (D) High dose (PGPE-400): Mild tissue damage and neutrophil infiltration, (E) Standard (Sulfasalazine treated): Less tissue damage and neutrophil infiltration

Histopathology

Histopathological studies also provided a supportive confirmation for biochemical analysis. The acetic acid intoxicated groups of animals showed inflammation, centrilobular degeneration and necrosis in colon region. Treatment with PGPE (200 and 400 mg/kg) found to reduce inflammation, centrilobular and bridging necrosis. Colon section of these groups revealed significant reduction in areas of necrosis when compared to toxic group. These changes showed protective effect of the extract against ulcerative colitis induced by acetic acid (Fig. 1).

Effect on MPO, LPO, CAT and GSH in TNBS induced colitis

It was observed that animals treated with TNBS developed a colonic damage, observed as increase in LPO, MPO and decrease in GSH, CAT, when compared to normal control. Animals treated with standard (Sulfasalazine) showed extremely significant ($p<0.001$) increase in GSH, CAT and decrease in LPO and MPO. Treatment with PGPE (200 and 400 mg/kg, p.o.) significantly ($p<0.05$) prevented the increase in LPO, MPO levels and brought them near to normal level, whereas GSH, and CAT levels were significantly raised, thus providing protection against TNBS toxicity (Table 2).

Histopathology

Histopathological studies also provided a supportive

confirmation for biochemical analysis. In TNBS intoxicated groups of animal inflammation, centrilobular degeneration and necrosis was observed in colon. Whereas treatment with PGPE (200 and 400 mg/kg) found to reduce inflammation, centrilobular and bridging necrosis. Colon section of these

groups revealed significant reduction in areas of necrosis when compared to toxic group. These changes showed protective effect of the extract against TNBS induced ulcerative colitis (Fig. 2).

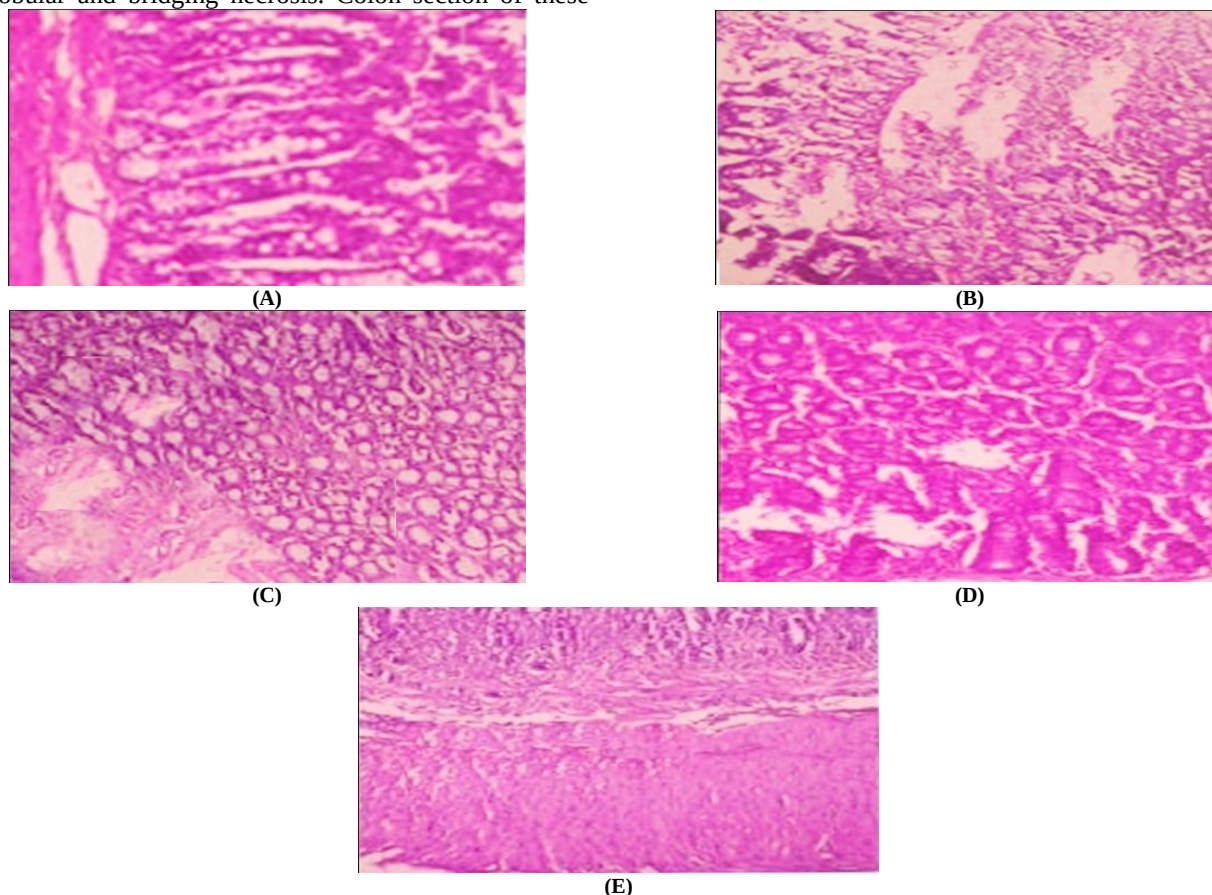


Fig. 2: Effect of Sulfasalazine and PGPE on colon histology in TNBS induced colitis

(A) Normal control: normal texture of colon tissue, (B) Positive control (TNBS treated): Severe tissue damage and neutrophil infiltration, (C) Low dose (PGPE-200): Moderate tissue damage and neutrophil infiltration, (D) High dose (PGPE-400): Mild tissue damage and neutrophil infiltration, (E) Standard (Sulfasalazine treated): Less tissue damage and neutrophil infiltration

DISCUSSION

The present study was undertaken to assess the protective effect of hydro-alcoholic extract of *P. granatum* fruit peels in colonic ulcer induced by toxic substances like acetic acid and TNBS. Intra rectal instillation of acetic acid in rats affected only the distal colon portion. The inflammation was not transmural. Massive necrosis of mucosal and sub mucosal layers were observed. This model shares many of the histological features of ulcerative colitis in human beings including mucosal edema, neutrophil infiltration of the mucosa and sub mucosal ulceration.^[17]

The mechanism by which acetic acid produces inflammation appears to involve the entry of the protonated form of the acid into epithelium, where it dissociates to liberate protons causing intracellular acidification that most likely account for the observed epithelial injury. The inflammatory response initiated by acetic acid includes activation of cyclooxygenase and lipoxygenase pathways and generation of inflammatory mediators like prostaglandins and leukotrienes. Excess production of reactive oxygen metabolites e.g. superoxide, hydroxyl radical, hydrogen peroxide, hypochlorous acid and oxidant derivatives such as N-chloramines are detected in inflamed mucosa and may be pathogenic in IBD. Also, there is an increase in proinflammatory cytokine TNF- α production in colonic mucosa after acetic acid instillation.^[18]

Colitis can be induced in animals by intra rectal instillation of the haptening substance TNBS. Massive necrosis of mucosal and sub mucosal layers were observed. This model showed many of the histological features of ulcerative colitis in human beings including mucosal edema, neutrophil infiltration of the mucosa and sub mucosal ulceration. TNBS dissolved in ethanol with or without a skin pre-sensitation step was used. Ethanol is required to break the mucosal barrier, whereas TNBS is believed to haptinize colonic autologous or microbiota proteins rendering them immunogenic to the host immune system.^[19]

The treatment with hydro-alcoholic extract of *P. granatum* fruit peel has showed a decrease in the macroscopic scores for the inflammation. Since the intestine is in a constant state of controlled inflammation, thus amplification of the inflammatory response activates infiltration of inflammatory cells that triggers pathological responses and symptoms of IBD.^[20]

MDA is considered as an important indicator of lipid peroxidation^[21], which is found to be increased in rats treated with acetic acid and TNBS. This might be due to lipid peroxidation. Rat pretreated with *P. granatum* fruit peel extract showed protection against lipid peroxidation characterized by significant decrease in MDA level.

The antioxidant enzyme, catalase is the first line defensive enzyme against free radicals. [22] In the present study, it is decreased with acetic acid and TNBS treated groups, where as in *P. granatum* fruit peel extract treated animals the antioxidant parameter (CAT) is significantly increased. This shows that the extract can reduce reactive free radicals that might cause oxidative damage to the tissues.

GSH is a natural antioxidant present in the body, which is found to be decreased in rats treated with acetic acid and TNBS. [23] In the present study, it was observed that pretreatment with *P. granatum* fruit peel extract exhibited elevated GSH level.

Myeloperoxidase (MPO) is an enzyme mainly found in azurophilic granules of neutrophils. It can serve as a good marker of inflammation, tissue injury and neutrophil infiltration in gastrointestinal tissues. It was increased with acetic acid and TNBS administration. Pretreatment with *P. granatum* fruit peels extract exhibited decrease in polymorphonuclear infiltration demonstrated by significant reduction in MPO activity. Oxidative damage may represent crucial pathogenic factor in IBD because intestinal inflammation is accompanied by increased production of reactive oxygen and nitrogen species. [24]

In the preliminary phytochemical screening, it was observed that the hydro-alcoholic extract of *P. granatum* fruit peels extract contained polyphenols like glycosides, flavonoids, tannins and steroids. Presence of these polyphenols in the extract might attribute to their antioxidant properties, in turn responsible for protective effect against colitis.

The present study concludes that hydro alcoholic extract of *P. granatum* fruit peel witnessed a significant dose dependent ulcerative colitis protective activity in both acetic acid and TNBS induced experimental models. The ulcerative colitis protective activity was found to be more significant in high dose PGPE 400 mg/kg compared to low dose PGPE 200 mg/kg. On the basis of above experimental data it can be concluded that the hydro-alcoholic extract of *P. granatum* fruit peel might be useful in treating ulcerative colitis in humans. However, the exact mechanism behind ulcerative colitis protective activity of hydro alcoholic extract of *P. granatum* fruit peel is still unclear, further study is needed to assess exact mechanism and characterize the active principles responsible for ulcerative colitis protective activity.

ACKNOWLEDGEMENT

The authors are thankful to the authorities of A. Shama Rao Foundation Mangalore, Karnataka, India for providing required facilities.

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