Prophylactic and therapeutic effect of *Punica granatum* in trinitrobenzene sulfonic acid induced inflammation in rats

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Abstract: Pomegranate (*Punica granatum L., Punicaceae)* contains varieties of antioxidants and phytochemicals; there are evidences that phytochemicals and antioxidants play a vital role in reducing inflammation. Hence this investigation was planned to assess the outcome of *Punica granatum* on trinitrobenzene sulfonic acid provoked colitis in rats at 2, 5 and 8ml/kg of the body weight. The effect of *P. granatum* was assessed in two group i.e. prophylaxis as pre-colitis and therapeutic as post-colitis. After completion of dosing in both the groups, macroscopic and histological examination of colon was carried out along with estimation of serum myeloperoxidase, glutathione, alkaline phosphate, fibrinogen and C-reactive protein. In prophylactic procedure *P. granatum* revealed significant ($P \leq 0.05$) changes in biochemical markers of inflammation at 5 and 8ml/kg doses. However in therapeutic procedure significant change was observed only at 8ml/kg. Thus results of the present study suggest that *P. granatum* have a role in prevention as well as treatment of inflammation.

Keywords: Trinitrobenzene sulfonic acid, myeloperoxidase, glutathione, alkaline phosphate, fibrinogen, C-reactive protein.

INTRODUCTION

Physiologically inflammation is a useful reaction in response to injury to tissues; delay in their perseverance may cause diseases like inflammatory bowel disease (IBD), rheumatoid arthritis and cancer. IBD is a chronic inflammatory condition, associated with rectal bleeding and diarrhea, introducing inflammatory cells in the neighboring stroma by interruption of the epithelial barrier, however exact pathogenesis of IBD remains obscure.

Despite considerable progress in the treatment of disease, drugs commonly used in the treatment such as cyclosporine and mercaptopurines are associated with various side effects. Thus drug induced toxicity and high recurrence rate of IBD is still a continuous challenge. There is increasing experimental data that support role of natural constituents, phytochemicals and antioxidants in various ailments (Grassi *et al*. 2010; Rosillo *et al* 2012; Khan and Riaz 2014; Riaz and Khan 2014; Riaz *et al*. 2014).

Interestingly plants, fruits, vegetables and their juices have been a valuable source of natural products in maintaining human health, thus can be of great importance in therapeutic management and prevention. Fruits consumption are directly related with improvements in cardiovascular function, maintaining physiological homeostasis (Gattuso *et al*. 2007; Ezeabara *et al*. 2013), inhibit prostate cancer (Hong *et al*. 2008), protect neurons against injury induced by neurotoxins, activate synaptic signaling and improve cerebrovascular blood flow (Spencer 2010; Jager and Saaby 2011; Nehlig 2012).

Pomegranate is a widely used ancient fruit rich in phytochemicals like polyphenols- punicalagin (PA), ellagic acid (EA), gallotannins, anthocyanins, apigenin, luteolin and flavonoids-quercetin, kaempferol and luteolin (Bhagwat *et al*. 2011; Heber 2011; Hollebeeck *et al*. 2012; Tehranifar *et al*. 2010). Furthermore, *Punica granatum* (PG) contains 85% water, 10% sugars and 1.5% pectin, glutamic acid, aspartic acid, Vitamin E, C, B_5, iron, potassium, calcium Ce, Cl, Co, Cr, Cs, Cu, Mg, Mn, Mo, Na, Rb, Sc, Se, Sn, Sr and Zn (Waheed *et al*. 2004; Stowe 2011). Several studies have reported PG's as hypoglycemic, hypolipidemic and antioxidant (Grassi *et al*. 2010; Stowe. 2011; Yin *et al*., 2011).

Estimation of biological markers is a non-invasive way to objectively measure inflammation which plays a primary role in the assessment of disease. Numerous studies have addressed local, injured, colonic inflammatory biomarker contents in IBD, but pro-inflammatory detection at systemic level is rare, that have prognostic value in evaluating the activity of disease (Garrido-Mesa *et al*. 2011). In view of these hypotheses that inflammation is controlled by various extra cellular mediators and regulators, including coagulation cascade (Davalos *et al*., 2012), cytokines, growth factors, eicosanoids, blood levels of C-reactive protein (CRP) and other markers of inflammation (Turner *et al*., 2014), we analyzed serum concentration of CRP, fibrinogen (Fb), as pro-

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inflammatory, glutathione (GSH), as biomarker of oxidative stress (Rose *et al*., 2012); myeloperoxidase (MPO), as pro-inflammatory as well as biomarker of oxidative stress (Haegens *et al*. 2008), CRP and alkaline phosphate (ALP), as marker of inflammation (de Medina *et al*. 2004) in TNBS induced inflammation model to observe the prophylaxis and therapeutic effect of PG in comparison with negative control rats.

MATERIAL AND METHODS

Animals

Investigations were conceded on adult male Wister rats bred in the animal house of Department of Pharmacology (180-200g) housed in precise condition of temperature 23±2°C and humidity 50-60%. Animals were preserved during the experiment on a 12/12h light and dark cycle with uninterrupted access to rat chow and water. Five rats were kept in each plastic cage measuring 81×46×41 cm. The use of animals was in agreement with the National Institute of Health (NIH) guide for the care and use of Laboratory Animals (National Research Council 1996) and approved by the Board of Advance Studies and Research University of Karachi.

Drug treatment

P. granatum was procured from local market of Gulshan-e-Iqbal Karachi and recognized by center of plant conservation, University of Karachi. The sample specimen no P.G 11-12 was placed in Department of Pharmacognosy, University of Karachi. The fruit were unpeeled and squeezed to yield fresh juice which was given after filtration in three doses i.e. 2, 5 and 8ml/kg, respectively as per body weight of rats. Prednisolone was used as standard anti-inflammatory drug in the dose of 0.7mg/kg (Ziesche *et al*. 1999).

Induction of inflammation

Colonic inflammation was induced within 24 h following administration of trinitrobenzene sulfonic acid (TNBS) as defined by Morris *et al* (1989). Inflammation was induced in all rats except animals of untreated control. Overnight fasted rats were lightly anesthetized with halothane and given rectally 10mg of TNBS dissolved in 0.25ml of 50% ethanol (v/v) 8 cm in the colon through Teflon cannula. After TNBS administration rats were detained in a head down position for 2-3 minutes in order to evenly distribute the agent in the entire colon. Two different dosing procedures were adopted to observe the prophylactic and therapeutic anti-inflammatory effects of *P. granatum*.

Dosing protocol for prophylactic procedure

Fifty rats were separated into five groups, each including ten animals. One group designated as untreated control was given phosphate buffer saline (PBS) without inducing inflammation, another group designated as negative control was given PBS before induction of inflammation and remaining three groups were given *P. granatum* before induction of inflammation. Juices and PBS were administered for 15 days before colitis induction and continued up to the day before sacrifice of the rats, that was 2 days after TNBS administration.

Dosing protocol for therapeutic procedure

Fifty rats were separated into five groups, each containing ten animals. One group designated as negative control was given PBS after induction of inflammation and three groups were given *P. granatum* after induction of inflammation, while fifth group of animals was considered as positive control, given standard drug prednisolone after induction of inflammation. Drugs and juices were administered to rats for 15 days from the day of colitis induction to the day before sacrifice of the animals. Untreated control without inducing inflammation was used for prophylactic as well as therapeutic procedure. Entire study was performed under NCCL guideline (Wayne, 1998). *P. granatum,* prednisolone and PBS administered orally in both prophylactic and therapeutic procedures.

Macroscopic examination

After completion of study, overnight fasted rats were sacrificed by decapitation. The colon was detached and opened longitudinally after placing in ice-cold physiological saline to remove fecal residues. Then colonic segments were weighed and lengths were measured under persistent load of 2gm. Macroscopic visible damage was assessed on a 0-10 scale by two observers unaware of the treatment, as per criteria formerly reported in detail (Bell *et al*., 1995). 0 score represents normal exterior, no ulcer, no inflammation; 1 shows local hyperemia, without ulcers; 2 indicate ulceration without inflammation, 3 shows ulceration, inflammation and adhesion at one side only; 4 represents two or more sites of ulceration and inflammation and a score of 5 shows ulceration extending more than 2cm.

Histological examination

After macroscopic observation, randomly selected intestinal colon samples from each test and control animals were secure in 4% para formaldehyde, then dehydrated and later embedded in paraffin and lastly segmented in 4µm thick sections. The samples were stained with hematoxylin and eosin (H&E) in agreement with the standard techniques for histological evaluation as described by Diab *et al*. (2012).

Biochemical determination

Blood samples were collected in 3.2% (9:1 v/v) sodium citrate tubes. Serum from blood samples in gel tubes was obtained through Humax 14 K centrifuge at 2000 rpm for 10 min to analyze biochemical parameters.

Myeloperoxidase assay

Myeloperoxidase (MPO) serum level was measured using the Anti-Myeloperoxidase ELISA (IgG) kit,

EUROIMMUN as per manufacturer instructions. The MPO activity was defined as the quantity of enzyme degrading 1 RU of peroxide per minute at 37°C and was expressed in unit per ml of serum.

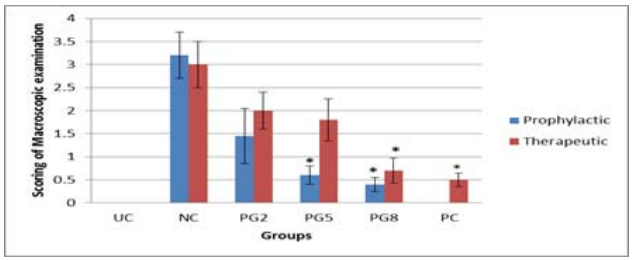

Fig. 1: Effect of *P. granatum* on macroscopic changes during prophylactic and therapeutic procedures.
n=10, Average values ± S.E.M.
Columns with (*) are significantly different, P≤0.05 as compare to negative control (U.C= untreated control; N.C= negative control; PG2=*P. granatum* 2ml/kg; PG5= *P. granatum* 5ml/kg; PG8= *P. granatum* 8ml/kg; P.C= Positive control treated with prednisolone)

Glutathione assay

Glutathione (GSH) serum level was determined using the Glutathione ELISA kit, Cusabio Biotech Co., Ltd. as per manufacturer instructions. The activity level of GSH in the samples was stated as percentage linked to the activity level of standard plasma.

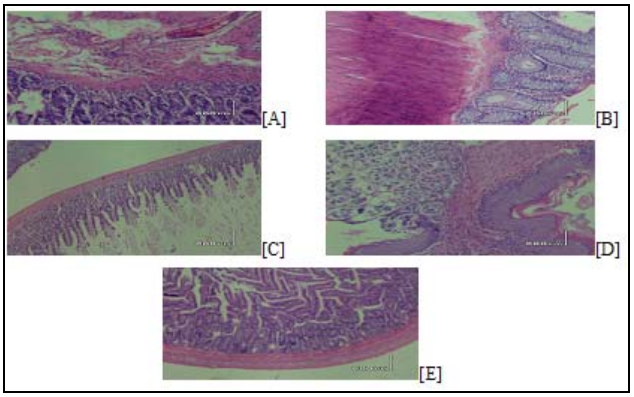

Fig. 2: Histopathological assay of intestinal tissues in TNBS induced colitis.
[A] Negative control showing inflamed colonocytes; [B] Colonic tissue showing lower degree of inflamed mucosal and submucosal tissue at 2ml/kg PG during prophylactic procedure; [C] Colonic tissue showing normal mucosa and submucosa at 8ml/kg PG during prophylactic procedure; [D] Colonic tissue showing less inflamed mucosal and submucosal tissues at 5ml/kg PG during therapeutic procedure; [E] Colonic tissue showing normal mucosa and submucosa at 8ml/kg PG during therapeutic procedure**.**

Alkaline phosphate assay

Alkaline phosphate (ALP) serum was measured by using commercial kit of ALP, Human Diagnostic, Germany, by HumaLyzer 3000, Human Germany, as per manufacturer instructions.

C - reactive protein assay

C-reactive protein (CRP) serum was measured using HumaTex CRP test kit, Human Diagnostic, using Latex agglutination slide tests, as per manufacturer instructions.

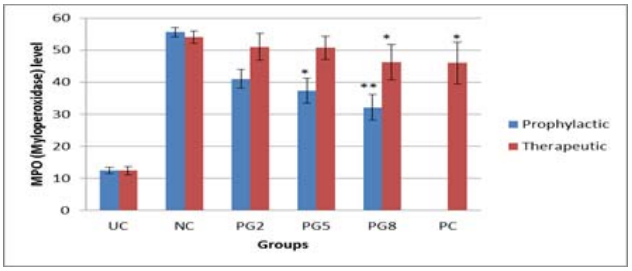

Fig. 3: Effect of *P. granatum* on MPO during prophylactic and therapeutic procedure.
n=10, Average values ± S.E.M.
Columns with (*) are significantly different, P≤0.05; as compare to negative control.
Columns with (**) are highly significant different, P≤0.005; as compare to negative control.

Fibrinogen assay

Fibrinogen (Fb) level was measured as described by McNerlan *et al*. (1997) using Clauss (1957) method. All parameters were measured by commercial kits of Human, Germany, as per manufacturer instructions.

STATISTICAL ANALYSIS

Data entry and analysis was accomplished by Statistical Package for the Social Sciences (SPSS) version 20. Data was shown as mean ± S.E.M with 95% confidence interval. ANOVA followed by post hoc was done for evaluation of values with control. Values of p≤0.05 were considered significant and p≤0.005 as highly significant.

RESULTS

Macroscopic changes

Fig. 1 reveals macroscopic changes during prophylactic and therapeutic procedure in animals treated with three doses of *P. granatum* in comparison to negative control. There was significant reduction in inflammation and necrosis in colon during prophylactic procedure at 5 and 8ml/kg of PG. However, during therapeutic procedure significant (p≤0.05) reduction in inflammation and necrosis was observed only at 8ml/kg PG, while there were no macroscopic changes in colon of untreated animals.

Histopathological changes

Histopathological assay during prophylactic and therapeutic procedures revealed significant expression of damage, shown in fig. 2 (A-E).

Biochemical changes

Changes in Myeloperoxidase Levels

Fig. 3 reveals the effect of *P. granatum* on Myeloperoxidase (MPO) levels during prophylactic and

therapeutic procedures in animals treated with three doses of PG. There was significant and highly significant decrease in MPO level at 5 and 8ml/kg respectively during prophylactic procedure as compared to negative control. However during therapeutic procedure reduction in serum MPO level was only significant at 8ml/kg PG in comparison to negative control.

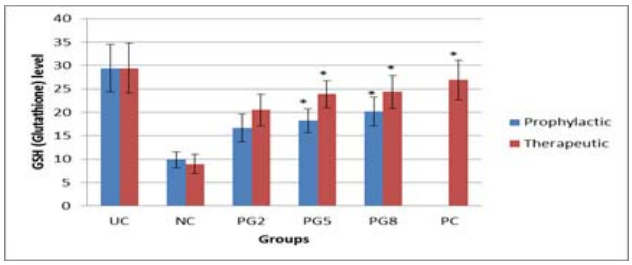

Fig. 4: Effect of *P. granatum* on GSH during prophylactic and therapeutic procedure.
n=10, Average values ± S.E.M.
Columns with (*) are significantly different, P≤0.05; as compare to negative control

Changes in glutathione levels

Fig. 4 shows the effect of *P. granatum* on glutathione (GSH) levels during prophylactic and therapeutic procedures in animals treated with three doses of PG. There was significant increase in GSH at 5 and 8ml/kg as compared to negative control in both procedures.

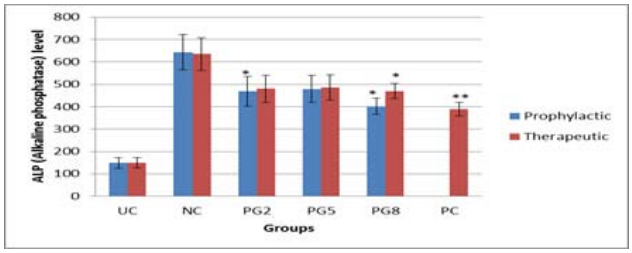

Fig. 5: Effect of *P. granatum* on ALP during prophylactic and therapeutic procedure.
n=10, Average values ± S.E.M.
Columns with (*****) are significantly different, P≤0.05; as compare to negative control

Columns with (******) are highly significant different, P≤0.005; as compare to negative control

Changes in alkaline phosphate levels

Fig. 5 shows the effect of *P. granatum* on alkaline phosphate (ALP) levels during prophylactic and therapeutic procedures in animals treated with three doses of PG. ALP was significantly reduced both at 2 and 8ml/kg in prophylactic procedure and significantly reduced at 8ml/kg in therapeutic procedure when compared to negative control.

Changes in C - reactive protein levels

Fig. 6 shows the effect of *P. granatum* on C-reactive protein (CRP) levels during prophylactic and therapeutic procedures in animals treated with three doses of PG. CRP was significantly reduced at 5 and 8ml/kg PG during prophylactic procedure. However no significant changes were observed during therapeutic procedure when compared to negative control.

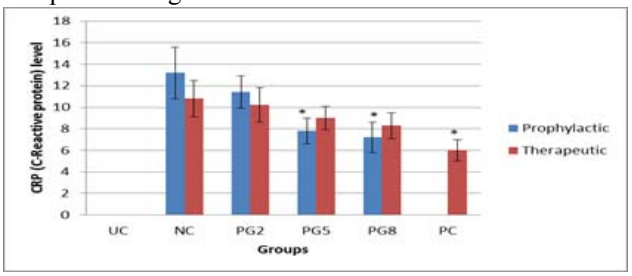

Fig. 6: Effect of *P. granatum* on CRP during prophylactic and therapeutic procedure.
n=10, Average values ± S.E.M.
Columns with (*) are significantly different, P≤0.05; as compare to negative control

Changes in fibrinogen levels

Fig. 7 shows the effect of *P. granatum* on fibrinogen (Fb) levels during prophylactic and therapeutic procedures in animals treated with three doses of PG. Fb was significantly reduced at all three doses of *P. granatum* in a dose dependent manner during prophylactic procedure. However significant change was only observed at 8ml/kg PG during therapeutic procedure when compared to negative control.

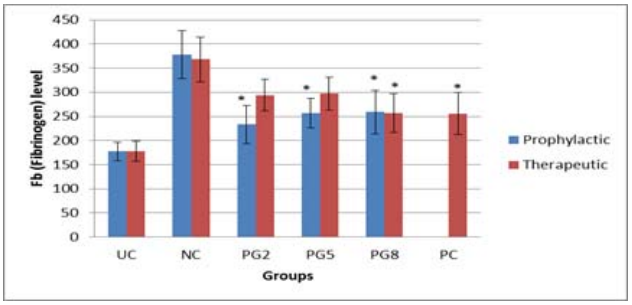

Fig. 7: Effect of *P. granatum* on Fb during prophylactic and therapeutic procedure.
n=10, Average values ± S.E.M.

Columns with (*) are significantly different, P≤0.05; as compare to negative control

DISCUSSION

The IBD model of colitis by TNBS was proposed on the principle that the ethanol vehicle would damage the colonic epithelium, thereby permitting entry of the hapten into the lamina propria where it would bind to tissue acts as an antigen inducing transmural inflammation. Since TNBS inflammation is due to the generation of oxidative stress and discharge of pro-inflammatory cytokines during tissue damage. Hence oxidative stress is a potent yet non-specific trigger for inflammation (Garrido-Mesa *et al*., 2011; Liu *et al*. 2011; Shi *et al*., 2011; Wallace *et al*., 1995).

Pomegranate (*Punica granatum* L.) has been used for medicinal purposes since ancient times, due to presence of phytochemicals full of antioxidant capacity (Grassi *et al*. 2010; Hollebeeck *et al*. 2012). Extensive research on phytochemicals has shown their potential in the prevention of common forms of cancer and other chronic diseases (Betanzos-Cabrera *et al*. 2011). Present study on *P. granatum* provides an evidence for its prophylaxis and therapeutic potential in inflammation since decreases levels of inflammatory biomarker and overcome epithelial dysfunction in pre and post TNBS induced colitis, possibly due to its high phytochemical contents.

Anti-oxidative efficacy of active compounds in PG may be impaired due to poor bioavailability of flavonoids in extract (Cerda *et al*., 2004, 2006; Lansky *et al*., 2007), hence freshly squeezed juices of PG in three doses, 2, 5 and 8ml/kg in were used in this study.

Histopathological examination of colon showed less mucosal damage in animals treated with 2ml/kg of PG, while normal cells at 5 and 8ml/kg during prophylactic procedure, suggesting protective anti-inflammatory effect of PG at both of these doses, while during therapeutic procedure normal mucosal cells in animals treated with 8ml/kg suggests PG as effective target molecule for therapy at this dose.

Histopathological changes in present study by *P. granatum* are supported by alterations of inflammatory biomarkers. There was significant and highly significant decrease in MPO level in TNBS induced inflammation in animals at 5ml/kg and 8ml/kg during pre-colitis procedure indicative of prophylactic anti-inflammatory effect of *P. granatum.*

Similarly there was a significant decrease in MPO level at 8ml/kg in post-colitis group showing therapeutic anti-inflammatory effect of *P. granatum*. Many trials have established that toxic colitis could increase MPO level; on the contrary various agents used in the treatment can decrease the contents of MPO in colonic tissues (Zamuner *et al*. 2003). Kaempferol in PG act as antioxidant and have ability to decrease MPO by decreasing expression of cytokines (Kowalski *et al*. 2005; Salawu *et al*. 2013).

In present study increase in glutathione levels in TNBS induced prophylactic and therapeutic procedure by PG was in dose-dependent manner at 5 and 8ml/kg. These results are identical with studies of Sudheesh *et al* (2005) and Rosenblat *et al* (2006). Glutathione is the chief endogenous antioxidant formed by the cells, contributing directly in the neutralization of free radicals and reactive oxygen compounds, as well as maintaining exogenous antioxidants such as vitamins C and E in their reduced (active) forms.

The reduction of MPO and increase GSH can be taken as expression of the anti-inflammatory effect of PG due to presence of procanthocyanidins and anthocyanidins as antioxidants (Bagchi *et al*., 2004). These finding suggest that PG has therapeutic and excellent prophylactic anti-inflammatory potential as reported in different studies (Gil *et al*., 2000; de Nigris *et al*., 2005).

Results of the present study have also shown significant decrease in alkaline phosphate activity at 2 and 8ml/kg in pre-colitis during prophylactic procedure, whereas there was significant decrease in ALP activity at 8ml/kg in post-colitis during therapeutic procedure. The beneficial effect of reduction in MPO activity may be associated with significant decrease in ALP activity, since it is present in many tissues including intestine (Sekiguchi *et al*. 2011) and has been established as a marker of intestinal inflammation (de Medina *et al*. 2004). The decrease levels of serum ALP by PG in pre and post colitis group is identical with several studies showing that dietary nutrients promotes protective role of ALP against toxins (Mahmood *et al*., 2003; Goldberg *et al*., 2008).

CRP is increased in blood in response to inflammation, infection, or tissue damage (Pepys *et al*., 2003), under normal situation less CRP is produced by hepatocytes, however during inflammation, hepatocytes rapidly increase production of CRP that rapidly decreases after resolution of the inflammation (Sohail *et al*., 2010; Tall 2004). Results of the prophylactic procedure showed significant decrease in serum CRP in animals treated with three doses of PG as compare to negative control, and this might be due to rich flavonoids contents of PG. Since several studies have reported that plasma CRP is related to flavonoid intake and diverse flavonoids may reduce CRP level in hepatic cells in a dose-dependent manner (Garcia-Mediavilla *et al*., 2007). Insignificant decrease in CRP level during therapeutic procedure by PG could be justified at present.

Fibrinogen the end product of the coagulation cascade, not only play an important role in hemostasis but also in reproduction, tissue repair and inflammatory responses related to infection or disease, since it has been identified as a significant risk factor and modulator of inflammatory processes in several pathologic conditions. Results of prophylactic procedure revealed decrease in Fb at all three doses of PG however results of therapeutic procedure showed decrease in Fb level at PG 8ml/kg.

Moreover novel molecular mechanisms linking coagulation and inflammation has highlighted factors of the coagulation cascade as new targets for therapeutic intervention in a wide range of inflammatory human diseases (Davalos *et al*., 2012; Riaz *et al*., 2016).

In recent years a dual role of thrombin has been revealed. It is not only involved in blood coagulation, but also associated with inflammatory response, cell-mediated immunity and cell death (Di Cera 2008; Jenkins *et al*.

2006). Hence it may be concluded that this fruit may retard the progression of inflammatory conditions and act as anti-inflammatory may be through its anti-coagulant affect. Since coagulation and inflammation, has been simultaneously reported as biological mediators of cardiovascular disease (Arid; Rallidis *et al*. 2004; Hamer *et al*. 2008). Therefore on the basis of data shown in current study it may be concluded that anti-inflammatory activity of this fruit may be supported by its potent antioxidant and anticoagulant effects (Pawlak *et al*. 2010).

Present results demonstrate that *P. granatum* have potential to produce anti-inflammatory effect prophylactically and therapeutically, it was found to be effective therapeutic agent at 8ml/kg with effects similar to prednisolone hence it may be suggested that *P. granatum* produce these effects by a mechanism similar to prednisolone (Farrell and Kelleher 2003).

P. granatum has also been effective in prophylaxis management of inflammation at 5 and 8ml/kg, the inhibitory actions of *P. granatum* against inflammatory biomarkers opens a new door for further investigations on this fruit juice.

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