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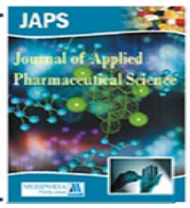

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Analgesic and Anti-inflammatory Activities of Flower Extracts of *Punica granatum* Linn. (Punicaceae)

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ABSTRACT

Punica granatum has been used for centuries to confer health benefits in a number of inflammatory diseases. Based on its usage in Ayurvedic and Unani medicine, dietary supplements containing pomegranate extract are becoming popular for the treatment and prevention of arthritis and other inflammatory diseases. Pet-ether, dichloromethane and methanol fractions of flower part were chosen for pharmacological screening and analgesic and anti-inflammatory activities in animal model. The anti-inflammatory activity was assessed using the carrageenan-induced rat paw edema model. The analgesic effect was measured in mice using the acetic acid-induced writhing test. In the acetic acid-induced writhing test in mice, pet-ether, dichloromethane and methanol fractions at 200 mg/kg doses level showed 75.77% (p<0.001), 68.56% (p<0.001), 54.64% (p<0.001) inhibition of writhing, respectively. In rat paw edema model induced by carrageenan, pet-ether, dichloromethane and methanol fractions were found to reduce significantly (p<0.001) the formation of edema at the 100 mg/kg dose level and showed 26.92% (p<0.001), 27.97%(p<0.001), 21.85%(p<0.001) inhibition respectively of edema volume at the end of 4 h. *Punica granatum* possesses evident analgesic and anti-inflammatory activities. The results signify the traditional uses of *Punica granatum* for inflammation and pain.

Keywords: *Punica granatum*, punicaceae, analgesic, anti-inflammatory and carrageenan.

INTRODUCTION

Punica granatum Linn., commonly known as pomegranate, is a small tree, belonging to the Punicaceae family. Pomegranate is grown mainly in Iran, India and the USA, but also in most Near and Far East countries. Pomegranate juice and wine have become increasingly popular because of the attribution to the plant of important biological actions (Schubert *et al.*, 2002), including antioxidant activity and cardiovascular protection (Aviram *et al.*, 2002). *Punica granatum*, popularly known as 'dadima' in Sanskrit and 'pomegranate' in English is described for its medicinal properties in Ayurveda. The dried flowers are used in hematuria, hemorrhoids, hemoptysis and dysentery. The powdered flower buds are used in bronchitis (Ross *et al.*, 2001). Flower juice is recommended as a gargle for sore throat, in leucorrhea, hemorrhages and ulcers of the uterus and rectum (Ali *et al.*, 2006).

The root barks as well as the stem bark of the plant are astringent and also used as anthelmintic specifically against tapeworms. The rind is valued as an astringent in diarrhea and dysentery. Pomegranate flowers have been prescribed in Unani and Ayurvedic medicines for the treatment of diabetes (Huang *et al.*, 2005[a]). Recently, it has been reported that the aqueous ethanolic (50% v/v) extract from pomegranate showed hypoglycemic activity in a diabetic animal model (Huang *et al.*, 2005[a]; Jafri *et al*., 2000). The seeds are considered to be stomachic and the pulp as cardiac and stomachic. The fruit rind is valued as an astringent and green leaves are made into a paste and applied in conjunctivitis (Li *et al.*, 2005). Modern uses of pomegranate derived products now include treatment of acquired immune deficiency syndrome (AIDS) (Lansky *et al.*, 2007). The biological activities, viz. antibacterial, antifungal, anthelmintic and antifertility, of the various extracts of different parts of this plant have also been reported. The extracts of flower of *Punica granatum* and rind of this plant have been reported to exert some sugar lowering action in animals (Jafri *et al*., 2000). Different parts of the plant have anti-hepatotoxicity, anti-tumour and anti-inflammatory activities (Lee *et al.*, 2010). Thus, the aim of this study was to characterize analgesic and anti-inflammatory activities of different fractions of flower part of *Punica granatum* plant.

MATERIALS AND METHODS

Plant collection

The flower part of fresh unadulterated *Punica granatum* was collected from Naogaon and taxonomically identified by the National Herbarium of Bangladesh, Mirpur, Dhaka. A voucher specimen has been deposited (accession number 35050) in the Herbarium for further reference. Fresh sample was dried at room temperature (25–30°C) for 10 days. The dried sample was then ground in coarse powder using high capacity grinding machine in the Phytochemical Research Laboratory, Faculty of Pharmacy, University of Dhaka and preserved in air tight container. The sample was weighed before extraction.

Extraction of the plant material

About 500 g of powdered flower material was taken in clean, round bottomed flask (5 liters) and mixed with 2 liters of pet-ether. The whole mixture was then filtered through cotton plug followed by Whatman No.1 filter paper and the filtrate thus obtained was concentrated at 40°C with a Heidolph rotary evaporation. The concentrated extract was then air dried to solid residue. The weight of the crude pet-ether extract of flower was obtained 70.5 g.

Solvent-solvent partitioning

Solvent–solvent partitioning was done using the protocol designed by Kupchan and modified by VanWagenen *et al*. (1993). The crude extract (35g) was dissolved in 10% aqueous methanol and partitioned between pet-ether, dichloromethane and methanol fractions. All the fractions were evaporated to dryness. These were collected for further analysis.

Drugs and Chemicals

Carrageenan and diclofenac were purchased from Sigma-Aldrich, Germany. Acetic acid was purchased from Merck, Germany.

Experimental animal

Swiss albino mice weighing 20-30 g and Long-Evans rats weighing 100-150 g were used in this study. They were obtained from the Animal Research Branch of the International Centre for Diarrheal Diseases and Research, Bangladesh (ICDDR, B). The animals were housed in polyvinyl cages with not more than six animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2^0C) and a 12/12 h dark/light cycle and received feed, formulated by ICDDR,B and water *ad libitum*. To keep the hydration rate constant, food and water were stopped 12 h before the experiments.

Analgesic activity

Acetic acid induced writhing test

The peripheral analgesic activity was measured by the acetic acid induced writhing test in mice (Koster *et al.*, 1959; Das *et al.*, 2012). The abdominal writhing was induced by intraperitoneal injection of acetic acid solution (0.7%) at a dose of 0.1 mL/10g of body weight to each mouse, a model of visceral pain. Diclofenac sodium at oral dose of 50 mg/kg was used as standard analgesic agent. Pet-ether, dichloromethane and methanol fractions of flower part were administered at 200 mg/kg body weight. The extracts, standard drug and control (normal saline solution, 10 mL/Kg) were orally administered 1 h prior to the injection of acetic acid. The number of writhing was calculated for 10 min, 10 min after the application of acetic acid.

Anti-inflammatory study

Carrageenan-induced rat hind paw edema

The anti-inflammatory potential was assessed by the carrageenan-induced right hind paw edema method (Winter *et al.*, 1962; Das *et al.*, 2012). Briefly, acute inflammation was produced by subplantar injection of 0.1 ml of 1% suspension of carrageenan in normal saline, in the right hind paw of the rats 1h after the oral administration of test materials. Paw volumes were measured up to a fox mark by mercury displacement as viewed by traveling microscope at 1, 2, 3, 4 and 5 hours after the administration of the standard drug and test extracts. Pet-ether, dichloromethane and methanol fractions of flower part were administered at 100 mg/kg body weight by gavage. Diclofenac at a dose of 50 mg/kg body weight was used as standard anti-inflammatory agent. The negative control group received 0.1% Tween-80 in saline solution. The anti-inflammatory effect of the extract was calculated by the following equation (Asif *et al.*, 2009):

$$\text{Anti-inflammatory activity (\%)} = (1-D/C)\ X100,$$

Where, C= Mean paw volume of control, D= Mean paw volume of test.

STATISTICAL ANALYSIS

The results obtained were expressed as mean ± S.E.M. The data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's *t* test to determine the level of significance. A value of $P<0.05$ was considered to be significant. The statistical analysis was carried out using the SPSS program (version 17.0).

RESULTS

Analgesic activity

Acetic acid induced writhing test

Pet-ether, dichloromethane and methanol fractions at the dose of 200 mg/kg b.w. and diclofenac sodium 50 mg/kg b.w have induced a significant ($p<0.001$) decrease in the number of writhes when compared to controlled untreated groups (Table 1). In the acetic acid induced writhing, the pet-ether, dichloromethane and methanol fractions of flower part exhibited very significant antinociceptive power (75.77%, 68.56%, 54.64% inhibition respectively) compared to diclofenac at dose of 50 mg/kg b.w. (61.86% inhibition) which was used as reference drug (Table 1).

Table. 1: Effect of different extracts of flowers of *Punica granatum* on acetic acid induced writhing response in mice.

Treatment[b]	Dose (mg/kg)	Writhing[a]	% inhibition
Control	10 mL/Kg	38.8 ± 1.71*	-
Diclofenac	50	14.8 ± 1.15*	61.86
PEF	200	9.4 ± 0.93*	75.77
DCMF	200	12.2 ± 1.46*	68.56
MeOHF	200	17.6 ± 1.43*	54.64

[a]values represent mean ± SEM (n=5).
***p*<0.001(One -way ANOVA and Dunnett's t test, significantly different from control). [b]PEF = Pet ether fraction, DCMF = Dichloromethane fraction and MeOHF = Methanol fraction of flowers of *Punica granatum.*

Anti-inflammatory activity

The anti-inflammatory activity of pet-ether, dichloromethane and methanol was measured at a dose of 100 mg/kg b.w. against acute paw edema induced by carrageenan. A strong inhibition of the paw edema was observed with the different fractions of flower part of the plant and with diclofenac. The pet-ether, dichloromethane and methanol fractions exhibited very significant anti-inflammatory activity (26.92%, 27.97% and 21.85% inhibition of paw edema, respectively at the end of 4 h) compared to diclofenac (38.11% inhibition of paw edema) which was used as reference anti-inflammatory drug (Table 2).

DISCUSSION

Pain and inflammation are associated with the pathophysiology of various clinical conditions such as arthritis, cancer and vascular diseases. Inflammatory reactions are not only the response of living tissues to injury and infection, but also are relevant to disease developments, such as asthma, multiple sclerosis, colitis, inflammatory bowel disease and atherosclerosis (Balkwill *et al.,* 2005). Physiological or acute inflammation is a beneficial host response to tissue damage, but when timely resolution is delayed, it may lead to such immune-associated diseases as rheumatoid arthritis, inflammatory bowel disease (IBD), and cancer (Balkwill *et al.,* 2005). Chronic inflammation can lead to early changes associated with the development of cancer through attraction of soluble pro-inflammatory mediators TNF-α, interleukins (e.g. IL-6 and IL-8), transcription activation factors (e.g. NF-$_k$B), and bioactive lipids such as eicosanoids (e.g. prostaglandin E2 and lipoxygenase derived products) (Lansky *et al.,* 2007). Many natural products are used in traditional medical systems to treat the relief of symptoms from pain and inflammation (Marrassini *et al*., 2010).

In the present study, the anti-inflammatory and analgesic activities of different fractions of flower part were investigated, applying experimental animal models, and phytochemical analysis. This study has shown that the different fractions possess a significant anti-edematogenic effect on paw edema induced by carrageenan. As the carrageenan-induced inflammation model is a significant predictive test for anti-inflammatory agents acting by inhibiting the mediators of acute inflammation (Mossa *et al.,* 1995), these results are an indication that pomegranate can be effective in acute inflammatory disorders.

The carrageenan-induced rat paw edema is a biphasic process. The release of histamine or serotonin occurs in the first phase and the second phase is associated with the production of bradykinin, protease, prostaglandin, and lysosome (Saha *et al.,* 2009). Therefore, the inhibition of carrageenan-induced inflammation by the extract of pomegranate could be due to the inhibition of the enzyme cyclooxygenase and subsequent inhibition of prostaglandin synthesis. In the acetic acid-induced writhing test, the constriction response of abdomen produced by acetic acid is a sensitive procedure for peripheral analgesic agents. This response is believed to be mediated by the prostaglandin pathways (Ronaldo *et al.,* 2000).

Table. 2: Effect of different extracts of flowers of *Punica granatum* on carrageenan-induced rat paw edema.

Treatment[b]	Dose (mg/kg)	Paw volume (mL)[a]				
		1 h	2 h	3 h	4 h	5 h
Control	-	77.83 ± 4.19	90.67 ± 2.29	105.17 ± 2.21	95.33 ± 2.80	90.83 ± 1.70
Diclofenac	50	53.00 ± 1.39*(31.90)	58.33 ± 1.35*(35.66)	59.66 ± 1.40*(43.26)	59.00 ± 1.34*(38.11)	58.17 ± 1.19*(35.96)
PEF	100	61.66 ± 1.85(20.77)	62.50 ± 1.91*(31.06)	65.16 ± 2.68*(38.03)	69.66 ± 2.84*(26.92)	70.17 ± 3.84*(22.75)
DCMF	100	58.66 ± 1.23*(24.63)	64.33 ± 0.88*(29.04)	67.00 ± 1.03*(36.29)	68.66 ± 0.42(27.97)	69.66 ± 1.02*(23.30)
MeOHF	100	61.83 ± 1.35*(20.55)	61.66 ± 2.24*(31.98)	81.50 ± 3.88*(22.50)	74.50 ± 2.78*(21.85)	77.00 ± 1.15(15.23)

[a]Values are mean ± SEM. (n=6).
***p*<0.001(One-way ANOVA and Dunnett's t test), significantly different from control. Figures in parentheses are the % inhibition of paw edema. [b]PEF = Pet ether fraction, DCMF = Dichloromethane fraction and MeOHF = Methanol fraction of flowers of *Punica granatum.*

The extracts produced antinociceptive activity and thus indicate the presence of analgesic components that might influence the prostaglandin pathways. Previous studies also showed that it has antipyretic analgesic activity (Lee *et al.,* 2010; Chakraborthy *et al*., 2008). The phytochemical analysis of flower extract revealed that it contains ursolic acid, oleanolic acid, maslinic acid, asiatic acid (Ahmed *et al.,* 1995; Huang *et al.,* 2005[b]). The flowers contain compounds also found in peels (e.g. gallic acid) and seed (e.g. ursolic acid), and quite possibly unique, distinctive compounds as well (Lansky *et al*., 2007). Maslinic acid inhibits NF-$_k$B, NO and peroxide formation in lipopolysaccharide induced murine macrophages (Martin *et al.,* 2006). Inhibition of inflammation by pomegranate components involves inhibition of both COX and LOX enzymes (Schubert *et al*., 1999) and a decline in prostaglandin release from cells (Polagruto *et al.,* 2003). The phytochemical analysis of flower extract also revealed that it contains flavonoids characterized as 5,6,7,8,2′,3′,5′-heptahydroxy-4′-methoxyflavanone (punicaflavanol) and 5,6,7,8,2′,5′-hexahydroxy-4′- methoxyflavanone -7-*β*-D- xylopyranoside (granatumfla vanyl xyloside) (Bagri *et al*., 2010). Flavonoids are well known for their ability to inhibit pain perception. Flavonoids also have anti-inflammatory properties due to their inhibitory effects on enzymes involved in the production of the chemical mediator of inflammation (Owoyele *et al.,* 2005). Flavone, its methoxy derivatives exhibited significant dose-dependent analgesic activity (Thirugnanasambantham *et al.,* 1993). So, different fractions of flower part of the plant have anti-inflammatory activity. In conclusion, our findings demonstrate that pomegranate has favorable analgesic and anti-inflammatory activities and thus gives scientific basis to its traditional uses.

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