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FREE RADICAL SCAVENGING AND ANTI-INFLAMMATORY ACTIVITIES OF *PUNICA GRANATUM* LINN. FRUIT RIND

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Research Article

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ABSTRACT

The methanolic extracts of *Punica granatum* fruit rind possess significance *in vitro* antioxidant and anti-inflammatory activities. The concentration needed for 50% inhibition of superoxide radical, hydroxyl radical generation, nitric oxide radical formation and lipid peroxidation were 183.3, 94.0, 53.0 and 88.5 µg ml-1 respectively. The anti-inflammatory activity of the extract was evaluated in carrageenan, BSA (Bovine Serum Albumin) and dextran induced acute and formalin induced chronic inflammatory models in balb/c mice. The extract showed significant anti-inflammatory activity in both the models comparable to control group.

INTRODUCTION

Inflammation is the immediate defensive mechanism or reaction to an injury, which may be caused by infection, chemical or physical agents [1]. It involves pain, heat redness and swelling and loss of function of affected part. In some cases the inflammation may lead to the development of chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel diseases, psoriasis etc. Reactive oxygen species (ROS) and free radicals are thought to act directly as cellular messengers and elicit an inflammatory response. ROS and free radicals also activate a series of enzyme system including protein kinases, protein phosphatases, transcription factors and heat shock proteins and increase the extend of inflammation. Supplementation of non-toxic antioxidants may have a protective role in these conditions. From ancient times medicinal plants have been proved to be powerful therapeutic agents for the treatment of cancer, ulcer, inflammation etc. Approximately 60% of the World's population almost relies entirely on plants for medication [2] and several medicinal plants have been proved to be useful as therapeutic agents, several other plants are still awaiting discovery.

Punica granatum L. (Punicaceae) commonly called 'Pomegranate' is a small tree used medicinally in Europe, Mauritania, Indo China, West Indies, Guiana, Brazil and La Reunion and South Africa [3]. This plant is used in folklore medicine for the treatment of various diseases such as hepatic damage, snake bite, ulcer, arthritis etc [4]. This plant also shows a potent *in vivo* antioxidant and gastro-protective activities against aspirin and ethanol induced gastric ulceration models [5]. The present study aims at investigating the free radical scavenging and anti-inflammatory (acute and chronic models) activities of methanolic extract of *P. granatum* fruit rind.

MATERIALS AND METHODS

Plant material and drug preparation: *P. granatum* fruits were purchased locally from Thrissur, Kerala. Fruit rinds were air dried and powdered material was extracted with 70% methanol by stirring at room temperature for 24 hours. The extract was filtered, concentrated and evaporated to dryness. The dried extract was suspended in distilled water and used for further studies.

Animals: The *in vivo* anti-inflammatory effect of fruit rind of *P. granatum* was assessed using Balb/c mice (22-26 g body weight) supplied by the Small Animal Breeding Station of Kerala Agriculture University, Mannuthy, Thrissur, Kerala. The animals were grouped in ventilated cages and maintained at 22-28^{0}C, 60-70% relative humidity and 12-hour light and dark cycle. The animals were fed with standard mouse chow (Lipton, India) and water ad libitum.

***In vitro* antioxidant activities**

Superoxide radical scavenging activity: The effect on the superoxide radical production was checked using the nitroblue tetrazolium (NBT) reduction method [6]. The reaction mixture contained: EDTA (6mM; with 3mg NaCN), riboflavin (2mM), NBT (50mM) plant extract (from 1 to 100µg ml-1) and phosphate buffer (67mM, pH=7.8) in a final volume of 3ml. The tubes were uniformly illuminated with an incandescent lamp for 15 minutes, and the optical density was measured at 530nm before and after illumination.

Hydroxyl radical scavenging activity: Hydroxyl radical scavenging was measured by studying the competition between deoxyribose and the test compounds for hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system. The reaction mixture contained: deoxyribose (2.8 mM), FeCl3 (0.1 mM), EDTA (0.1 mM), H_2O_2 (1 mM), ascorbate (0.1 mM), KH_2PO^{4-} - KOH buffer (20 mM, pH=7.4) and the extract (from 1 to 100 µg ml^{-1}) in a final volume of 1ml. After incubation for 1 hour at 37^{0}C, the deoxyribose degradation was measured by the method of Ohkawa et al. [7].

Nitric oxide radical inhibition activity: Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which were measured by Griess reaction [8,9]. The reaction mixture (3ml) contained sodium nitroprusside (10mM) in phosphate buffered saline (PBS) and the compound (from 1 to 100 µg ml^{-1}) was incubated at 25^{0}C for 150 minutes. After incubation, 0.5ml of the reaction mixture was removed and 0.5ml of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthyl ethylene diamine dichloride) was added. The absorbance of the chromatophore formed was reviewed at 546nm.

Inhibition of lipid peroxide formation (induction by Fe2+/ascorbate system): The reaction mixture containing rat liver homogenate (0.1 ml, 25% w/v) in Tris-HCl (30mM), ferrous ammonium sulphate (0.16 mM), ascorbic acid (0.06 mM) and different concentrations of the compound (from 1 to 100 µg ml-1) in a final volume of 0.5 ml was incubated for 1 hour at 37^{0}C and resulting thiobarbituric acid reacting substance (TBARS) was measured by the method of Ohkawa et al. [7]. A 0.4ml aliquot of the reaction mixture was treated with sodium dodecyl sulphate (SDS) (0.2 ml, 8.1%), thiobarbituric acid (1.5 ml, 0.8%) and acetic acid (1.5 ml, 20%; pH=3.5), made to a total volume of 4 ml by adding distilled water, and kept in a water bath at 95^{0}C for 1 hour. After cooling, distilled water (1 ml) and 5 ml of n-butanol/pyridine 15:1 (v/v) were added. After shaking and centrifugation, the organic layer was separated and the absorbance was measured at 532 nm.

***In vivo* anti-inflammatory activities**

Acute and chronic anti-inflammatory activity was evaluated. The former was done by the method of carrageenan, BSA and dextran induced paw oedema in mice and later by formalin induced oedema in mice hind paw.

Acute models: For each model, the animals were divided into three groups of five animals in each group. In all groups, acute inflammation (carrageenan, dextran and BSA) was induced by subplantar injection of 0.02ml of freshly prepared 15% suspension of carrageenan, dextran and BSA in normal saline in right hind paw of mice respectively. In each acute model, one group was kept as the control, the second group received 250 mg kg^{-1} and the third group 500 mg kg^{-1} of methanolic extract of *P. granatum* orally 1 hour prior to the sub plantar injection. The paw thickness for each model was measured using vernier calliper at the initial and three hours after sub plantar injection.

Chronic model: Animals were divided into three groups of five animals in each group. In all groups, inflammation was induced by sub plantar injection of 0.02 ml of 2% formalin in the right hind paw of mice. One group was kept as the control while the second group received 250 mg kg^{-1} and the third group 500 mg kg^{-1} of methanolic extract orally one hour prior to formalin injection and the administration of the extract was continued for six consecutive days. Degree of inflammation was measured using vernier calliper before and six days after formalin challenge.

Statistical analysis: The data were statistically analyzed using Student's t test and P values less than 0.05 were considered significant. All data were represented as mean ± SD.

RESULTS

***In vitro* antioxidant activities**

Inhibition of superoxide radical production

The methanolic extract of *P. granatum* fruit rind was found to scavenging the superoxide generated by riboflavin photo reduction method. The concentration of the extract needed for 50% inhibition (IC50) of superoxide radicals was found to be 61.1 µg ml^{-1} (Table 1). The concentration of known antioxidant such as curcumin needed for the same effect was 6.5 µg ml^{-1} which indicates the antioxidant activity of *P. granatum* fruit extract is quite potent.

Inhibition of hydroxyl radical production: Degradation of deoxyribose by hydroxyl radicals generated by Fe^{3+}/ascorbate/EDTA/H_2O_2 system was found that a concentration of 94 µg ml^{-1} of the extract is needed for 50% inhibition whereas the concentration of curcumin needed for the same effect was 2.8 µg ml^{-1} **(Table 1).**

Table 1. Value of IC_{50} (concentration required for 50% inhibition) of methanolic exract of *P. granatum* fruit rind and a standard drug curcumin.

	IC_{50} values in µg ml^{-1}			
	Superoxide	**Hydroxyl**	**Nitric oxide**	**Lipid peroxidation**
P. granatum extract	61.1 ± 2.7[a]	94.1 ± 3.2[a]	53.0 ± 5.0[a]	88.5 ± 7.5[a]
Curcumin	6.5 ± 0.8[b]	2.8 ± 0.5[b]	9.2 ± 3.2[b]	6.4 ± 1.2[b]

Inhibition of nitric oxide radical production: Nitric oxide radicals generated from sodium nitroprusside at physiological pH was found to be inhibited by the methanolic extract of *P. granatum* fruit rind. The concentration of the extract needed for 50% was found to be 53 µg ml^{-1} **(Table 1).**

Anti-inflammatory activities: The result of carrageenan, dextran, BSA and formalin induced mice oedema which indicate that the anti-inflammatory activity of alcoholic extract of *P. granatum* fruit rind are presented in the **(Table 2).** It was found that the extract significantly inhibited the oedema formation in dose related manner. At the dose of 500mg kg-1 body weight shows inhibitory effect (70.7, 39.2, 79.7 and 87.8% for carrageenan, dextran, BSA and formalin induced models respectively) on the oedema formation.

Table 2. Effect of methanolic extract of *P. granatum* fruit rind on anti-inflammatory activities (acute and chronic models).

Groups	Treatment (mg kg-1)	Initial paw thickness (cm)	Paw thickness after 3 hr (cm)	Increase in paw thickness (cm)	Inhibition (%)
	Carrageenan model				
Control	Vehicle	0.194± 0.008	0.360±0.023	0.164±0.010	-
P.granatum	250	0.196±0.011	0.278±0.013	0.082±0.008*	50.0
P.granatum	500	0.196±0.008	0.244±0.011	0.048±0.013*	70.7
	Dextran model				
Control	Vehicle	0.188±0.009	0.306±0.014	0.112±0.008	-
P.granatum	250	0.178±0.021	0.268±0.007	0.090±0.006*	19.6
P.granatum	500	0.144±0.005	0.212±0.088	0.068±0.010*	39.2
	BSA model				
Control	Vehicle	0.186±0.011	0.334±0.018	0.148±0.015	-
P.granatum	250	0.158±0.013	0.254±0.011	0.096±0.009*	35.1
P.granatum	500	0.168±0.020	0.198±0.013	0.030±0.008*	79.7
	Formalin model**				
Control	Vehicle	0.194±0.088	0.334±0.023	0.140±0.045	-
P.granatum	250	0.196±0.011	0.301±0.014	0.096±0.009*	27.7
P.granatum	500	0.196±0.008	0.213±0.006	0.017± 0.004*	87.8

*P values <0.05 compared to control group.
**Paw thickness was measured after 6 days.

DISCUSSION

The present study for the first time reports the antioxidant and anti-inflammatory activity of methanolic extract of *P. granatum* fruit rind. The study showed that the extract could inhibit carrageenan, dextran, BSA and formalin induced acute and chronic inflammation in a dose dependent manner. The administration of the extract showed a significant decrease (P<0.05) in carrageenan, BSA and formalin induced inflammation models whereas the extract showed little effect in dextran induced paw oedema in mice (P>0.05).

Free radicals have been demonstrated to be involved in the triggering of several diseases such as atherosclerosis, cancer, ulcer, inflammatory diseases [10]. Superoxide radical regulates metabolites capable of signalling and communicating important information to the cellular genetic machinery. Over production of superoxide radical takes place in various chronic inflammatory cases, induced by drug, toxin, stress, tissue injury and heavy excercise. Hydroxyl radicals are also involved in inflammatory processes. Oxygen derived free radicals may be released extracellularly from leukocytes after exposure to chemotactic agents, immune complexes or a phagocytic challenge. Extracellular release of low levels of these potent mediators can increase the expression of chemokines (e.g., IL-8), cytokines and endothelial leukocyte adhesion molecules, amplifying the cascade that elicits the inflammatory response [11].

Inflammation is mainly caused by the generation of free radicals. Hence, the administration of antioxidants may have a protective role in these conditions. Most of the anti-inflammatory drugs act as antioxidant and scavenge free radicals generated during inflammatory processes. It is also reported that the administration of superoxide dismutase or the other scavengers of free radicals has been observed to decrease inflammation in some animal models [12]. The present study revealed that methanolic

extract of *P. granatum* fruit rind shows potent activity in scavenging superoxide, hydroxyl and nitric oxide *in vitro*. We have also reported that the administration of methanolic extract *P. granatum* increases the superoxide dismutase, catalase and GSH levels in aspirin and ethanol induced ulcer [5].

P. granatum is reported to contain alkaloids such as pelletriene, pseudopelletriene and the preliminary phytochemical screening of the plant showed the presence of flavanoids and terpenes [13]. These compounds have several biological properties including protective effects through several mechanisms such as antioxidant effects [13]. Hence, the present study proved that the anti-inflammatory activity of *P. granatum* fruit rind may be due to its potent antioxidant properties. In conclusion, the findings of our study provide some scientific basis for the traditional use of *P. granatum* fruit rind for managing inflammatory pains. However, further experiments are necessary to isolate the active principle and to understand the correct mechanism of action of the compound.

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