



## Research Article

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### EVALUATION OF *IN VIVO* IMMUNOMODULATORY ACTIVITY OF *PUNICA GRANATUM* LINN.

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#### ABSTRACT

The aim of the present study was to evaluate the immunomodulatory activity of ethanolic extract of the peels, as well as the fruit juice of *P. granatum*. The *in vivo* studies were conducted using Wistar albino rats. The effect on cell-mediated immunity was determined by neutrophil adhesion test and Cyclophosphamide induced myelosuppression. HA (Hemagglutination Antibody) titer was used to determine the effect on antibody mediated immunity. Statistical analysis was done using one way ANOVA, followed by Tukey-Kramer multiple comparison test. The results were expressed as Mean  $\pm$  SEM. The peel extract and the fruit juice were found to have significant effect on the percentage neutrophil adhesion, cyclophosphamide induced myelosuppression and HA titer, when compared to the standard (Levamisole) group. It was concluded that, the peel extract and the fruit juice had significant effect on cell-mediated and humoral immunity.

**Keywords:** Neutrophil adhesion, TLC, DLC, HA titer, SRBC.

#### INTRODUCTION

The immune system is involved in the etiology as well as pathophysiologic mechanism of many diseases. Immunomodulation is the process that can alter the immune system of an organism by interfering with its function. Modulation of the immune system denotes, any change in the immune response that can involve induction, expression, amplification or inhibition of any part or phase of the immune response. There are generally two types of immunomodulators based on their effects: immunosuppressants and immunostimulators. Immunomodulatory agents may selectively activate either cell mediated or humoral immunity. Among the suppressive synthetic substances, cyclophosphamide has been extensively studied. However, the major drawback of this drug is myelosuppression, which is undesirable<sup>1</sup>. Nevertheless, there are major limitations to the general use of these agents, such as increased risk of infection and generalized effect throughout the immune system<sup>2</sup>. An increasing number of people are adopting alternative systems of medicine owing to the irreversible effects of modern drugs and therapies. Indian medicinal plants are a rich source of substances which are claimed to induce para-immunity, the non-specific immunomodulation of especially granulocytes, macrophages, natural killer cells and competent functions. The natural resistance of the body against infection can be enhanced by the use of herbal drugs<sup>3</sup>. Phytopharmacologic studies of various medicinal plants have revealed many compounds like flavonoids, alkaloids, saponins, terpenoids, monoterpenoids (linalool), glycoproteins, polysaccharides, tannins, essential fatty acids, phenolic compounds and vitamins having pronounced antioxidant, antineoplastic, antiulcer, anti-inflammatory and immunostimulating potential<sup>4</sup>. *Punica granatum* L. (family Punicaceae) is cultivated around the world in

subtropical and tropical regions such as in Iran, California, Turkey, Egypt, Italy, India, Chile and Spain. The world *P. granatum* production amounts to approximately 1,500,000 tons, where the peels (pericarp, rind or hull) amount to approximately 60 % of the fruit weight. *P. granatum* fruit extracts, rich in ellagitannins (ETs) proved their efficacy as antioxidant and anticancer agents, especially against breast and colon cancer<sup>5</sup>. But the immunomodulatory potential of the different parts of the fruit has not been scientifically proven. The immunomodulatory activities of ethanolic extract of *P. granatum* peels, and the fruit juice, rich in polysaccharides, flavonoids, triterpenoids and steroids have not yet been explored<sup>6</sup>. The present study is mainly focusing on the edible portion of *P. granatum* and the extract of its peels; to prove its efficacy in immunodeficiency cases, such as cancer, AIDS etc.

#### MATERIALS AND METHODS

##### Collection of *P. granatum* plant and fruit

*P. granatum* fruits were purchased from an open market of Ettumanoor, Kottayam, Kerala, India in the month of May 2012 and authenticated.

##### Experimental animals

The animals used in this study consisted of 24 Wistar albino rats, weighing 150-200 g, obtained from animal house, University College of Pharmacy, Cheruvandoor campus, Ettumanoor P.O, Kottayam, Kerala, India. The study was conducted after obtaining the approval from Institutional Animal Ethics Committee (IAEC No:003/MPH/UCP/CVR/12), and the experimental procedure was in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)<sup>7</sup>, India.

### Preparation of alcoholic extract of the peels of *P. granatum* fruit

The fruits were peeled, and the peels were separated from the fruits. The peels were air-dried, pulverized into coarse powder, using a mixer grinder and subjected to extraction. The air dried powdered peels were subjected to extraction in soxhlet assembly. 70 g of the powdered material was packed in the column of soxhlet assembly and about 400 ml of ethanol was taken in a round bottom flask. The apparatus was set up in the mantle and the extraction was carried out for 8 h at 46-70°C<sup>6</sup>.

### Preparation of *P. granatum* fruit juice

#### Procedure

The fresh *P. granatum* fruits, free of blemishes or obvious defects were purchased in May 2012. The fruits were washed and manually peeled, without separating the seeds. Juice was obtained using a mixer grinder, and immediately diluted with distilled water to a volume of 1:3 and stored at 20°C for no longer than 2 months<sup>8</sup>.

### Drugs and Doses

Drugs used in the study were Cyclophosphamide injection-200 mg/10 ml (Cipla Ltd.), Levamisole-150 mg tablets (Cipla Ltd.). Doses of drugs have been adopted from previous studies<sup>4,6</sup>. Cyclophosphamide injection was reconstituted with sterile water for injection (10 ml) and administered intraperitoneally (30 mg/kg i.p) for 3 consecutive days for inducing myelosuppression. Levamisole tablet was suspended in Carboxy methyl cellulose (CMC) (0.5 g/100 ml) and administered at the dose of (50 mg/kg/p.o.).

### Grouping of animals and treatment schedule

Healthy Wistar albino rats, of either sex were used for the study. 4 groups of 6 rats each (n = 24) were approved for the study. The treatment schedule was as follows:

Group I: Control

Group II: *P. granatum* peel extract treated (200 mg/kg p.o)<sup>9</sup>

Group III: *P. granatum* fruit juice treated (3 ml/kg p.o)<sup>10</sup>

Group IV: Standard (Levamisole) treated (50 mg/kg/p.o.)

According to the study, the treatment period varied.

### Experimental Design

#### Neutrophil Adhesion Test

The group I received vehicle, while that of the other groups received treatment, as per the schedule for 14 days respectively. On the 14<sup>th</sup> day of the treatment, blood samples from all the groups were collected by puncturing retro-orbital plexus. Blood was collected in vials, pre-treated by disodium EDTA and analyzed for total leukocyte count (TLC) and differential leukocyte count (DLC) by fixing blood smears and staining with Leishman's stain (Nice Chemicals Pvt Ltd.). After initial counts, blood samples were incubated with nylon fiber (80 mg/ml of blood sample.) for 15 minutes at 37°C. The incubated blood samples were again analyzed for TLC and DLC under a microscope (Labomed, CXL, Mono). The product of TLC and percentage neutrophil gives neutrophil index (NI) of the blood sample. Percentage neutrophil adhesion was calculated as follows,

$$\text{Neutrophil adhesion (\%)} = \frac{\text{NIu} - \text{Nit}}{\text{NIu}} \times 100$$

Where, NIu: Neutrophil Index before incubation with nylon fiber,  
Nit: Neutrophil Index after incubation with nylon fiber.

### Cyclophosphamide-induced myelosuppression

Animals were divided into 5 groups containing 6 animals each. The negative control group I and the positive control group V received vehicle, while that of the treated groups were given the test samples for 13 days. Positive and negative control received 1.0 % Sodium carboxy methyl cellulose in water. On days 11, 12 and 13, all animals except in the negative control group were given cyclophosphamide injection (30 mg/kg i.p), 1 h after administration of extract. Blood samples were collected on day 14 and the total white blood cell (WBC) count was determined<sup>11</sup>.

### Haemagglutination Antibody (HA) Titer

#### Preparation of sheep RBCs (SRBCs)

Sheep blood was collected in sterile Alsevere's solution in 1:2 proportion of Alsevere's solution (freshly prepared). Blood was kept in the refrigerator and processed, for the preparation of SRBC batch, by centrifuging at 2000 rpm for 10 minutes and washing with physiological saline 4-5 times and then suspending into buffered saline for further use.

#### Procedure for HA titer

The animals were given the vehicle, extract, juice or the standard accordingly, for 21 days. On the 7<sup>th</sup> and 14<sup>th</sup> day of the study, rats from all the groups were immunized and challenged respectively, with SRBCs in normal saline (0.1 ml of 20 % SRBCs) intraperitoneally. Blood was withdrawn on 14<sup>th</sup> and 21<sup>st</sup> day from retro-orbital plexus from all antigenically sensitized and challenged rats respectively. Blood was centrifuged to obtain serum, normal saline was used as a diluent and SRBCs count was adjusted to (0.1 % of SRBCs). Each well of a micro titre plate was filled initially with 20 µl of saline and 20 µl of serum was mixed in the first well of micro titer plate. Subsequently the 20 µl diluted serum was removed from first well and added to the next well to get two fold dilutions of the antibodies present in the serum. Further two fold dilutions of this diluted serum were similarly carried out till the last well of the second row (24<sup>th</sup> well), so that the antibody concentration of any of the dilutions is half of the previous dilution. 20 µl SRBCs (0.1 % of SRBCs) were added to each of these dilutions and the plates were incubated at 37°C for one hour and then observed for haemagglutination. The highest dilution giving haemagglutination was taken as the antibody titer. The antibody titers were expressed in the graded manner, the minimum dilution (1/2) being ranked as 1, and mean ranks of different groups were compared for statistical significance. Antibody titer obtained on 14<sup>th</sup> day after immunization (on 7<sup>th</sup> day) and on 21<sup>st</sup> day after challenge (on 14<sup>th</sup> day) with SRBCs was considered as primary and secondary humoral immune response respectively.

### Statistical Analysis

Statistical analysis was performed using Graph pad Prism software version 6.00. All the results were expressed as mean  $\pm$  Standard error mean (SEM). Data were analyzed using one-way Analysis of Variance (ANOVA) followed by Tukey-Kramer multiple comparison test. P-values < 0.05 were considered as statistically significant.

## RESULTS AND DISCUSSION

### Extraction

Percentage yield of ethanolic extract of *P. granatum* peel was found to be 7.4 % w/w.

Results of neutrophil adhesion, Cyclophosphamide induced myelosuppression and HA titer expressed as mean  $\pm$  SEM and shown in Table 1, 2 and 3. The results of one way- ANOVA are shown in Table 4.

Immunopharmacology is a comparatively new and developing branch of pharmacology, aiming at the search for immunomodulators. The relevance of neutrophil function is usually in host defense, in bacterial and fungal infections. Intravascular neutrophils sense the focus of infection, slow down and adhere to the endothelium of capillaries and venules adjacent to the inflammatory locus, migrate through the vessel wall and the interstitial tissues to the infectious site, engulf, kill and digest the invading microorganisms<sup>12</sup>. In neutrophil adhesion test, adhesion of neutrophils to the nylon fiber, describes the migration of polymorphonuclear lymphocyte through the blood vessel walls and the number of macrophages reaching the site of inflammation, under the influence of neutrophils<sup>12</sup>. This showed the effect on the stimulation of neutrophils towards the site of inflammation. Both the *P. granatum* peel extract ( $44.54 \pm 0.33$ ) and the fruit juice ( $37.61 \pm 0.54$ ) showed a significant ( $P < 0.001$ ) increase in the neutrophil adhesion, when compared to the standard group ( $70.57 \pm 1.05$ ). According to Srikumar *et al.*, 2005, the adhesion of neutrophils to nylon fiber might be associated with the up-regulation of the  $\beta_2$  integrins<sup>13,14</sup> which helps in adhesion of neutrophils to the blood vessel wall. Further work is needed in this direction, to prove its role in the present study. Cyclophosphamide induces myelosuppression in the experimental animals<sup>15</sup>. It belongs to nitrogen mustard subclass of alkylating agents, and acts as an immunosuppressive agent by causing alkylation of DNA, in turn by interfering in DNA synthesis and function. Cyclophosphamide at the dose of 30 mg/kg, i.p. was experimentally proved to cause a significant reduction in the synthesis of WBCs in the bone marrow. The immunostimulatory role of peel extract and the fruit juice, resulting in a restoration of bone marrow activity, was determined by this experiment. There was no complete restoration of the Cyclophosphamide induced myelosuppression, but a reduction in the myelosuppression produced by the drug, was seen in the peel extract ( $2121 \pm 8.32$ ) and fruit juice ( $2261 \pm 5.26$ ) treated groups, which was found to be significant ( $P < 0.001$ ) when compared to the standard group (2628

$\pm 11.38$ ). According to Diwanay *et al.*, 2004, this shows the effect of *P. granatum* on the haemopoietic system<sup>16</sup>. The effect of *P. granatum* peel and fruit juice on stimulation of the cell-mediated immunity could be understood from neutrophil adhesion test and Cyclophosphamide induced myelosuppression. Cell-mediated immunity (CMI) involves effectors mechanisms carried out by T- lymphocytes and their products (lymphokines)<sup>17</sup>. The humoral immunity involves interaction of B-cells with the antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cells. Antibody functions as the effectors of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells. To evaluate the effect of *P. granatum* on humoral response, its influence was tested on sheep erythrocyte (SRBC) specific HA titer in rats. If the serum contains antibodies to the SRBC, there will be agglutination because of the formation of antibody bridges with the neighboring erythrocytes and these settle at the bottom as latex. Unagglutinated red blood cells appear in the well bottom as a button<sup>18</sup>. The humoral immunity involves interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cells. The increased antibody titre for both primary and secondary immune responses, by both the peel extract and juice treated group is indicative of the enhanced responsiveness of macrophages, T and B lymphocyte subsets involved in antibody synthesis. When the *P. granatum* peel extract and the fruit juice were subjected to haemagglutination antibody titer, the titer value of circulating antibodies were increased significantly ( $P < 0.001$ ) when compared to the standard. The HA titer studies revealed that, the circulating levels of antibodies were increased by both the peel extract and the fruit juice of *P. granatum*<sup>19</sup>. Hence it is concluded that both *P. granatum* peel extract and its fruit juice possess immunomodulatory activity, i.e., it stimulates both cell-mediated and antibody mediated immune response. Haemagglutination antibody titer method has proved the significant effect of the extract and the juice on antibody production. Neutrophil adhesion test and Cyclophosphamide induced myelosuppression proves the effect on cell-mediated immunity, whereas HA titer shows the effect on antibody mediated immune response.

### CONCLUSION

The peel extract and the fruit juice of *P. granatum* have significant effect on cell-mediated and humoral immunity. Hence it can be used as an immunostimulant drug. The mechanism of action and the cell mediators responsible for this action, role of  $\beta_2$  integrins in adhesion of neutrophils to the blood vessels, its regulation and formation, provides space for wider areas of research in immunology. This emphasizes the importance, and future scope of this study.

**Table 1: Determination of percentage Neutrophil adhesion of various treatment groups**

Sl.No.	Treatment group	Percentage Neutrophil Adhesion
1.	Control	29.4 ± 0.67
2.	Peel extract of <i>P.granatium</i>	44.54 ± 0.33***,###
3.	<i>P.granatium</i> fruit juice	37.61 ± 0.54***,###
4.	Levamisole	70.57±1.05###

(n=6, Values expressed as mean±SEM \* denotes p<0.05, \*\* denotes p<0.01,\*\*\*denotes P<0.001, when compared to the Standard group and ###P<0.001, when compared to the control group)

**Table 2: Effect of Cyclophosphamide (30mg/kg i.p) treatment on the TLC of Wistar albino rats in various treatment groups**

Sl.No.	Treatment groups	TLC count(cells/mm <sup>3</sup> )
1.	Negative control	7148 ± 51.41
2.	Peel extract of <i>P.granatium</i>	2121 ± 8.32 ***,###
3.	<i>P.granatium</i> fruit juice	2261 ± 5.26***,###
4.	Levamisole	2628 ± 11.38 ###
5.	Positive control	1926 ± 35.82

(n=6, values are expressed as mean±SEM,\*\*\*P<0.001, when compared to the Standard group and ###P<0.001, when compared to the positive control group)

**Table 3: Determination of primary and secondary antibody titer of various treatment groups of Wistar albino rats by HA titer method**

Sl.No.	Treatment group	HA titer (Primary)	HA titer (Secondary)
1.	Control	6.91 ± 0.15	6.91 ± 0.15
2.	PJ	8.01 ± 0.12***,###	8.42 ± 0.08***,###
3.	PPEE	7.83 ± 0.11 ***,###	8.25 ± 0.11***,###
4.	Levamisole	9.75 ± 0.11	12.75 ± 0.13

(n=6, values are expressed as mean±SEM, \*\*\*P<0.001 when compared to the Standard group and ###P<0.001, when compared to the positive control group)

**Table 4: ANOVA summary for primary and secondary antibody titer determination in HA titer method**

ANOVA Summary	Neutrophil adhesion test	Cyclophosphamide induced myelosuppression	Primary HA Titer	Secondary HA titer
F	647.8	5895	118.6	462.7
P value	< 0.001	< 0.001	< 0.0001	< 0.0001
DF	3, 8	4, 8	3, 8	3, 8

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