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Research Article

# EVALUATION OF ANTINOCICEPTIVE AND ANTI-INFLAMMATORY EFFECT OF THE HYDROALCOHOLIC EXTRACTS OF LEAVES AND FRUIT PEEL OF *P. GRANATUM* IN EXPERIMENTAL ANIMALS.

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**ABSTRACT**

**Objective:** We investigated antinociceptive, acute and chronic anti-inflammatory activity of hydroalcoholic extracts of leaves and fruit peel of *P. granatum* and compared it with standard drug Ibuprofen.

**Materials and Methods:** Hydroalcoholic extracts of leaves and fruit peel of *P. granatum* was prepared using Soxhlet apparatus. Swiss albino mice weighing 25 ± 5 g and Wistar albino rats weighing 120 ± 10 g of either sex were used in this study. The antinociceptive property of both the extracts was screened by acetic acid-induced writhing in mice while acute and chronic anti-inflammatory property was studied using the carrageenan-induced paw edema and cotton pellet induced granuloma model respectively in rats. The percent inhibition of the writhing response, paw edema and dry weight of cotton pellet induced granuloma was noted.

**Results:** Leaves and fruit peel extracts of *P. granatum* exhibited a significant reduction in writhing, carrageenan induced paw edema and weight of cotton pellet induced granuloma when compared to control. Fruit Peel extract of *P. granatum* at higher dose was found better than leaves extract of *P. granatum* at higher dose. Antinociceptive and acute and chronic anti-inflammatory activity of the standard drug Ibuprofen treatment was better than all the extracts, but there was no significant difference between the activities at higher doses of both plant extracts and standard drug. No toxicity was observed even when both extracts were administered at 10 times of highest dose used in this study.

**Conclusion:** The results obtained indicate that fruit peel and leaves of *P. granatum* has favorable analgesic and anti-inflammatory activities and thus support the traditional use of fruit peel and leaves of *P. granatum* as analgesic and anti-inflammatory agent.

**Keywords:** Antinociceptive, Anti-inflammatory, Acetic acid, Carrageenan, Cotton pellet, *Punica granatum*.

## INTRODUCTION

Inflammation is the first response of the immune system to infection or irritation that helps the body to protect itself against an injurious stimulus. It can be evoked by a variety of noxious agents, e.g., infections, antibodies or physical injuries.

Inflammation may be acute or chronic, depending on the nature of the stimulus and the effectiveness of the initial reaction in eliminating the stimulus or the damaged tissues. Acute inflammation is rapid in onset (typically minutes) and is of short duration, Chronic inflammation may follow acute inflammation or be insidious in onset. It is of longer duration and is associated with the presence of lymphocytes, macrophages, proliferation of blood vessels, fibrosis, and tissue destruction [1, 2].

Non steroidal anti inflammatory drugs (NSAIDs), Opioids and corticosteroids are the group of drugs which are used presently for management of pain and inflammatory conditions [3]. Out of these, NSAIDs are the most commonly used drugs as they do not depress CNS or produce any physical dependence like Opioids [4]. The adverse drug reactions associated with NSAIDs are the gastrointestinal problems due to direct and indirect irritation of the gastrointestinal (GI) tract, the most common being gastrointestinal bleeding and peptic ulcers. Therefore, despite of the currently available anti-inflammatory drugs, there is need for safer alternatives [5].

The use of natural remedies for the treatment of inflammatory and painful conditions has a long history in Ayurveda and other systems of traditional medicine [6]. *Punica granatum,* commonly known as pomegranate, is one of the plants that have long been used in traditional herbal medicine. It is widely cultivated in Indian subcontinent. It is one of the oldest known fruits, found in writings and artefacts of many cultures and religions. In the past decade, numerous studies on the antioxidant, anticarcinogenic, and anti-inflammatory properties of pomegranate constituents have been published [7].

Many scientific studies have reported anti-inflammatory activity of seeds and flower of the *P. granatum* [8, 9, 10]. However, only few studies have evaluated the anti-inflammatory property of fruit peel and leaves of *P. granatum*.

Hence, the present study was designed to scientifically validate anti-inflammatory activity of fruit peel and leaves of *P. granatum* and to compare its anti-inflammatory activity with Ibuprofen.

## MATERIALS AND METHODS

### Chemicals

Acetic acid was obtained from Merck Pvt Ltd (Germany). Carrageenan was obtained from Sigma-Aldrich Chemicals (Dorset, UK). Ibuprofen was obtained from Cipla Ltd (India). The solvent and other chemicals used were of analytical grade.

### Plant material and extraction.

### Collection of the plant materials

The leaves of *P. granatum* were collected in the month of October from Nagpur, Maharashtra, India and were then authenticated by botanist of Science College. The fruits of *P. granatum* were purchased from local fruit shop and peel was removed from it after authentication by the Botanist.

### Preparation of extract of leaves of *P. granatum*

Fresh leaves of *P. granatum* were carefully cleaned, shade dried, powdered and stored in airtight containers. Hydroalcoholic extract was prepared according to the procedure reported by Mahanta and Mukharjee [11]. A total of 40 g of dried powder was packed in the thimble of Soxhlet apparatus and was extracted using 95% ethanol refluxing at 50-70°C which yielded a dark brown extract. The stock extract was preserved in airtight glass container and stored at 4°C.

**Preparation of extract of fruit peel of *P. granatum***

Fruit peel of *P. granatum* was carefully removed, cleaned, shade dried, powdered and stored in airtight containers. Hydroalcoholic extract of fruit peel of *P. granatum* was prepared by the same procedure used for leaves and was preserved in airtight glass container and stored at 4°C.

**Animals**

Adult Swiss albino mice weighing 20 ± 5 g and Wistar albino rats weighing 120 ± 10 g of either sex were used for the study. The animals were maintained under standard laboratory conditions (12 h dark-light cycle 8:00 am–8:00 pm and temperature 27 ± 2°C) with free access to water *ad libitum*. The animals were divided into groups of 6 for all experiments.

**Ethical clearance**

Ethical clearance was taken from the institutional animal ethics committee (IAEC) and all the experiments were done under its strict guidance.

**Acute toxicity study and dose selection**

Healthy adult male albino Wistar rats were used for this study. Pilot study was performed using three doses 500 mg/kg body weight, 1000 mg/kg body weight and 2000 mg/kg body weight of the leaves (LEPG) and fruit peel extract *of P. granatum* (PEPG). Acute toxicity studies showed the non-toxic nature of the leaves as well as fruit peel extract up to dose of 2000 mg/kg body weight. So doses 100 and 200 mg/kg, p.o., which were $1/20^{th}$ and $1/10^{th}$ than 2000 mg/kg were chosen for further studies

**Antinociceptive Activity**

**Acetic acid-induced writhing**

The antinociceptive activity of leaves and fruit peel of *P. granatum* was assessed using the acetic acid-induced writhing test in mice [12]. Control, Test and standard drug were given orally. After 60 minutes writhing was induced by intraperitoneal injection of 1% acetic acid in volume of 0.1 ml/10 g body weight. Group 1 received 1% Carboxyl methyl cellulose (CMC) as vehicle orally and was considered as control. Groups 2 and 3 received LEPG orally at a dose of 100 and 200 mg/kg respectively. Groups 4 and 5 received PEPG orally at a dose of 100 and 200 mg/kg respectively; Group 6 received Ibuprofen 100 mg/kg and served as the standard control. In all the groups, writhing episodes i.e. stretching movements consisting of arching of the back, elongation of body and extension of hind limbs were counted for 30 minutes. All the groups received the same volume of preparations. Antinociceptive activity was expressed as the percentage inhibition of abdominal constrictions between control animals and mice pre-treated (n=6) with the extract or standard drug using the ratio: (Control mean – Treated mean) /Control mean x 100

**Acute anti-inflammatory activity**

**Carrageenan-induced paw edema**

The study of the acute anti-inflammatory activity of leaves and fruit peel *of P. granatum* was assessed using the carrageenan-induced paw edema in rats [13]. Animals were divided into four groups of six animals each. Control, Test and standard drug were given orally. After 30 minutes of oral administration of the drugs, animals of all the groups were injected with 0.1 ml of 1% suspension of carrageenan in 0.9% normal saline, under the plantar aponeurosis of the right hind paw. Group 1 received 1% CMC as vehicle orally and was considered as control. Groups 2 and 3 received LEPG orally at a dose of 100 and 200 mg/kg respectively. Groups 4 and 5 received PEPG orally at a dose of 100 and 200 mg/kg respectively. Group 6 received Ibuprofen 100 mg/kg and served as the standard control. All the groups received the same volume of preparations. The paw volume from each rat from all groups was measured at 1 h, 2 h, 3 h, 4 h and 5 h after carrageenan injection using Plethysmograph. Acute anti-inflammatory activity was expressed as the percentage inhibition of paw volume between control animals and mice pre-treated (n=6) with the extract or standard drug using the ratio: (Control mean – Treated mean) /Control mean x 100

**Chronic anti-inflammatory activity**

**Cotton pellet induced granuloma**

The effect of ethanolic extract of leaves and fruit peel of *P. granatum* on chronic inflammation was assessed using cotton pellet induced granuloma model. Method adopted by Hicks R. (1969) was carried out by using sterilized cotton pellet implantation in rats [14]. Under light ether anesthesia subcutaneous tunnel was made using blunted forceps and sterilized cotton pellets (10 ± 1 mg) were implanted in the pits and groin region of the rat. After recovering from anesthesia, animals were treated orally with vehicle control, Test and standard drug consecutively for 7 days, once per day. Group 1 received 1% CMC as vehicle orally and was considered as control. Groups 2 and 3 received LEPG orally at a dose of 100 and 200 mg/kg respectively. Groups 4 and 5 received PEPG orally at a dose of 100 and 200 mg/kg respectively. Group 6 received Ibuprofen 100 mg/kg and served as the standard control. All the groups received the same volume of preparations. They were sacrificed on $8^{th}$ day by cervical dislocation and the cotton pellets were removed, freed from extraneous tissue and dried at 50 $^0$C for 24 hrs. The dry weight of the granuloma (i.e. the amount of actual granulation tissue formed) was calculated by noting the difference in the dry weight of the cotton pellets recorded before and after implantation. The percentage inhibition of the dry weight of the granuloma were calculated and compared. Chronic anti-inflammatory activity was expressed as the percentage inhibition of dry weight of the granuloma between control animals and mice pre-treated (n=6) with the extracts or standard drug using the ratio: (Control mean – Treated mean) /Control mean x 100

**Statistical analysis**

Data was analyzed using SPSS statistical software version 17.0 produced by SPSS Inc. Results are expressed as Mean ± Standard Deviation. Statistical analysis was performed using one way analysis of variance followed by post-hoc test *Bonferroni*. *P value* < 0.05 was considered as statistically significant.

**RESULTS**

**Effect of leaves and fruit peel of *P. granatum* on acetic acid-induced writhing**

The hydroalcoholic extract of leaves and fruit peel of *P. granatum* in both doses showed a significant dose-dependent reduction in the number of writhing when compared with control.

LEPG 100, LEPG 200, PEPG 100 and PEPG 200 exhibited a writhing inhibition percentage of 27.80, 43.25, 30.11 and 49.80 respectively as compared to control group. Maximum inhibition was observed at a dose of PEPG 200 among all plant extracts. Standard drug Ibuprofen was found better than all plant extracts in inhibition of acetic acid-induced writhing, but there was no significant difference between the higher doses of both plant extracts and standard. [Table 1]

**Effect of leaves and fruit peel of *P. granatum* on carrageenan-induced paw edema**

The hydroalcoholic extract of leaves and fruit peel of *P. granatum* in higher doses showed a significant dose-dependent reduction in the volume of paw edema when compared with control at the end of 2 h, 3 h, 4 h and 5 h while in lower doses at the end of 4 h and 5 h.

LEPG 100, LEPG 200, PEPG 100 and PEPG 200 showed significant reduction (31.01%, 44.96%, 31.78% and 50.38% respectively) in paw edema of respectively at the end of 5 h as compared to control group. The maximum reduction was observed at a dose of 200

mg/kg of fruit peel extract of *P. granatum* among all extracts. Standard drug Ibuprofen showed 59.69% reduction in paw edema which was greater than that showed by all extracts. There was no significant difference between the higher dose of both plant extracts and standard drug at the end of 5 h. [Table 2 and 3]

**Effect of leaves and fruit peel of *P. granatum* on the Cotton pellet induced granuloma**

The mean weight of the cotton pellet induced granuloma in vehicle treated group was 66 ± 12.8 mg. The hydroalcoholic extract of leaves and fruit peel of *P. granatum* at both doses showed a significant dose-dependent reduction in the weight of granuloma induced by cotton pellet when compared with control.

**Table 1: Effect of oral administration of leaves and fruit peel extract of *P. granatum* and Ibuprofen on acetic acid-induced writhing in mice. (n=6)**

| Groups | Treatment (mg/kg) | No. of writhes in 30 min | Percent inhibition |
|---|---|---|---|
| Group 1 | Vehicle | 43.17 ± 8.57 | - |
| Group 2 | LEPG 100 | 31.17 ± 3.19** | 27.80 |
| Group 3 | LEPG 200 | 24.50 ± 6.6** | 43.25 |
| Group 4 | PEPG 100 | 30.17 ± 5.08** | 30.11 |
| Group 5 | PEPG 200 | 21.67 ± 4.03** | 49.80 |
| Group 6 | Ibuprofen 100 | 18.50 ± 1.88** | 57.15 |

*Results were expressed in Mean ± SD. * significant* p-*value (<0.05) with one-way ANOVA followed by* post-hoc *test Bonferroni when compared with control. ** Significant* p-*value (<0.001) with one-way ANOVA followed by* post-hoc *test Bonferroni when compared with control. LEPG: Leaf extract of* P. granatum, *PEPG: Fruit peel extract of* P. granatum, *100: 100 mg/kg, 200: 200 mg/kg*

**Table 2: *Effect of* oral administration of *leaves and fruit peel extract of* p. granatum *and ibuprofen* on carrageenan-induced paw edema in rats. (n=6)**

| Groups | Treatment (mg/kg) | Paw Edema (ml) | | | | |
|---|---|---|---|---|---|---|
| | | 1 hr | 2 hr | 3 hr | 4 hr | 5 hr |
| Group 1 | Vehicle | 0.77 ± 1.07 | 0.84 ± 1.63 | 0.88 ± 0.79 | 1.10 ± 0.15 | 1.29 ± 0.81 |
| Group 2 | LEPG 100 | 0.75 ± 0.76 | 0.81 ± 0.62 | 0.83 ± 0.67 | 0.95 ± 1.26* | 0.89 ± 0.80** |
| Group 3 | LEPG 200 | 0.72 ± 0.59 | 0.78 ± 0.50* | 0.77 ± 0.48* | 0.74 ± 0.41** | 0.71 ± 1.05** |
| Group 4 | PEPG 100 | 0.74 ± 1.04 | 0.80 ± 0.64 | 0.82 ± 0.67 | 0.92 ± 0.15* | 0.88 ± 1.3** |
| Group 5 | PEPG 200 | 0.71 ± 0.82 | 0.75 ± 0.50* | 0.74 ± 0.54* | 0.72 ± 0.50** | 0.64 ± 0.67** |
| Group 6 | Ibuprofen | 0.68 ± 0.48* | 0.63 ± 0.49** | 0.60 ± 0.28** | 0.53 ± 0.66** | 0.52 ± 0.61** |

*Results were expressed in Mean ± SD. * significant* p-*value (<0.05) with one-way ANOVA followed by* post-hoc *test* Bonferroni *when compared with control. ** Significant* p-*value (<0.001) with one-way ANOVA followed by* post-hoc *test* Bonferroni *when compared with control. LEPG: Leaf extract of* P. granatum, *PEPG: Fruit peel extract of* P. granatum, *100: 100 mg/kg, 200: 200 mg/kg*

**Table 3: Effect of oral administration of leaves and fruit peel extract of *p. granatum* and ibuprofen on percentage reduction of carrageenan-induced paw edema in rats. (n=6)**

| Groups | Treatment (mg/kg) | Paw Edema (Percent inhibition) | | | | |
|---|---|---|---|---|---|---|
| | | 1 h | 2 h | 3 h | 4 h | 5 h |
| Group 1 | Vehicle | - | - | - | - | - |
| Group 2 | LEPG 100 | 2.59 | 3.57 | 5.68 | 13.63* | 31.01** |
| Group 3 | LEPG 200 | 6.5 | 7.14* | 12.5* | 32.72** | 44.96** |
| Group 4 | PEPG 100 | 3.9 | 4.76 | 6.81 | 16.36* | 31.78** |
| Group 5 | PEPG 200 | 7.79 | 10.71* | 15.91* | 34.54** | 50.38** |
| Group 6 | Ibuprofen | 12.99* | 25** | 31.81** | 51.81** | 59.69** |

** significant* p-*value (<0.05) with one-way ANOVA followed by* post-hoc *test Bonferroni when compared with control. ** Significant* p-*value (<0.001) with one-way ANOVA followed by* post-hoc *test Bonferroni when compared with control. LEPG: Leaf extract of* P. granatum, *PEPG: Fruit peel extract of* P. granatum, *100: 100 mg/kg, 200: 200 mg/kg*

**Table 4: Effect of oral administration of leaves and fruit peel extract of *P. granatum* and Ibuprofen on Cotton Pellet induced Granuloma in Rats. (n=6)**

| Groups | Dose (mg/kg) | Dry weight of granuloma (mg) | Percent inhibition |
|---|---|---|---|
| Group 1 | Vehicle | 66.00 ± 12.8 | |
| Group 2 | PEPG 100 | 48.17 ± 4.67* | 27.02 |
| Group 3 | PEPG 100 | 37.67 ± 10.01** | 42.92 |
| Group 4 | LEPG 100 | 46.33 ± 7.84* | 29.80 |
| Group 5 | LEPG 100 | 33.33 ± 6.09** | 49.5 |
| Group 6 | Ibuprofen 100 | 28.00 ± 3.1** | 57.58 |

** significant* p-*value (<0.05) with one-way ANOVA followed by* post-hoc *test Bonferroni when compared with control. ** Significant* p-*value (<0.001) with one-way ANOVA followed by* post-hoc *test Bonferroni when compared with control. LEPG: Leaf extract of* P. granatum, *PEPG: Fruit peel extract of* P. granatum, *100: 100 mg/kg, 200: 200 mg/kg*

LEPG 100, LEPG 200, PEPG 100 and PEPG 200 showed significant reduction (27.02%, 42.92%, 29.80% and 49.5% respectively) in weight of cotton pellet induced granuloma respectively on 8$^{th}$ day as compared to control group. Maximum reduction was observed by peel extract of *P. granatum* at a dose of 200 mg/kg. Standard drug Ibuprofen treatment resulted in a 57.58% reduction in cotton pellet induced granuloma. There was no significant difference between the higher doses of both plant extracts and standard. [Table 4]

**DISCUSSION**

In the present study, analgesic and anti-inflammatory activities of leaves as well as fruit peel extracts of *P. granatum* were investigated by experimental animal models.

The Antinociceptive activity was studied by the acetic acid-induced writhing test using Swiss albino mice. Intraperitoneal administration of acetic acid in rats irritates serous membranes and provokes a stereotyped behavior in mouse known as writhing.

The number of writhes counted for 30 minutes following acetic acid injection. The percentage inhibitions of writhing produced by different pretreatments were calculated. The percent reduction in the number of abdominal contractions (writhing) indicates the level of analgesia in the acetic acid writhing reflex model [15].

Leaves as well as fruit peel extract of *P. granatum* in both doses of 100 mg/kg and 200 mg/kg produced significant inhibition of writhing. This showed that leaves as well as fruit peel extracts of *P. granatum* have a significant analgesic property. The higher dose of both extracts exhibited better analgesic effect.

The writhing induced by acetic acid is a sensitive procedure to evaluate peripherally acting analgesics. Intraperitoneal injection of acetic acid causes pain by liberating endogenous substances such as prostaglandins (PGs), serotonin, histamine, bradykinins and substance P, which stimulate nerve endings [16]. Local peritoneal receptors are postulated to be involved in the abdominal constrictions response [17].

Acute anti-inflammatory activity of leaves and fruit peel *of P. granatum* was assessed using the carrageenan-induced paw edema in rats. Carrageenan is a widely used irritant or a phlogistic agent. Injection of carrageenan under the plantar aponeurosis of the hind paw of rat produces edema by local inflammation. The carrageenan-induced inflammation model is a test for evaluation of acute anti-inflammatory activity [18].

Leaves as well as fruit peel extracts of *P. granatum* in both doses produced significant anti-edematogenic effect on paw edema induced by carrageenan. The higher doses of both extracts exhibited better anti-inflammatory effect.

The inflammation induced by carrageenan injection is biphasic in nature [19]. The first phase observed during the first hour is attributed to the release of serotonin, histamine and kinin while the second phase is related to the release of prostaglandin [20, 21]. Leaves and fruit peel extracts of *P. granatum* are effective after first hour, suggesting that both the extracts inhibit the second phase of carrageenan induced inflammation.

The cotton-pellet induced granuloma model is based on the foreign body granuloma which is provoked in rats by subcutaneous implantation of pellets of compressed cotton and is widely used to assess the transudative and proliferative components of chronic inflammation [22, 23]. The weight of dry pellet correlates with the amount of granulomatous tissue.

In the present study, administration of leaves and fruit peel extracts of *P. granatum* exhibited inhibition of inflammation close to the inhibitory effect of Ibuprofen in a dose dependent manner. This suggests that leaves and fruit peel extracts have potential to inhibit the chronic inflammation.

Fruit peel and leaves of *Punica granatum* possess antinociceptive and anti-inflammatory effect which could be due to the presence of phytochemicals such as Tannins and Flavonoids [24]. Qualitative phytochemical screening showed that content of Flavonoids in leaves and fruit peel are same but tannins are more in fruit peel. Flavonoids are well known for their ability to inhibit pain perception [25]. Flavonoids also have anti-inflammatory properties due to their inhibitory effects on enzymes involved in the production of the chemical mediator of inflammation. Gallic acid is the major tannin found in both fruit Peel and leaves, but the other tannins like Gallagyldilacton and Granatin B is found in Fruit peel but not found in leaves of *P. granatum.* Among tannins, Granatin B more strongly inhibited COX-2 and this could be the reason for better activity of fruit peel extract than the Leaves extract of *P. granatum* [26, 27, 28].

## CONCLUSION

From this study, we can conclude that leaves and fruit peel extracts *of P. granatum* has significant Antinociceptive, acute anti-inflammatory and chronic anti-inflammatory activity. Further elaborative work is necessary for the better understanding of the mechanism of their Antinociceptive and anti-inflammatory activity. Detailed clinical studies in this direction are required to potentiate this claim in human beings.

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