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Research Article

ANALGESIC AND ANTI-INFLAMMATORY ACTIVITIES OF ETHANOLIC EXTRACT OF LEAVES OF *PUNICA GRANATUM L.* ON EXPERIMENTAL ANIMAL MODELS

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ABSTRACT

Objective: The aim of the study is to evaluate the analgesic and anti-inflammatory activities of the ethanolic extract of *Punica granatum L.* (EEPG) on experimental animal models.

Methods: Tail-flick method was used to test the central analgesic activity, using Pethidine as standard drug. The tail flick latencies or the basal reaction time of the animals were assessed using an analgesiometer. Glacial acetic acid induced writhing response was used to test the peripheral analgesic activity, using Aspirin as standard drug. Number of writhing responses was counted for 20 min in each group and the percentage protection was calculated. And Carrageenan induced rat paw edema method was used to test anti-inflammatory activity of EEPG against acute inflammation, using Aspirin as standard drug. The inhibition of rat paw edema was calculated in percentage.

Results: In central analgesic activity, the extract and pethidine showed significant increase in the reaction time. In peripheral analgesic activity, the extract and aspirin significantly reduced the number of writhes induced by acetic acid. And in anti-inflammatory activity, the extract produced significant reduction of the carrageenan induced paw edema.

Conclusion: The EEPG has demonstrated significant analgesic and anti-inflammatory activity.

Keywords: Analgesic, Anti-inflammatory, Pethidine, Aspirin, *Punica granatum*.

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INTRODUCTION

Pain is a type of unpleasant sensation localized to a part of the body. It is often described in terms of a tissue destructive or penetrative process or of a bodily or emotional reaction [1]. Inflammation is a process that occurs after an infection or tissue injury. The inflammatory response, leading to tissue damage due to fibroplasia, leukocytosis, excessive production of various mediators such ascytokines, tumor necrosis factor-α-alpha, interleukin (IL)-6, and IL-8, is essential in maintaining homeostasis. Inflammation is also an important physical factor that triggers the immune reaction. Superficial pain arises from nociceptive receptors in the skin and mucous membranes and is often associated with inflammation and tissue swelling [2].

Punica granatum is also known as pomegranate. It is a fruit-bearing deciduous shrub commonly found in Asia. Different parts of the plant have medicinal significance. *P. granatum* has been extensively used for the treatment of diarrhea, helminthiasis, hemorrhage, respiratory pathologies, etc., as traditional medicine across the globe. In addition, this plant is reported to have excellent antibacterial, antifungal, and antiprotozoal activity [3].

Commonly known as Dalim, it is cultivated almost throughout Assam [4]. Although only recently has pomegranate (*P. granatum*) been acclaimed for its health benefits, this fruit has long been cultivated and consumed, as fresh fruit or in the form of beverage, especially in the Mediterranean region. Pomegranate fruit, juice and peel possess a marked antioxidant capacity [5] with a high content in polyphenols, in particular, ellagitannins, condensed tannins, and various types of anthocyanin [6].

Furthermore, in recent times *P. granatum* has demonstrated antioxidant activity accompanied with radio protective and anti-fibrotic property [7]. Conventionally, to treat painful and inflammatory conditions, decoction of leaves has been used. However, there is no scientific data available on this. That is why, present study was done to evaluate the analgesic and anti-inflammatory activities of the ethanolic extract of *P. granatum L.* (EEPG) on animal model.

METHODS

Plant material

Leaves of *P. granatum* were collected from the plants in the campus of Assam Medical College, Dibrugarh. Prof. M. Islam has done authentication of the plant, who works in department of Life Sciences, in University of Dibrugarh.

Plant extract

The leaves were plucked from the plant and were air-dried in shade. These were then powdered and ethanol extracts were prepared using 95% ethanol by percolation method [8]. Followed by evaporation in a rotary evaporator under controlled temperature, and reduced pressure. After percolation, dry powder of 500 g of leaves yielded 90 g (18%) of dry powder.

Animals

Central Animal House, Assam Medical College and Hospital, Dibrugarh, Assam was the procurement site of the animals used in the study. The study conducted was in accordance with Committee for the Purpose of Control and Supervision of Experiment on Animals guidelines and approval was taken by the Institutional Animal Ethical Committee. Standard diet was fed to the animals with water *ad libitum*. Healthy albino rats (*Rattus norvegicus*) of either sex weighing 150–200 g and healthy albino mice of the species *Mus musculus* of either sex weighing 20–30 g were used as animal experimental models.

Table 1: Central analgesic activity

Groups	Drug Dose mg/kgs.c.	Reaction time before drug administration (In sec) Mean±SEM	15 min	30 min	60 min	90 min	120 min	150 min	180 min
Group A	Gum acacia (5 ml/kg)	3.6±0.08	3.55±0.04	3.6±0.14	3.55±0.16	3.51±0.16	3.55±0.12	3.7±0.15	3.75±0.12
Group B	EEPG (500 mg/kg)	3.3±0.08	3.5±0.06	3.8±0.06	4.15±0.07[a]	4.5±0.06[a]	4.2±0.12[a]	3.7±0.13	3.5±0.05
Group C	Naloxone (1 mg/kg)	3.3±0.02	3.1±0.06[a]	3.0±0.06[a]	2.9±0.06[a]	2.6±0.10[a]	2.6±0.11[a]	2.7±0.12[a]	3.0±0.05[a]
Group D	EEPG (500 mg/kg) +Naloxone (1 mg/kg)	0.09	0.04[a]	0.06[a]	0.07[a]	0.07[a]	0.06[a]	0.07[a]	0.09[a]
Group E	Pethidine (5 mg/kg)	3.7±0.15	4.1±0.15[a]	5.0+/- 0.7	5.05±0.09[a]	5.88±0.12[a]	5.6±0.17[a]	4.75±0.19[a]	4.15±0.08[a]
One-way ANOVA	F	3.96	21.76	97.68	86.9	284.5	112.7	34.05	23.27
	Df	25.4	25.4	25.4	25.4	25.4	25.4	25.4	25.4
	p	>0.01	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

One-way ANOVA followed by Dunnett's [a]=p < 0.05 versus Control, ANOVA: Analysis of variance, EEPG: Ethanolic extract of *Punica granatum* L.

Chemicals and drugs

This experimental study used following chemicals and drugs: 3% gum acacia suspension, normal saline, pethidine, naloxone, aspirin, and carrageenan.

Acute toxicity study

By following OECD 425 guidelines, acute toxicity test was done for EEPG [9]. An arbitrary dose 500 mg/kg was selected for the study, as the extract was found to be safe and no sign of toxicity and no mortality reported during the study period even if more than 2000 mg/kg dose was administered.

Method for central analgesic activity

Healthy albino rats (either sex) weighing 150–200 g were divided into five groups with six animals in each group formed for the study. Following were the groups with their respective treatments -

- Group A - Control, vehicle gum acacia 5 ml/kg subcutaneously (s.c.)
- Group B - EEPG 500 mg/kg (s.c.)
- Group C - Naloxone 1 mg/kg (s.c.)
- Group D - EEPG 500 mg/kg (s.c.)+Naloxone 1mg/kg (s.c.)
- Group E - Pethidine 5 mg/kg (s.c.).

Tail-flick method was used to test the central analgesic activity [10]. The tail flick latencies or the basal reaction time of the animals were assessed using an analgesiometer (Elite). By placing the tip (last 2 cm) of the tail on the radiant heat source, basal reaction time of radiant heat was taken. A cutoff time period of 10 s was observed to prevent the tail from damage. Reaction time was recorded at pre-drug, 15, 30, 60, 90, 120, 150, and 180 min after administration of vehicle or drugs. Here, the standard drug used was pethidine and naloxone was used to determine the mechanism of action.

Method for peripheral analgesic activity

Healthy albino mice (either sex) weighing 20–30 g were taken and divided into three groups with six animals in each group. Following were the groups with their respective treatments -

- Group A - Control, gum acacia 5 ml/kg per orally
- Group B - EEPG 500 mg/kg per orally
- Group C - Aspirin 100 mg/kg per orally.

Glacial acetic acid induced writhing response was used to test the peripheral analgesic activity [11]. One hour after administration of drugs, induction of writhing was done in mice by giving intraperitoneal injection of acetic acid at a dose of 5 ml/kg body weight. Number of writhing responses was counted for 20 min in each group and the percentage protection was calculated. Here, the standard drug used was Aspirin in the dose of 100 mg/kg per orally.

Table 2: Peripheral analgesic activity

Groups	Drug dose (mg/kg) per orally	Number of writhing movements (Mean±SEM)	Percentage of protection S.C. (%)
GROUP A	Gum acacia 10 ml/kg	67.5±0.46	----------
GROUP B	EEPG 500 mg/kg	47±0.86[a]	30.93
GROUP C	Aspirin 100 mg/kg	7±0.25[a]	89.92
One Way ANOVA	F	2611	
	df	15.2	
	P	<0.0001	

[a]=p < 0.05 versus Control; one-way ANOVA followed by Dunnett's Multiple Comparison Test, ANOVA: Analysis of variance, EEPG: Ethanolic extract of *Punica granatum* L.

Method for evaluation of anti-inflammatory activity

Healthy albino rats weighing 150–200 g of either sex were taken and divided into three groups having six animals each. Following were the groups with their respective treatments -

- Group A - Control 3% gum acacia-5 ml/kg orally
- Group B - EEPG 500 mg/kg orally
- Group C - Aspirin 100 mg/kg orally

Carrageenan induced rat paw edema model was used to test the anti-inflammatory activity of EEPG against acute inflammation. Carrageenan induced rat paw edema is very simple and the most widely used model for evaluation of the new compounds having promising anti-inflammatory activity. 0.1 ml of 1% carrageenan in normal saline was injected into the sub plantar region of the rat hind paw. The animals were treated with 3% gum acacia, EEPG and aspirin in the respective groups 1 h before carrageenan injection.

Just before carrageenan injection, the paw volume was measured plethysmometrically, at 0 h then at 1st, 2nd, 3rd, and 4th h after carrageenan injection [12]. Increase in paw volume was measured as the difference between the paw volume at "0" h and paw volume at the respective hour. The percentage inhibition of rat paw edema was calculated after each hour of carrageenan injection up to 4 h by the formula described by Agus [13].

% inhibition=(Control mean–Treated mean)/Control mean×100

Statistical analysis

Statistical analysis was performed by software using One-way analysis of variance followed by Dunnett's multiple comparison

Table 3: Carrageenan induced paw edema

Drug	Drug Dose	Mean increase in paw volume (Mean±SEM) (mL) (% Inhibition within parentheses)			
		1st h	2nd h	3rd h	4th h
A (Control)	5 mg/kg	0.24±0.04	0.30±0.05	0.57±0.01	0.36±0.02
B (EEPG) (%)	500mg/kg	0.13±0.01[a] (36.54)	0.17±0.01[a] (47.24)	0.254±0.01[a] (55.58)	0.15±0.01[a] (48.48)
C (Aspirin) (%)	100 mg/kg	0.12±0.01[a] (46.82)	0.13±0.17[a] (55.15)	0.21±0.02[a] (62.06)	0.13±0.01[a] (57.00)
ANOVA	F	10.12	46.84	170.7	60.81
	df	17,2	17,2	17,2	17,2
	P	<0.05	<0.05	<0.05	<0.05

Values are expressed as Mean±SEM, [a]=p < 0.05 versus Control, One way ANOVA followed by Dunnett's Multiple Comparison Test were done, ANOVA: Analysis of variance, EEPG: Ethanolic extract of *Punica granatum* L.

test. Level of significance <0.05 is required for consideration as significant [14].

RESULTS

The LD_{50} of EEPG is more than 2000 mg/kg, in acute oral toxicity tests.

There was significant increase in the latency time as shown in Table 1. EEPG is shown to have significant central analgesic activity when compared with control group. On comparison with control group, significant peripheral analgesic action was observed with group which received EEPG and aspirin as shown in Table 2, because there was inhibition of abdominal writhes produced by acetic acid. There was also significant reduction in carrageenan induced paw edema by EEPG and aspirin, as shown in Table 3.

DISCUSSION

The results of our study show that EEPG produced significant analgesia, at central and peripheral level. The extract (500 mg/kg s.c.) and pethidine showed significant increase in the reaction time. The reaction time was significantly decreased by pre-treatment with Naloxone, producing hyperalgesia while combined treatment of EEPG (500 mg/kg s.c.) and naloxone (1 mg/kg s.c.) produced significant decrease in the reaction time as compared to EEPG alone. The competitive antagonist at all types of opioid receptors is Naloxone. The actions of endogenous opioid peptides are also blocked by Naloxone [15]. In the face of a variety of physical (pain) or psychological stressors, an increased release of a variety of opioid peptides occurs [16]. This indicates the involvement of endogenous opioid peptides in mediation of analgesic activity of *P. granatum*, reflecting its probable central mechanism of action. However, since there is almost complete inhibition of analgesic activity of EEPG after naloxone, opioid mechanisms may also be involved.

The number of writhes induced by acetic acid was significantly reduced by the extract (500 mg/kg orally) and aspirin (100 mg/kg orally). Endogenous substances such as serotonin, histamine, prostaglandins, bradykinin and substance P, which stimulate pain nerve endings are released by acetic acid which causes algesia. Partial involvement of local peripheral receptors is postulated to be in the abdominal constriction (writhing response). Sensitization of nociceptive receptors to prostaglandins is responsible for the abdominal constriction [17]. Standard NSAIDs like aspirin offer relief from inflammatory pain by suppressing the formation of pain substances in the peripheral tissues, where prostaglandins and bradykinin were supposed to play an important role in the pain producing process. Direct stimulation of sensory nerve endings to other pain provoking stimuli by prostaglandins will elicit pain [18]. Therefore, it is likely that EEPG suppresses the formation of these substances or antagonizes the action of these substances that may suggest analgesic activity through peripheral mechanism.

Carrageenan induced paw edema is a suitable test for evaluating anti-inflammatory drugs which has frequently been used to assess the anti-edematous effect of natural products. After injection of carrageenan the development of edema in the paw of the rat, is a biphasic event. Release of histamine and serotonin, is responsible for the initial phase during the 1st h. Release of prostaglandins, protease and lysosome, is responsible for the second phase of the edema. EEPG (500 mg/kg) produced significant reduction of the carrageenan induced paw edema suggesting its anti-inflammatory activity, as shown by the study done. The maximum inhibition was seen at the end of 3rd h. Flavonoids are known to target prostaglandins that are involved in the late phase of acute inflammation and pain perception [19]. Therefore, flavonoids present in the leaves of *P. granatum* may be responsible for its analgesic and anti-inflammatory activities.

The present study conducted by us showed that the EEPG possess significant analgesic and anti-inflammatory activity. Thereby its traditional use in inflammatory and painful conditions is justified. However, further studies and development of more purified product of leaves of *P. granatum* are required for proper clinical use.

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AUTHORS' CONTRIBUTIONS

All the authors have contributed equally in the finalization of manuscript and during the study.

CONFLICTS OF INTEREST

None of the author has any financial/non-financial interest attached with this study.

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