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Pharmacological and Biochemical Evaluation of Anti-arthritic Activity of Punica Granatum Extracts in FCA Induced Arthritis in Wistar Rats

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ABSTRACT

The present studies were executed on Freund's complete adjuvant (0.1 ml) induced arthritic Wistar rats to explore the folkloric use of the seeds. This study describes the effect of *Punica granatum* Linn's Ethanolic (PGSE) and Chloroform (PGSC) extract within the FCA-induced arthritis rat paw oedema, vagaries in behaviour, haematological and alterations in biochemical parameters in the developed and progression of arthritis phases. There was a significant rise in the paw swelling (volume) of rats and reduction in (BW) body weight, with FCA-induced arthritic rats. In contrast, PGSE and PGSC with the dose of 200mg/kg and 400mg/kg and Diclofenac (20mg/kg) treated group showed a substantial decrease in paw volume and the normal improvement in body weight to the positive control group. The altered level of haematological parameters, including, Hb, RBC, WBC, and ESR, in arthritic rats, have been substantially regained to normal by PGSE and PGSC treatment at the dosage of 200 mg/kg/p.o and 400 mg/kg/p.o in both the developed and progression of arthritis phases. In this study, Anti-inflammatory action of the PGSE and PGSC with carrageenan-induced paw oedema has also been investigated. Thus, percentage (%) inhibition of PGSE and PGSC were found to be 91.8 % and 86.71, respectively, concerning Diclofenac sodium (93.14%), this gave the evidence of dose-dependent action potential of *Punica granatum* as anti-inflammatory activity.

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INTRODUCTION

Plants have always been used for its medicinal tenacities since the primaeval period, and the medicinal plants and their parts play an essential role in developing countries for potent therapeutic agents. Rheumatoid arthritis is a known autoimmune, musculoskeletal, infectious ailment, inflammatory, metabolic, affecting all the joints protected by synovium contributing to destructive polyarthritis and other body organs and inflammation is the human/body's immune response to injury, wound or other damage to start healing processes and eliminate harmful stimuli (Guo *et al.*, 2018).

The root cause of the autoimmune disease (RA) remains unclear, but it has stimulated the production of new medications and revolutionized care. Rheumatoid arthritis induction of the immune response involves specific CD4 + T cells, most perspective as a response to an unfamiliar exogenous or endogenous antigen and the recruited monocytes, macrophages, and fibroblasts, therefore, contain cytokines, including, tumour necrosis factor-

a (TNF-a) and synovial cavity interleukin-1 and these cytokines are crucial to the destructive cascade, which ultimately triggers the development of, MMP, matrix metalloproteinases and osteoclasts, resulting in bone and soft tissue irreversible damage (Olsen and Stein, 2004).

The pomegranate is the contemplate "a pharmacy for itself" in Ayurvedic medicine, the roots as well as bark thought to possess an anthelmintic and vermifuge property. Still, the peels are potent astringent, remedy for diarrhoea and oral aphthae (Ali and Sharma, 2006). The dried and extracted flowers are commonly used in hematuria, haemorrhoids, dysentery and hemoptysis, but the powdered buds were used to treat bronchitis (Ross *et al.*, 2001).

Punica granatum L. (Anar or Pomegranate) is an identified deciduous shrub or, can say, small tree, growth of 1.8-4.6m tall, belonging to the family Punicaceae. The fruits are globose, shiny berry types, and 5–7.6 cm in diameter, but when get matured, the colour is reddish or yellowish-green. The seeds are crunchy having acidic pulp enclosed in a membranous skin (Qnais *et al.*, 2007).

There have even been much preliminary toxicity studies and approved data in mice/rats (rodents) to report *Punica granatum* as non-toxic at all concentrations/doses, especially at a high dose (Braga *et al.*, 2005), and there are various compounds present in *P.granatum* fruits; among these compounds, the most therapeutic phytochemicals are polyphenolics, flavonoids, alkaloids, ellagic, as well as gallic acid both (Anibal *et al.*, 2013).

Consequently, this study was intended to explore and estimate the pharmacological and biochemical parameters of the *Punica granatum* ethanolic and chloroform seed extract in FCA-induced arthritis in Wistar rats. These current studies were also done to determine/evaluate an anti-inflammatory potential of the pomegranate seed extracts (ethanolic & chloroform) in Carrageenan-induced paw oedema in Wistar rats.

MATERIALS AND METHODS

Experimental Animal

Wistar rats of either sex weighing 150- 240g were acquired from College Animal House Facility, KIET School of Pharmacy (KSOP), Ghaziabad. In KSOP, the animal house temperature was maintained at 24-25 $\pm$ 2o C, and the animals were kept under the standard laboratory condition, i.e. 12 hours light and 12 hours dark cycle, in polypropylene cages. The animals had free access to good diet pellets (Pranave Agro Industries Ltd, New Delhi) and water ad libitum. The IAEC, Institutional Animal Ethics Committee, approved the protocol of KIET School of Pharmacy, Ghaziabad, with the Registration number (1099/PO/Re/S/07/CPCSEA).

Plant Material

The seeds of *Punica granatum* were purchased from Chawla & Co, Chandini Chowk, Delhi, and they were authenticated from the CSIR - National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India and the authentication number or Ref. No. for the plant is NSCAIR/RHMD/Consult/2019/3540-41.

Preparation of *Punica granatum* seeds extracts

The P. granatum seeds were obtained from an authorized vendor, dried mainly in the shade or dark, and then pulverized with an (electric) blender (Wang *et al.*, 2019).

A portion of the powder (250 g) was thoroughly extracted for 16-18 hours in a Soxhlet apparatus with 400 ml of absolute ethanol and chloroform. The extract filtrates were concentrated to dryness using the distillation process and stored at the appropriate temperature (Gautam *et al.*, 2013).

Morphological and Standardization of Plant

The Plant material was evaluated for its morphological characteristics, and all the standardization parameters such as foreign matters, moisture content, extractive values as well as ash value. These parameters were necessary for the standard objectives of the plant to determine its safety, efficacy, quality, quantity, and stability.

Phytochemical Screening

Phytochemicals are the broad range of secondary metabolites that are formed by plants, and these are classified as active plant chemical constituents (Kota *et al.*, 2018). There are tannins, alkaloids, saponins, flavonoids, phytosterols, triterpenoids, and glycosides (Nainwani, 2014; Bhandary *et al.*, 2012).

The existence of these active chemical components in plant extracts is determined by preliminary qualitative phytochemical analysis according to the procedure described in (Lee *et al.*, 2010; Yadav *et al.*, 2014).

Evaluation of In-vitro anti-arthritic activity

Two in-vitro models were selected for the study, and these are:

Inhibition of Protein Denaturation Model

A reaction mixture (50ml) was prepared with a 28ml phosphate buffer (pH 6.4, PBS), 2 ml fresh egg albumin, and 20 ml of varying extract and Diclofenac

sodium concentrations as a reference so that the final concentrations were 50, 100, 200, 400, 800, 1000, 2000μg/ml. The double-distilled water with parallel concentrations was used in control, and the above reaction mixture was mainly incubated (BOD incubator) at 36-37 ºC ($\pm$2) for 15 minutes.

After that, it was heated for 5 minutes at 70 ºC[9,13]. Keep it aside for cooling, and their absorbance was considered at 660nm using UV visible spectrophotometer. All of the above solutions go for viscosity test using Ostwald viscometer, and percentage inhibition was determined using the formula (Mittal *et al.*, 2013; Bensaad *et al.*, 2017).

$$Percentage\ (\%)\ Inhibition\ =\ \left(\frac{Vt}{Vc}-1\right)\ \times\ 100$$

where, Vt = absorbance of the test samples; Vc = absorbance of control.

HRBC Membrane Stabilization Method

Preparation of reagents

Alsevers solutions were prepared with the addition of sodium citrate (0.8%), 0.42% (NaCl) sodium chloride, 2% dextrose and citric acid (0.05%), in distilled water to mark up the volume up to 100ml with some distilled water and sterilized. 0.85 gm of sodium chloride (NaCl) was dissolved with 100ml distilled water, and this above solution was used as an isotonic saline solution. The hypotonic solution was prepared with adding 0.36 gm NaCl (sodium chloride) to 100ml of distilled water. Phosphate buffer containing 0.19gm potassium dihydrogen phosphate, 8gm of sodium chloride, 2.38 gm disodium hydrogen phosphate and pH at 7.4.

Assay of membrane stabilizing activity

The HRBC method was done to assess or evaluate the in-vitro anti-inflammatory action potential in the samples. A person who had not taken NSAIDs 14 days before the experiment was preferred for blood collection and collected blood was mixed with Alsevers solution in equal volume, and then centrifugation was done for 12-15 minutes at 3000rpm.

After centrifugation, decanted the supernatant liquid using a micropipette and washed the remaining packed cells with isosaline solution 3-4 times, then made a 10% suspension solution from it. The hydroalcoholic doses of extracts were prepared (100,200, 400, 800 and 1600μg/mL) respectively with distilled water. A solution was prepared to have a concentration of 1ml phosphate buffer, 0.5ml of HRBC suspension, 2ml hyposaline and 0.5 ml of drug extract. It was placed in an incubator on 37°C ($\pm$2) for 25-30 minutes and then centrifuged at 3000rpm for 20 min, and then the supernatant liquid was again decanted. Its haemoglobin content estimated using spectrophotometrically at 560 nm (Mittal *et al.*, 2013).

Diclofenac sodium having (50, 100, 200, 400, 800 and 1600μg/mL) concentration were taken for standard drugs and a control part was done by excluding all extracts, and distilled water in place of hyposaline. The percentage protection or HRBC membrane stabilization percentage and % hemolysis was calculated using the below formula (Kota *et al.*, 2018; Gautam *et al.*, 2013).

$$Percent\ \ Protection\ \ =\ \ \frac{100\ -\ [O.D\ of\ Sample]}{O.D\ of\ control}\ X\ 100$$

Evaluation of In-vivo anti-arthritic activity

Carrageenan-induced paw oedema in rats

The carrageenan-induced right hind paw oedema approach has already been carried out for determining the anti-inflammatory action. In short, acute inflammation was caused through a sub plantar injection of 0.1 ml of 1 per cent carrageenan suspension in the normal saline, in rats' right hind paw, 1h after an oral administration of test material (Sarker *et al.*, 2012; Kavitha *et al.*, 2011). Water Plethysmometer or Vernier calliper was used to determine oedema volume before and 1, 3 and 5 hours after the carrageenan injection. An hour earlier, the carrageenan injection, sample or test substance was administered, and the control group with the vehicle only (Bhandary *et al.*, 2014; Kamble and Nazia, 2017).

Diclofenac was taken as reference anti-inflammatory agent, with a dosage of 20 mg/kg bodyweight (Ratheesh and Helen, 2007; Sarker *et al.*, 2012). The control group (vehicle) was used to compare the inhibitory effect on the oedema formation to calculate the percentage inhibition. The formula that is used for the calculation of the above mention parameter is as (Lin *et al.*, 1999; Kavitha *et al.*, 2011) and the protocol followed for the same study were taken as Table 1.

$$\%\ Inhibition\ =\ \frac{Ec\ -\ Et}{Ec}\ \times\ 100$$

where, Ec = Edema rate of the control group; Et = Edema rate of the treated group.

Freund's Complete Adjuvant (FCA) induced arthritis in rats

Arthritis was induced with a single 0.1 mL intradermal injection of Freund's Complete Adjuvant (FCA) containing 1 mg.mL-1 Mycobacterium tuberculosis H37Ra suspension in a sterile paraffin oil into the footpad of Wistar rats' left hind paw for all animal groups, except for normal control group rats. The rats were carefully anaesthetized with the ether

Table 1: Protocol for Carrageenan-induced paw oedema in Wistar rats

S.No.	Group	Dose Schedule	References
1.	Normal Control	Normal Saline	Kavitha *et al.* (2011)
2.	Positive Control	Carrageenan (0.1ml of 1% in 0.9% Saline) + Normal Saline	Paval *et al.* (2009)
3.	Standard	Diclofenac Sodium (20mg/kg/b.w.p.o) + Normal Saline	Paval *et al.* (2009)
4.	Test Group	Carrageenan + PGSE	Kothari *et al.* (2011)
5.	Test Group	Carrageenan + PGSC	Kothari *et al.* (2011)

Table 2: Protocol for the FCA-induced Arthritis in rats

S.No.	Group (6animals/group)	Dose Schedule	References
1.	Normal Control	Normal Saline	Kavitha *et al.* (2011)
2.	Positive Control	FCA(0.1ml) + Normal Saline	Kothari *et al.* (2011)
3.	Standard	Diclofenac Sodium (20mg/kg/ b.w.p.o 21 days) + Normal Saline	Paval *et al.* (2009)
4.	PGSEH	400 mg/kg/ b.w.p.o 28 days	Kothari *et al.* (2011)
5.	PGSEL	200 mg/kg/ b.w.p.o 28 days	Kothari *et al.* (2011)
6.	PGSCH	400 mg/kg/ b.w.p.o 28days	Kothari *et al.* (2011)
7.	PGSCL	200 mg/kg/ b.w.p.o 28 days	Kothari *et al.* (2011)

Table 3: Morphological Parameters of *Punica granatum* dried seeds

S.No	Features	Observations
1.	Colour	Red- Purple
2.	Odour	Characteristic
3.	Taste	Sour, sweet-sour
4.	Size	1.05-2.09 mm (approx.)
5.	Shape	Rounded to oval

Table 4: Standardization parameters of *Punica granatum* seeds

S.No.	Parameters	Values Obtained
1.	Foreign Matter	0.09 %
2.	Moisture Content	16.26%
3.	Extractive Value in Ethanol	42.11%
4.	Extractive Value in Chloroform	29.6%
5.	Total Ash Value	9.01%
6.	Water Soluble Ash	4.27%
7.	Acid Insoluble Ash	1.02%

inhalation before and during the adjuvant injection because the adjuvant's very viscid nature exerts difficulty while being injected (Petchi *et al.*, 2015).

Treatment with *Punica granatum* extracts, Diclofenac and normal control (normal saline) was started on the 14th day after induction of arthritis and continued till 28 days (Wang *et al.*, 2019; Kothari *et al.*, 2011). After Freund's complete adjuvant injection, the paw volume for all taken animal group was measured by plethysmograph or Vernier calliper at 0, 7, 14, 17, 21 and 28 days (Mittal *et al.*, 2013; Das *et al.*, 2012).

On the 28th day (last day), blood was withdrawn using the retro-orbital process to the evaluation of the haematological parameters, i.e. RBCs, WBCs, Hb, and ESR (Kumar *et al.*, 2018; Umar *et al.*, 2012) and the protocol followed for the same were as Table 2.

Table 5: Phytochemicals screening

Phytochemicals	Tests	Ethanolic Extract	Chloroform Extract	Aqueous Extract
Carbohydrates	Molisch test	-ve	+ve	+ve
	Fehling test	-ve	+ve	+ve
Flavonoids	Ferric Chloride test	+ve	-ve	+ve
	Lead acetate test	+ve	-ve	+ve
Alkaloids	Mayer's test	+ve	-ve	+ve
	Hager's test	+ve	-ve	-ve
Saponin	Foam test	-ve	-ve	-ve
Tannins/Phenolic compounds	Gelatin test	+ve	-ve	+ve
Glycosides	Killer Killani test	-ve	-ve	+ve
Protein	Biuret test	-ve	-ve	-ve
Terpenoids	test	+ve	+ve	-ve
Steroids	Salkowski test	+ve	-ve	+ve

Table 6: Effects of Diclofenac sodium on protein denaturation and viscosity

Concentration (μg/ml)	% Inhibition (Diclofenac sodium)	Viscosity (cps)
Control	—	1.43
50	61.23	0.74
100	88.08	0.88
200	122.79	0.92
400	256.63	0.96
800	302.44	1.08
1000	464.25	1.16
2000	657.08	1.32

Table 7: Effects of ethanolic and chloroform seeds extract of *Punica granatum* on protein denaturation method and viscosity

Concentration (μg/ml)	% Inhibition (PGSE)	Viscosity (PGSE) (cps)	% Inhibition (PGSC)	Viscosity (PGSC) (cps)
50	78.52	0.82	64.7	0.89
100	175.5	0.88	147.05	0.93
200	235.29	0.92	220.58	0.99
400	388.23	0.97	294.11	1.19
800	421.17	1.04	385.29	1.08
1000	501.01	1.09	482.35	1.12
2000	756.86	1.11	514.7	1.19

Statistical Analysis

All data were depicted as mean ± standard value deviation error, (SEM), and its statistical significances were achieved using common one-way variance analysis (ANOVA), also followed by Dunnett's Multiple Comparison Test, where P<0.001 was considered statistically relevant using Graph Pad Prism version 8 (Hasan *et al.*, 2015).

RESULTS AND DISCUSSION

Morphological Evaluation of *Punica granatum* seeds

It was done to compared, weighed, counted and listed to determine differences or similarities in plant taxa by using these characters to define, classify and characterize plants. The results of all possible morphological parameters are observed and noted (Table 3).

Table 8: Effect of ethanolic seeds extract of *P. granatum* (PGSE) on HRBC membrane stabilization with standard, Diclofenac sodium

Concentration (μg/ml)	% Hemolysis (PGSE)	% Protection (PGSE)	% Hemolysis (Diclofenac)	% Protection (Diclofenac)
50	36.21	10.01	35.78	21.10
100	34.78	65.21	33.77	66.22
200	31.43	68.56	29.76	70.23
400	27.09	72.9	26.08	73.91
800	19.39	80.6	14.71	85.28
1600	10.7	89.29	9.69	90.3

Table 9: Effect of Chloroform (seeds) extract of *P. granatum* (PGSC) on HRBC membrane stabilization with standard, Diclofenac sodium

Concentration (μg/ml)	% Hemolysis (PGSC)	% Protection (PGSC)	% Hemolysis (Diclofenac)	% Protection (Diclofenac)
50	38.21	12.23	35.78	20.01
100	36.12	63.87	33.77	66.22
200	33.11	66.88	29.76	70.23
400	29.09	70.90	26.08	73.91
800	20.73	79.26	14.71	85.28
1600	16.05	83.94	9.69	90.3

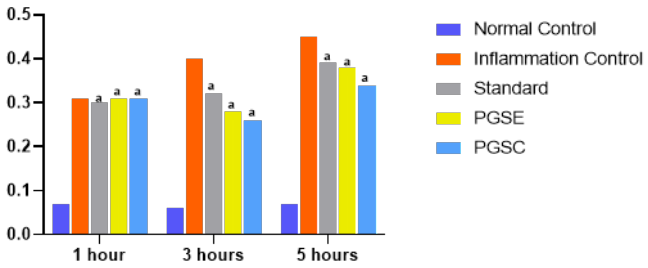

Figure 1: Effects of standard (Diclofenac sodium), PGSE and PGSC on change in Carrageenan-induced paw oedema (mm), and compared with the inflammatory control group at aP < 0.05. Data was taken in mean ± SEM, (n = 6).

Standardization Parameters of *Punica granatum* seeds

The standardization study was conducted to ensure consistency and the purity, protection and efficacy of medicinal plants and the observed results were recorded (Table 4).

Phytochemical Screening of Punica granatum

The phytochemical study was performed using aqueous, ethanolic and chloroform seeds extracts of *Punica granatum*, and it has given the preliminary confirmation of all compounds present in the extracts (Table 5).

In-Vitro Study

Protein Denaturation Study

Anti-inflammatory activity of Diclofenac and seeds' extracts of *Punica granatum* on protein denaturation inhibition test was recorded. It has shown the concentration-dependent potential for anti-inflammatory activities when compared against standard Diclofenac drug (Table 6, Table 7).

Human red blood cell (HRBC) membrane stabilization method

The anti-inflammatory action of Diclofenac and extracts of *Punica granatum* on human red blood cell (HRBC) membrane stabilization method were recorded. It has shown the concentration-dependent potential for anti-inflammatory activity when compared against Diclofenac sodium drug (Table 8, Table 9).

In-Vivo Study

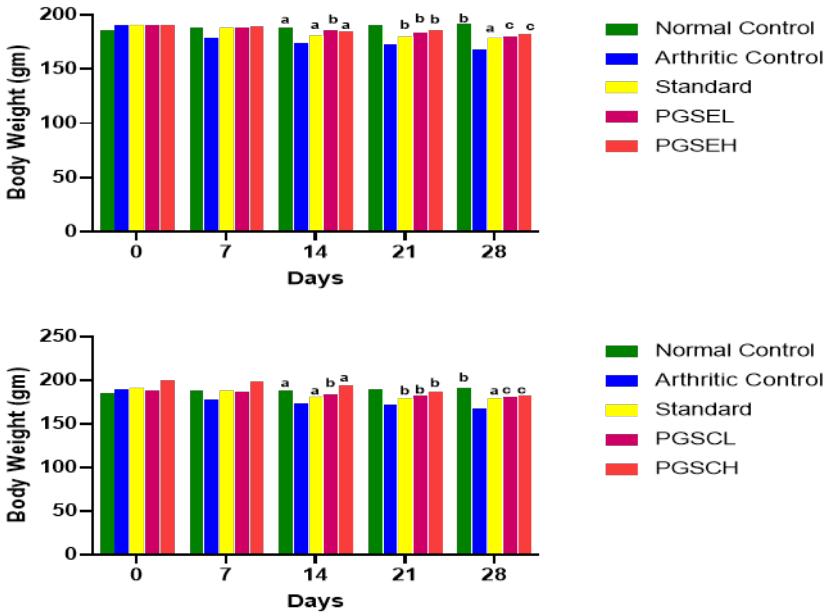

Figure 2: Effects of Ethanolic and Chloroform extracts of *Punica granatum* change in body weight of rats injected with FCA, compared with the negative control group as aP < 0.05, P<0.01, cP<0.001. Data was taken in mean ± SEM (n = 6).

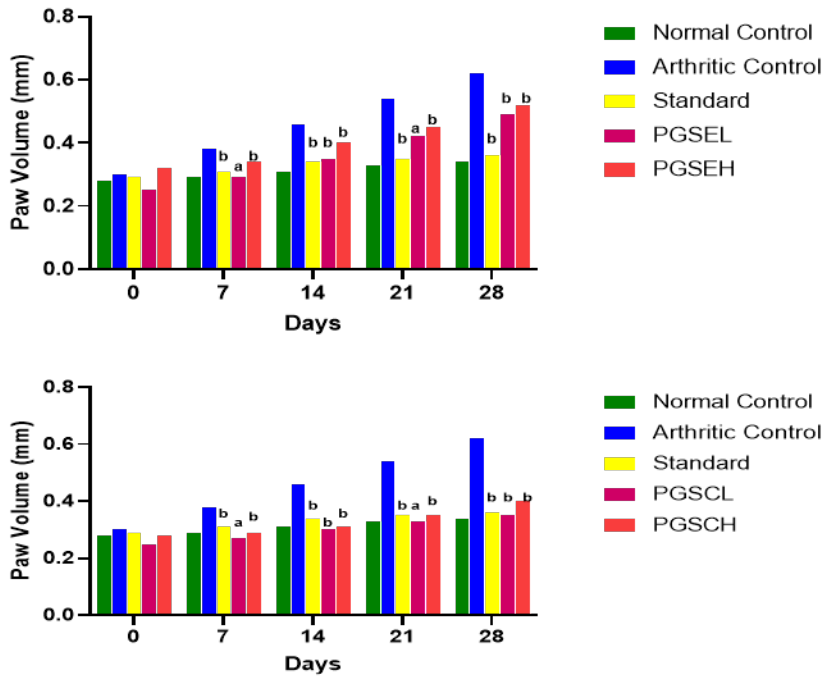

Figure 3: Effects of Ethanol and Chloroform extracts of *Punica granatum* (dose 200 and 400 mg/kg) on change in paw volume of rats injected with FCA, compared with the negative control group as aP < 0.05, P<0.01, cP<0.001. Data was taken in mean ± SEM (n = 6).

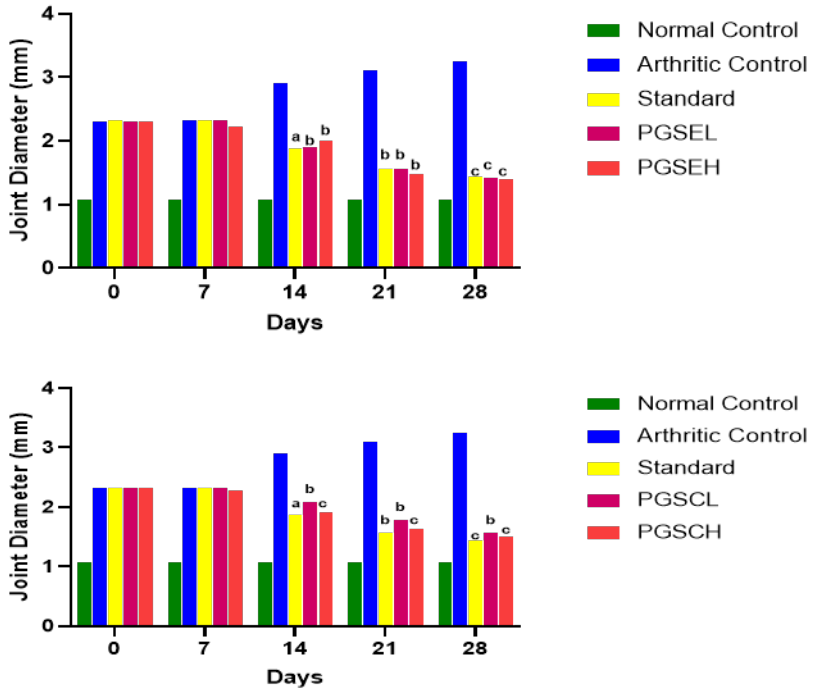

Figure 4: Effects of Ethanolic and Chloroform extract of *Punica granatum* (dose 200 and 400 mg/kg) on change in joint diameter of rats injected with FCA, compared with the negative control group as aP < 0.05, P<0.01, cP<0.001. Data was taken in mean ± SEM (n= 6).

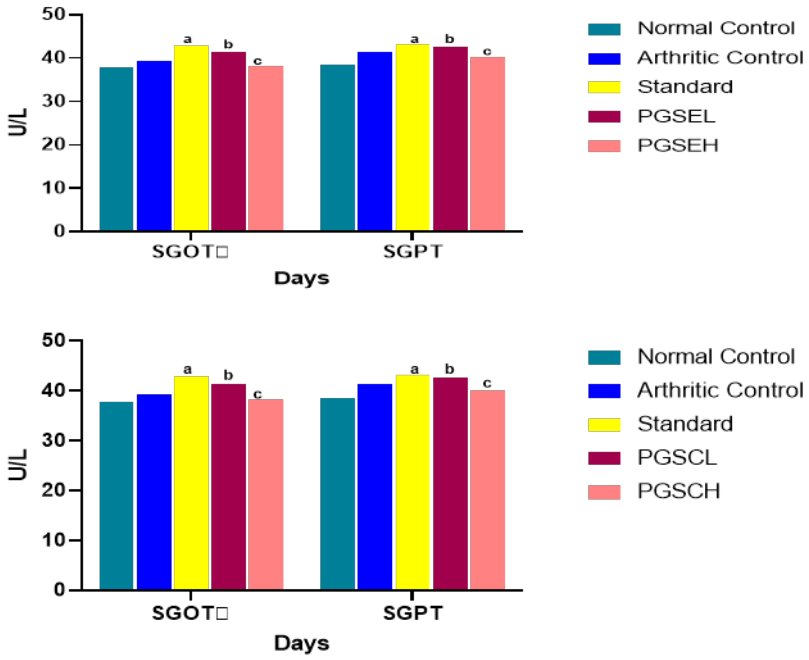

Figure 5: Effects of Ethanolic and Chloroform extracts of *Punica granatum* on change in a biochemical parameter of rats injected with FCA, compared with the negative control group as aP< 0.05, P<0.01, cP<0.001. Data was taken in mean ± SEM (n = 6).

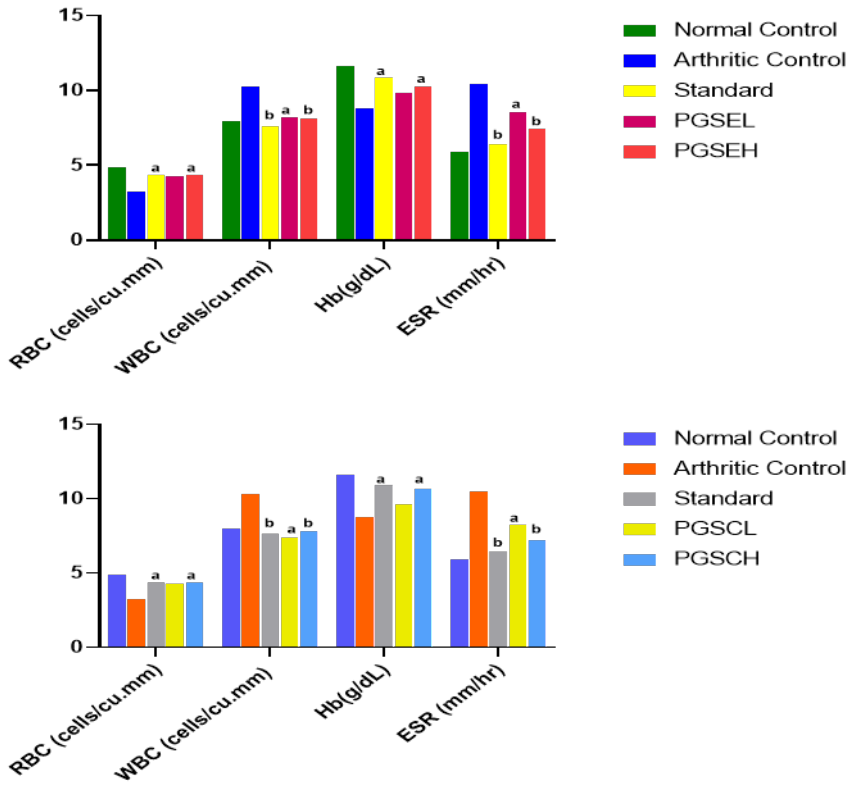

Figure 6: Effects of Ethanolic and Chloroform extracts of *Punica granatum* on change in a haematological parameter of rats injected with FCA, compared with the negative control group as aP < 0.05, P<0.01, cP<0.001. Data was taken in mean ± SEM (n = 6).

Carrageenan-induced Paw oedema

Carrageenan (0.1%) administration caused inflammation just within 1 hr, and after injection of this irritant, the peak effects were reached around 4-5 hours. The effects of the ethanolic, as well as chloroform seeds, extract of *Punica granatum* seeds with the dosage of 200mg/kg on the carrageenan-induced paw oedema was then recorded. It has shown the time-dependent action against the control group and shown similar effects as evaluated against standard, Diclofenac sodium (20mg/kg), drug (Figure 1). The data was statistical analysis using one-way ANOVA test that is followed by the Dunnett' test.

FCA- induced Arthritis Study

Bodyweight

The body weight was therefore measured at each of these days (0, 7, 14, 21, 28 days) and PGSE and PGSC (200 and 400 mg/kg), produced substantial increases (P< 0.05) and dose-dependent increases from day 14th till day 28th compared with the arthritic rats. Thus, results were shown in Figure 2.

Paw Volume

The decrease in paw swelling or volume is a constraint used to examine an anti-arthritic property of many drugs. PGSEL and PGSCH, produced significant dosage-dependent decreases from day 14th to day 28th, when compared to the standard and arthritic rats (Figure 3).

Joint Diameter

PGSE and PGSC (200 and 400mg/ kg) treatment showed significant with dose-dependent decreases (P < 0.05) from day 14^{th} to day 28^{th} compared to control and arthritis group. Hence, the results were summarized in Figure 4.

Biochemical Parameter

In this study, the arthritic control groups' biochemical parameters showed a slight rise in both the SGOT and SGPT levels and standard drug treatment group, it was increased significantly, but PGSE and PGSC treatment (PGSEL and PGSCH), produced significant decreases in the both SGOT and SGPT levels. The results were presented in Figure 5.

Haematological Parameters

The haematological parameters of arthritic control group rats showed lessening in the both RBC count and haemoglobin (Hb) levels and with an increase in the WBC and ESR levels. PGSE and PGSC treatment

(PGSEL & PGSEH) produced significant increases in RBC and Hb, with substantial decreases in WBC and ESR (Figure 6).

DISCUSSION

In the present investigation, the pharmacological and biochemical evaluation of *Punica granatum* seeds extracts (ethanolic and chloroform) in FCA-induced arthritis and Carrageenan-induced paw oedema in Wistar rats were studied. As found in some studies, several biologically vigorous and therapeutic active phytocompounds, namely, terpenoids, flavonoids, steroids, alkaloids, tannins, glycosides, and phenolic compounds, mainly are accountable for important anti-arthritic as well as anti-inflammatory activity inside numerous plant extracts. Increases in test sample absorbances against control indicate protein stabilization. Viscosities of protein solution has been stated to increase on denaturation. Thus, in the followed present study, both the seeds extract of *Punica granatum* has shown concentration-dependent percentage inhibition of tissue protein denaturation with % protection of membranes, suggesting its therapeutic anti-inflammatory activity to diclofenac sodium and control group. The results for the in-vitro study were confirmed by a practical evaluation of PGSE and PGSC in the in-vivo model. This activity was due to the availability of active phytochemicals and their ability to reduce inflammatory cytokine concentrations.

CONCLUSION

PGSE and PGSC have shown the highest concentration-dependent action in protein denaturation and HRBC membrane stabilization at the dosage of 2000μg/ml and 1000μg/ml, respectively. *Punica granatum* at the given 200 mg/kg dose level and 400mg/kg, p.o was shown to decrease the volume of rats' paw oedema and could normalize behavioural, haematological as well as biochemical irregularities in adjuvant-induced arthritic rats in the both developed and developing of FCA-induced arthritis, indicating momentous recovery in rheumatoid arthritis.

Future Aspects

The exact mechanism of action of *Punica granatum* on adjuvant-induced arthritis is not evident with these studies. The activity of *Punica granatum* on proinflammatory mediators including TNF-an Interleukins and other related mediators will be carried out in future to study its mechanisms.

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Conflict of Interest

The authors announce that there are no conflicts of interest for this study.

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