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Tannin Rich Fraction of *Punica granatum* Linn. Leaves Ameliorates Freund's Adjuvant Induced Arthritis in Experimental Animals

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ABSTRACT

Background: Rheumatoid Arthritis (RA) is a chronic, relapsing autoimmune disorder. *Punica granatum* Linn. (Punicaceae) is traditionally used herbal plant, which is reported to have immuno-modulatory and anti-inflammatory activity. In view of its reported activities, present investigation was designed to evaluate anti-arthritic potential of *Punica granatum* L. leaves in Freund's Complete Adjuvant (FCA) induced arthritis in laboratory animals along with identification of possible phytoconstituents responsible for the proposed activity. **Materials and Methods:** Arthritis was induced in experimental animals by subplanter injection of 0.1 mL of FCA (heat killed *Mycobacterium tuberculosis* in sterile paraffin oil 10 mg mL^{-1}) suspension into the left hind paw. After 14 days of FCA immunization, rats were treated with *Punica granatum* L. leaves tannin fraction (PGTF) orally at a dose of 50, 100 and 200 mg $kg\ day^{-1}$ for 28 days. Indomethacin (2 mg kg^{-1}) was used as a standard. The severity of arthritis was evaluated by symptoms, biochemical, hematological, radiological and histopathological assessment. **Results:** Therapeutic treatment with PGTF in FCA-induced arthritic rats exhibited significant reduction in serum levels of C-reactive Protein (CRP), Rheumatoid Factor (RF), Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS) and lysosomal enzymes. Furthermore, PGTF treated arthritic rats revealed significant reduction in paw edema formation, arthritic index, Erythrocyte Sedimentation Rate (ESR) and urinary hydroxyproline along with normalization of hematologic changes and body weight. The treatment with PGTF also markedly ameliorated radiologic and histopathologic changes in arthritic joint as compared with arthritic control group. The qualitative, quantitative and HPTLC analysis reveals the presence of gallotannins in PGTF which may be responsible for the proposed activity. **Conclusion:** The experimental data demonstrates that PGTF potentially ameliorates the symptoms and inhibits the progression of arthritis in experimental animals. The anti-arthritic potential of PGTF can be attributed to its hydrolysable tannin component gallotannin.

Key words: Rheumatoid arthritis, *Punica granatum*, gallotannins, Freund's complete adjuvant

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterized by pain, synovial membrane inflammation and restricted joint movement due to tissue damage. In RA inflammatory process is targeted towards the synovium which causes destruction of the articular cartilage, peri-articular bone erosion and eventual alteration of joint integrity and function (Yuan *et al*., 2012). Recent studies have demonstrated that non-enzymatic factors like Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS) and other inflammatory mediators largely contribute in the degeneration of the cartilage and bone (Hemshekhar *et al*., 2012). Therapeutic management of RA is based on two principle approaches, symptomatic and disease modifying anti-rheumatic treatment with non-steroidal anti-inflammatory agents (NSAIDS) and disease modifying anti-rheumatic drugs (DMARDS), respectively (Wang *et al*., 2011). However, severe adverse effects, potential toxicity and high cost of NSAIDS and DMARDS limit their effectiveness (Shen *et al*., 2011). Therefore, screening of new therapeutic agents from natural products which usually have less side effects and low cost have gained interests for developing new therapy for arthritis.

Punica granatum Linn. (Punicaceae) is considered "a pharmacy unto itself" in Ayurvedic medicine system, the bark and root is used as an anthelmintic and vermifuge respectively, the peels are used for treatment of colitis, headache, aphthae, diarrhea, dysentery and ulcers (Dipak *et al*., 2012). In Ayurvedic, Unani and Egyptian medicine system, pomegranate is used for treatment of

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inflammatory disorders, cough and infertility (Shukla *et al*., 2008). Studies by various researchers have reported that *Punica granatum* Linn. (Punicaceae) leaves extract possesses immunosuppressant (Lee *et al*., 2008), anti-inflammatory (Parminder *et al*., 2011) and anti-oxidant (Elfalleh *et al*., 2012) activity. Since immune system stimulation, inflammation and oxidative stress play a crucial role in the pathogenesis of RA, *Punica granatum* leaves may be helpful in management of RA. Some tannins, most of which have gallic acid and ellagic acid as their parent nucleus, such as punicalin, punicalagin, casuarinin, gallagyldilacton, pedunculagin, tellimagrandin, 1, 2, 3-tri-O-galloyl-β-glucose, 1, 2, 4, 6-tetra-O-galloyl-β-D-glucose, granatin A and granatin B present in *Punica granatum* leaves (Lansky and Newman, 2007) are considered to be its main active principles. Based on these findings, the present study was undertaken to explore therapeutic effect of *Punica granatum* L. leaves tannin fraction (PGTF) on FCA-induced arthritic rats with the aim to focus on the underlying mechanism(s) involved and to identify the phytoconstituents responsible for the proposed activity.

MATERIALS AND METHODS

Collection and identification of plant material: The leaves of the *Punica granatum* Linn. (Punicaceae) were procured from their natural habitat in the Solapur region of Maharashtra during the month of August. The plant was identified and authenticated by the Botanical Survey of India, Pune. Voucher specimen (V. no. USP-1) has been deposited in the herbarium for future reference. The leaves were shade dried without exposing them to direct sunlight and pulverized in grinder and stored in airtight container for further use.

Preparation of the tannin fraction: Crude extract was prepared by using hydroalcoholic extract (methanol: water, 70:30) by cold maceration technique. The fractionation of hydroalcoholic extract of *Punica granatum* Linn. leaves was carried out by partition coefficient method (Naczk and Shahidi, 2004). The hydroalcoholic extract was subsequently dissolved in water at 40-60°C, cooled and extracted with n- hexane in separating funnel (Chen *et al*., 2011) and was later re-extracted with dichloromethane (DCM) (Tiwari *et al*., 2011) and ethyl acetate (Das *et al*., 2012). The remaining aqueous layer was evaporated to obtain the tannin fraction, PGTF.

Preliminary phytochemical screening: PGTF was screened for the presence of various phytoconstituents like steroids, alkaloids, flavonoids and tannins (Tiwari *et al*., 2011).

Determination of total tannin content of the fraction: Total tannin content was determined by hide powder test according to the procedure described by World Health Organistaion (WHO, 1998) in which weight difference between tanned and untanned hide powder was used for quantitative determination of tannins.

High performance thin layer chromatography (HPTLC) analysis of PGTF: PGTF was analyzed for the presence of tannin by spectral comparison with co-chromatographic standard compound gallic acid. In the HPTLC procedure, PGTF at the concentration of 1 μg mL^{-1} and standard marker at the concentration of 1.2 μg mL^{-1} were spotted with the Linomat 5 (automatic applicator) syringes on the precoated aluminum plates (E-Merck) of size of 4×10 cm and flow rate was controlled using nitrogen gas cylinder. The sample and standard solutions were prepared in methanol. After application of bands, the plates were developed in twin trough chamber consisting of toluene: ethyl acetate: formic acid at a ratio of 7: 5: 1. The developed plates were dried with the help of hot hair dryer and scanned at the 560 nm with Camag U.V. scanner (Patel and Telange, 2011).

Experimental animals: Male Wistar rats (130–150 g) and female Swiss albino mice (18-22 g) were obtained from National Institute of Bioscience, Chaturshrungi, Pune. Animals were housed in standard polypropylene cages lined with raw husk. The animal house was maintained on 12 h light/dark cycle at approximately 22±2°C, relative humidity 60-70% and the animals were provided with standard laboratory diet and water *ad libitum*. The animals were randomly assigned to different groups and a minimum period of 7 days was allowed for adaptation on each experiment. The animals described as fasting were deprived of food for 24 h before experimentation but allowed free access to water throughout. The study protocol was approved by Institutional Animal Ethical Committee (IAEC) of Modern College of Pharmacy in accordance with the regulations of Committee for the Purpose of Control and Supervision of Experiments on Animals CPCSEA (884/ac/05/CPCSEA).

Drugs and chemicals: Freund's Complete Adjuvant (FCA) was obtained from Sigma-Aldrich Ltd. (Germany). Serum Glutamatic Oxaloacetic Transaminase (SGOT), Serum Glutamic Pyruvic Transaminase (SGPT) and Alkaline Phosphatase (ALP) kits were purchased from Coral Biosystems Ltd. (Goa). All standard chemicals used in the present study were freshly prepared and of analytical grade.

Acute toxicity study: Acute toxicity study was performed according to Organisation for Economic Co-operation and Development (OECD) guidelines no. 423 (OECD, 2008). Animals selected by random sampling technique were employed in this study. The

animals were fasted overnight with free access to water. PGTF was administered orally to different groups at increasing dose levels of 50, 100, 300 and 2000 mg kg^{-1} b.wt. After dosing, the animals were observed for 2 h and then intermittently for further 4 h for changes in behavioral (alertness, restlessness, irritability and fearfulness), neurological (spontaneous activity, convulsion, gait, bleeding, touch and pain response), autonomic (defecation and micturition) profiles and mortality up to 24 h till 14 days.

Experimental design

Experimental induction of arthritis: Arthritis was induced in rats according to the previously described method of Ekambaram *et al*. with slight modification (Ekambaram *et al*., 2010). Briefly, arthritis was induced by subplanter injection of 0.1 mL of FCA (heat killed *Mycobacterium tuberculosis* in sterile paraffin oil 10 mg mL^{-1}) suspension into the left hind paw. Treatment with PGTF, indomethacin and vehicle was started from the 14th day after arthritis induction and continued for 28 days.

Experimental groups: Experimental animals were divided into 6 groups, each consisting of 6 animals:

Group I: Saline treated normal control
Group II: FCA injected arthritic control
Group III: Arthritic animals treated with indomethacin (2 mg kg day^{-1})
Group IV: Arthritic animals treated with PGTF (50 mg kg day^{-1})
Group V: Arthritic animals treated with PGTF (100 mg kg day^{-1})
Group VI: Arthritic animals treated with PGTF (200 mg kg day^{-1})

Anti-arthritic activity of PGTF was determined by evaluating body weight, paw swelling and arthritic index score on day 14, 21 and 28 days, respectively. On the 28th day, ankle joint was X-rayed and urine was collected from overnight fasted rats of all groups. On the next day blood was collected by cardiac puncture and the animals were sacrificed. Thymus, liver and spleen were removed and weighed. Collected blood was allowed to coagulate at room temperature for 45 min and serum was separated using cooling centrifuge (REMI C-24BL) at 7500 rpm for 15 min. Separated serum was analyzed for change in levels of different biochemical parameters. Paw and joint were separated and processed for histological assessment.

Evaluation of physical parameters

Assessment of body weight and paw swelling: The severity of arthritis was determined by the change in body weight and paw swelling. The diameter of tibotarsal joint was measured by digital verniercalliper. The body weight and paw diameter were measured on 14th, 21st and 28th day of study.

Assessment of arthritic index: Arthritic score was evaluated on 14th, 21st and 28th day using macroscopic scoring which is carried out by independent observers using scale from 0 (no sign of arthritis), 1 (mild swelling and redness of the paw), 2 (moderate swelling and redness of the paw) and 3 (severe swelling and redness of the paw).The arthritic index was calculated by adding the scores for each individual paw (Ekambaram *et al*., 2010).

Assessment of dorsal flexion pain test: The ankle joint was gently flexed dorsally until the toes touched the front of the leg for 5 times with an inter-test interval of 5 sec. Pain was scored zero when the animal showed neither squeaking nor quick leg-withdrawal. The scores was 1 when either reaction appeared and scored 2 when both reactions appeared. All the groups were evaluated in this manner on 14th, 21st and 28th day of treatment period.

Assessment of stair climbing activity test: Overnight fasting animals were trained for one week to climb a staircase with steps at a height of 5, 10 and 15 cm having water at the second and food at the third step. Climbing ability of the rats in above groups was scored 0 if the rats did not climb; 1, if the rats climbed onto step-1; 2, if the rats climbed onto step-2 and 3, if the rat could climb all the three steps. All the groups were evaluated in this manner on 14th, 21st and 28th day of treatment period.

Assessment of motility test: The motility pattern of the rats was observed for a period of 5 min on a plane surface and scored 0, if the rat walked easily, scored 1, if rat walked with little difficulty and scored 2, if rat walked with more difficulty and avoided touching the toes of the inflamed paw to the floor. All the groups were evaluated in this manner on 14th, 21st and 28th day of treatment period (Kumar and Roy, 2009).

Evaluation of oxidative stress parameters

Estimation of serum nitric oxide (NO) concentration: Serum level of nitric oxide was measured using Griess reagent according to method described by Feng *et al*. (2001).

Estimation of serum superoxide dismutase (SOD): SOD activity was measured according to method of Marklund (1985) with slight modification. Assay mixture consisted of 2.95 mL Tris-HCl buffer, 25 μL of pyrogallol and 0.05 mL of serum in total volume of 3 mL. The difference between the optical densities obtained at 1.30 and 3.30 min was determined and expressed as U mg^{-1} protein.

Estimation of serum catalase: Catalase was measured according to method described by Sahreen *et al*. (2011) with slight modification. Briefly, sample readings were

Table 1: Effect of PGTF on paw swelling in rats with FCA-induced arthritis

Groups	Paw swelling (mm)		
	14th day	21st day	28th day
Normal control	4.70±0.20	4.7±0.21	4.7±0.20
Arthritic control	10.53±0.32##	11.10±0.37##	11.60±0.41##
Indomethacin (2 mg kg^{-1})	10.63±0.82	8.87±0.57**	7.13±0.63**
PGTF (50 mg kg^{-1})	10.47±0.48	8.09±0.28**	7.71±0.29**
PGTF (100 mg kg^{-1})	10.48±0.46	8.05±0.31**	7.46±0.29**
PGTF (200 mg kg^{-1})	10.54±0.46	7.96±0.4**	7.41±0.34**

Data is expressed as Mean±SEM (n = 6), ## and **p<0.01 as commpared to normal and arthritic control system, respectively, Data analyzed by one-way ANOVA followed by Dunnet's multiple range test for comparison, FCA: Freund's Complete Adjuvant, PGTF: *Punica granatum* L. leaves tannin fraction, ESR: Erythrocyte Sedimentation Rate

Table 2: Effect of PGTF on body weight in rats with FCA-induced arthritis

Groups	Body weight (g)		
	14th day	21st day	28th day
Normal control	178.33±4.33	182.5±5.27	184.5±5.05
Arthritic control	160.83±7.28##	147±2.16##	152.83±2.92##
Indomethacin (2 mg kg^{-1})	168.83±11.38	189.16±4.4**	188.5±6.83**
PGTF (50 mg kg^{-1})	170.5±3.36	181.83±4.6**	195.5±8.7**
PGTF (100 mg kg^{-1})	167.16±9.71	186.83±10.36**	218.83±6.18**
PGTF (200 mg kg^{-1})	161.33±10.18	193.16±4.92**	209.16±6.77**

Data is expressed as Mean±SEM (n = 6), ## and **p<0.01 as commpared to normal and arthritic control system, respectively, Data analyzed by one-way ANOVA followed by Dunnet's multiple range test for comparison, FCA: Freund's Complete Adjuvant, PGTF: *Punica granatum* L. leaves tannin fraction, ESR: Erythrocyte Sedimentation Rate

taken by placing 1 mL of phosphate buffer and 5 μL of serum in the reference cuvette and test cuvette. Hydrogen peroxide (10 μL) was then added in the test cuvette in the spectrophotometer. Reading was taken at 240 nm, 1 min after placing the cuvettes in the spectrophotometer and expressed as U mg^{-1} protein.

Biochemical parameters: The level of serum C-reactive Protein (CRP), Rheumatoid Factor (RF) and serum lysosomal enzymes such as Serum Glutamate Pyruvate Transaminase (SGPT), Serum Glutamate Oxaloacetate Transaminase (SGOT) and Alkaline Phosphatase (ALP) was determined using commercial assay kit (Coral biosystems, Goa, India). Concentration of urinary hydroxylproline in urine was measured according to Neuman and Logan (1950) and expressed in terms of mg mg^{-1} of hydroxyl proline/day.

Haematological parameters: The haematological parameters such as haemoglobin (Hb), RBC, WBC and ESR were determined by standardized laboratory method (Mythilypriya *et al*., 2008).

Radiological analysis of joints: Radiologic analysis of the joint was carried out according to the method described by Cai *et al*. (2005).

Histopathological assessment of joints: The ankle joint was subjected to histopathological study for assessment of cellular infiltration, joint space narrowing, synovial hyperplasia, pannus formation, bone and cartilage erosion according to the method described by Cai *et al*. (2007).

Statistical analysis: All the results were expressed as Mean±SEM. Data were analyzed by a one-way ANOVA, followed by Dunnet's multiple test of comparison using Graph Pad Instat (version-3) software. Value of $p<0.01$ was considered statistically significant.

RESULTS

Preliminary phytochemical analysis: Qualitative phytochemical screening of PGTF showed presence of tannins. Further, the total tannin content was found to be 80% (w/w) in PGTF.

HPTLC profile of PGTF: HPTLC analysis confirmed the presence of gallic acid (0.45 R_f value), as evident from the spectral overlay (Fig. 1).

Effect of PGTF on paw swelling: As shown in Table 1, PGTF (50, 100 and 200 mg kg^{-1}) significantly ($p<0.01$) inhibited paw swelling as compared to arthritic control group. The effect of PGTF was dose dependent from 14th to 28th day with peak effect produced at the dose of 200 mg kg^{-1} on the 28th day. This effect shown by PGTF on 28th day was similar to that produced by standard drug indomethacin (2 mg kg^{-1}).

Effect of PGTF on body weight: As shown in Table 2, PGTF (50, 100 and 200 mg kg^{-1}) significantly ($p<0.01$) inhibited loss of weight in treated rats as compared to arthritic control group. The effect of PGTF was dose dependent from 14th to 28th day with peak effect produced at the dose of 200 mg kg^{-1} at the 28th day.

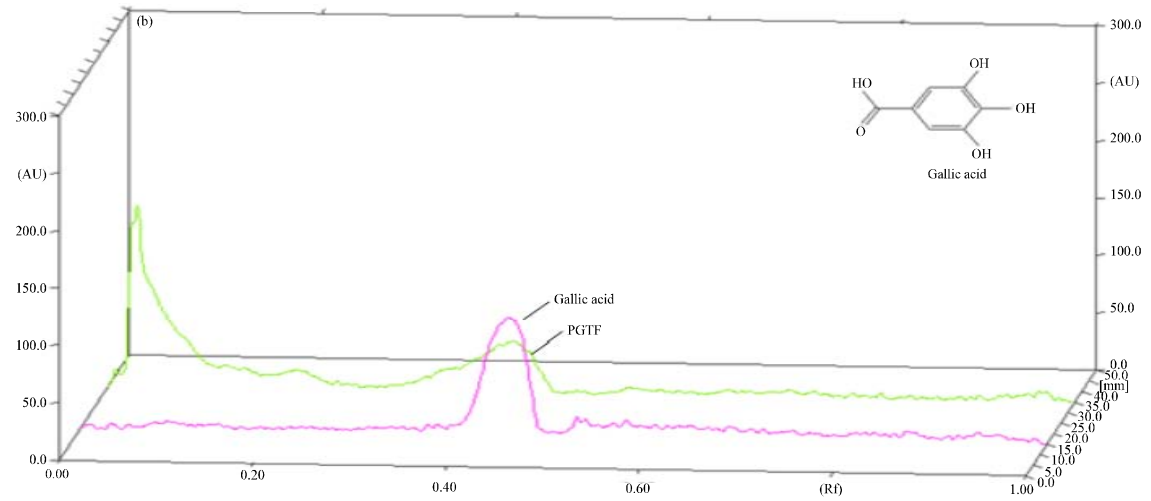

Fig. 1: HPTLC chromatographic overlay spectra of PGTF with standard biomarker gallic acid at 560 nm. PGTF: *Punica granatum* L. leaves tannin fraction

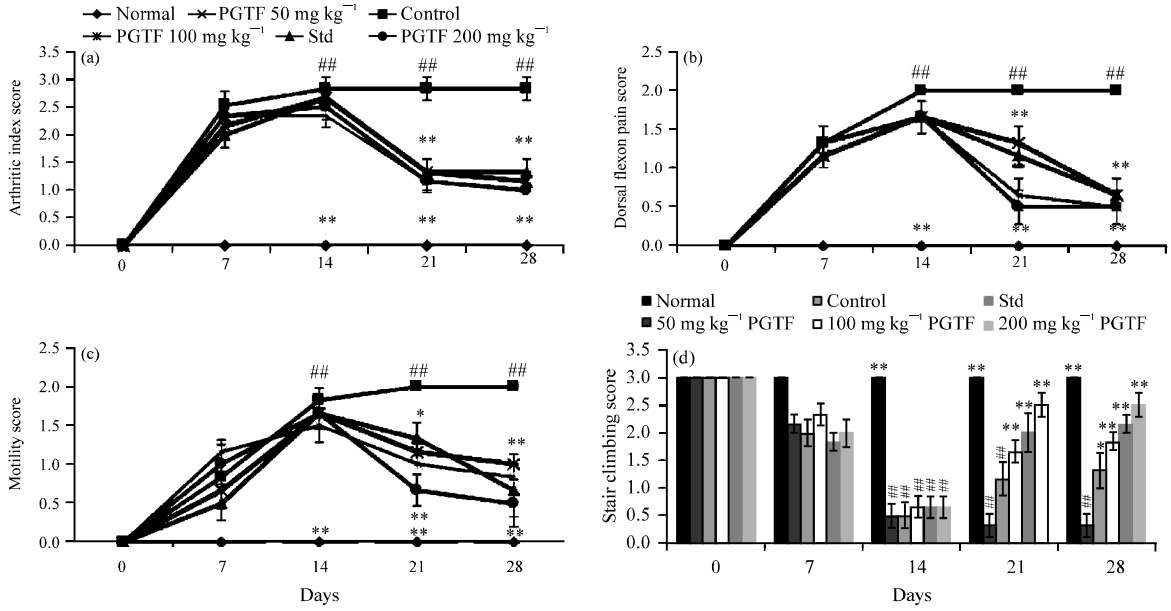

Fig. 2(a-d): Effect of PGTF on (a) Arthritic index (b) Dorsal flexion pain (c) Motility test (d) Stair climbing in FCA-induced arthritic rats. Data are expressed as Mean±SEM (n=6). #p<0.05, ##p<0.01 as compared to normal control and *p<0.05, **p<0.01 as compared to arthritic control. Data analyzed by one-way Analysis of Variance (ANOVA) followed by Dunnet's multiple test for comparison. FCA: Freund's Complete Adjuvant, PGTF: *Punica granatum* L. leaves tannin fraction

Effect of PGTF on arthritic index: As shown in Fig. 2a, arthritic index was significantly elevated in the arthritic control group as compared to normal control group. Treatment with PGTF for 4 weeks exhibited a significant ($p<0.01$) decrease in arthritic index in PGTF treated arthritic rats as compared to arthritic control group.

Effect of PGTF on dorsal flexon pain: As shown in Fig. 2b, pain conduction was significantly elevated in the arthritic control group as compared to normal control group. Treatment with PGTF for 4 weeks exhibited a significant ($p<0.01$) decreased pain sensation in FCA induced arthritic rats as compared to arthritic control group.

Table 3: Effect of PGTF on oxidative stress parameters in rats with FCA-induced arthritis

Groups	Nitric oxide (μM)	Superoxide dismutase (U mg^{-1})	Catalase (U mg^{-1})
Normal control	11.04±1.87	12.16±0.69	168.19±5.72
Arthritic control	33.49±2.47$^{\#\#}$	4.27±0.41$^{\#\#}$	92.72±7.66$^{\#\#}$
Indomethacin (2 mg kg^{-1})	19.73±1.18**	8.94±0.46**	146.07±5.64**
PGTF (50 mg kg^{-1})	24.95±1.05**	5.37±0.72	103.91±4.25
PGTF (100 mg kg^{-1})	21.90±0.78**	5.44±0.67	117.18±4.03*
PGTF (200 mg kg^{-1})	20.67±1.00**	8.16±0.58**	143.37±5.61**

Data is expressed as Mean±SEM (n = 6), $^{\#\#}$ and **p<0.01 as commpared to normal and arthritic control system, respectively, Data analyzed by one-way ANOVA followed by Dunnet's multiple range test for comparison, FCA: Freund's Complete Adjuvant, PGTF: *Punica granatum* L. leaves tannin fraction, ESR: Erythrocyte Sedimentation Rate

Table 4: Effect of PGTF on various biochemical parameters in rats with FCA-induced arthritis

Groups	RF (mg L^{-1})	CRP (mg L^{-1})	Urinary hydroxyproline (mg mg^{-1})
Normal control	-	-	10.05±2.74
Arthritic control	4.33±0.49$^{\#\#}$	2.31±0.35$^{\#\#}$	20.29±4.12$^{\#\#}$
Indomethacin (2 mg kg^{-1})	1.55±0.41**	1.61±0.14*	11.21±3.74**
PGTF (50 mg kg^{-1})	3.66±0.66	1.71±0.13*	13.37±3.37**
PGTF (100 mg kg^{-1})	2.33±0.42*	1.43±0.13**	12.17±2.99**
PGTF (200 mg kg^{-1})	1.66±0.33**	1.28±0.07**	10.34±1.61**

Data is expressed as Mean±SEM (n = 6), $^{\#\#}$ and **p<0.01 as commpared to normal and arthritic control system, respectively, Data analyzed by one-way ANOVA followed by Dunnet's multiple range test for comparison, FCA: Freund's Complete Adjuvant, PGTF: *Punica granatum* L. leaves tannin fraction, ESR: Erythrocyte Sedimentation Rate

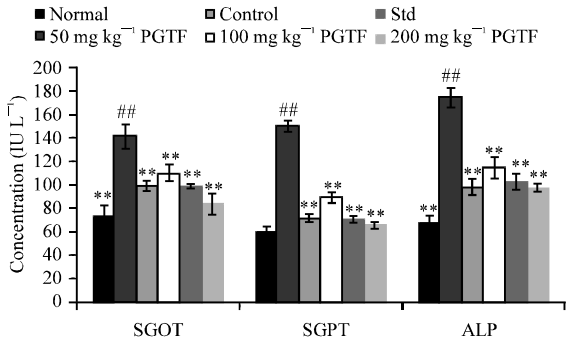

Fig. 3: Effect of PGTF on serum lysosomal enzymes concentration in rats with FCA-induced arthritis. Data are expressed as Mean±SEM (n = 6), #p<0.05, ##p<0.01 as compared to normal control and *p<0.05, **p<0.01 as compared to arthritic control. Data analyzed by one-way Analysis of Variance (ANOVA) followed by Dunnet's multiple test for comparison. FCA: Freund's Complete Adjuvant, PGTF: *Punica granatum* L. leaves tannin fraction, SGOT: Serum Glutamate Oxaloacetate Transaminase, SGPT: Serum Glutamate Pyruvate Transaminase, ALP: Alkaline Phosphatase

Effect of PGTF on motility pattern: As shown in Fig. 2c, arthritic control group shows impaired motility pattern as compared to normal control group. Treatment with PGTF for 4 weeks normalized mobility pattern of treated rats as compared to arthritic control group.

Effect of PGTF on stair climbing test: As shown in Fig. 2d, stair climbing score was significantly decreased in the arthritic control group as compared to normal control group. Treatment with PGTF for 4 weeks significantly (p<0.01) increased stair climbing score in FCA induced arthritic rats as compared to arthritic control group.

Effect of PGTF on oxidative stress parameters: As shown in Table 3, arthritis resulted in significant decrease in antioxidant enzymes like Superoxide dismutase (SOD) and catalase. Moreover the levels of Nitric oxide (NO) were significantly increased in arthritic rats. PGTF exhibited improvement in antioxidant enzymatic activity compared to arthritic control group and nearly normalized the levels of NO, SOD and catalase.

Effect of PGTF on various biochemical parameters: As shown in Table 4, RF and CRP were significantly elevated in the serum of arthritic control group as compared to normal control group. Treatment with PGTF significantly (p<0.01) reduced serum RF as compared to arthritic control group. CRP in serum was also significantly (p<0.01) reduced by PGTF treatment. Urinary hydroxyproline was significantly (p<0.01) decreased in PGTF treated arthritic rats as compared to arthritic control group.

Effect of PGTF on serum lysosomal enzymes concentration: As shown in Fig. 3, arthritic control group increase in serum lysosomal enzymes such as SGOT, SGPT and ALP in control group. Treatment with PGTF significantly (p<0.01) inhibited the serum lysosomal enzymes as compared to the arthritic control group.

Effect of PGTF on hematological parameters: As shown in Table 5, Hb and RBC increased significantly

Table 5: Effect of PGTF on hematological parameters in rats with FCA-induced arthritis

Groups	Hb (g dL^{-1})	RBC(millions mm^{-3})	WBC (thousands mm^{-3})	ESR (mm)
Normal control	15.83±0.73	8.38±0.27	11.58±0.37	3.16±0.98
Arthritic control	9.78±1.09##	4.99±0.43##	22.11±1.75##	10.66±1.85##
Indomethacin (2 mg kg^{-1})	13.9±0.56**	7.95±0.58**	14.93±0.41**	4.16±0.9**
PGTF (50 mg kg^{-1})	12.66±0.62**	6.5±0.15**	17.60±1.46**	8±1.36**
PGTF (100 mg kg^{-1})	13.46±0.56**	7.39±0.36**	17.36±0.85**	4.66±1.05**
PGTF (200 mg kg^{-1})	14.63±0.55**	7.97±0.33**	15.95±0.73**	4.33±1.05**

Data is expressed as Mean±SEM (n = 6), ## and **p<0.01 as commpare to normal and arthritic control system, respectively, Data analyzed by one-way ANOVA followed by Dunnet's multiple rangetest for comparison, FCA: Freund's Complete Adjuvant, PGTF: *Punica granatum* L. Leaves tannin fraction, ESR: Erythrocyte Sedimentation Rate

Table 6: Effect of PGTF on organ weight in rats with FCA-induced arthritis

Groups	Thymus (g)	Spleen (g)	Liver (g)
Normal control	0.42±0.02	0.76±0.02	10.3±0.38
Arthritic control	0.24±0.01##	1.13±0.07##	6.71±0.24##
Indomethacin (2 mg kg^{-1})	0.41±0.03**	0.91±0.03**	9.26±0.41**
PGTF (50 mg kg^{-1})	0.35±0.01**	0.86±0.06**	8.41±0.55**
PGTF (100 mg kg^{-1})	0.39±0.03**	0.79±0.03**	9.90±0.66**
PGTF (200 mg kg^{-1})	0.42±0.03**	0.77±0.02**	9.97±0.79**

Data is expressed as Mean±SEM (n = 6), ## and **p<0.01 as commpare to normal and arthritic control system, respectively, Data analyzed by one-way ANOVA followed by Dunnet's multiple rangetest for comparison, FCA: Freund's Complete Adjuvant, PGTF: *Punica granatum* L. Leaves tannin fraction, ESR: Erythrocyte Sedimentation Rate

(p<0.01) whereas WBC and ESR decreased steeply in arthritic control rats as compared to normal control group indicating a stimulation of immune response towards FCA in arthritic rats. Treatment with PGTF (200 mg kg^{-1}) significantly (p<0.01) inhibit the stimulation of immune response towards FCA by decreasing blood WBC, ESR along with increasing in Hb and RBC compared to arthritic control group.

Effect of PGTF on organ weight: As shown in Table 6, thymus and liver weight of FCA-induced arthritic control group was found to be significantly (p<0.05) less compared to normal control group. After 4 weeks of treatment with PGTF thymus and liver weight significantly (p<0.01) increased compared to arthritic control groups. Spleen weight was significantly high in arthritic control rats as compared to normal control rats. At the end of 28 days of treatment spleen weight of treated groups significantly (p<0.01) decreased as compared to diabetic control group.

Histopathological analysis of ankle joint: As shown in Fig. 4, histopathological study of ankle joint of arthritic control group exhibited dense cellular infiltration, joint space narrowing, synovial hyperplasia, pannus formation and cartilage erosion. Treatment with PGTF reduced infiltration of inflammatory cells, joint space narrowing, synovial hyperplasia, pannus formation and cartilage erosion as evidenced from the histopathology sections of PGTF treated rats.

Radiological study of ankle joint: As shown in Fig. 5, radiological study of ankle joint of arthritic control group exhibited soft tissue swelling and bone erosion. Treatment with PGTF reduced swelling of soft tissue and bone erosion as evidenced from the X-rays of PGTF treated rats.

Acute toxicity study: Acute toxicity study revealed the non-toxic nature of the PGTF fraction. There was no lethality or any toxic reaction in animals at a single large dose of 2000 mg kg^{-1}. No mortality was recorded within the 14 days of observation.

DISCUSSION

Freund's Complete Adjuvant (FCA) induced arthritis in rats is widely used experimental model for inflammatory arthritis sharing several features of human rheumatoid arthritis (Phadke *et al*., 1985). The subplanter injection of FCA in rat paw results in generation of inflammatory arthritis by acute periarticular inflammation with synovial mononuclear infiltration followed by synovial hyperplasia and damage to particular bone and cartilage just as in the case of arthritis in human (Kaur and Sultana, 2012). Treatment with PGTF significantly inhibited the progression of arthritis by suppressing inflammation, leukocyte infiltration, cartilage destruction and bone damage.

In rheumatoid arthritis (RA), inflammatory mediators stimulate inflammation of the synovial tissues which causes soft tissue swelling along with fluid exudation and cellular influx in the synovium (Jin *et al*., 2010). Results showed that PGTF at higher dose reduced soft tissue swelling in arthritic rats which indicated that PGTF suppress inflammation and thus provide symptomatic relief in arthritis. The ability of PGTF to suppress inflammation was further evaluated by arthritic score. Arthritic score depends on severity of ankle joint

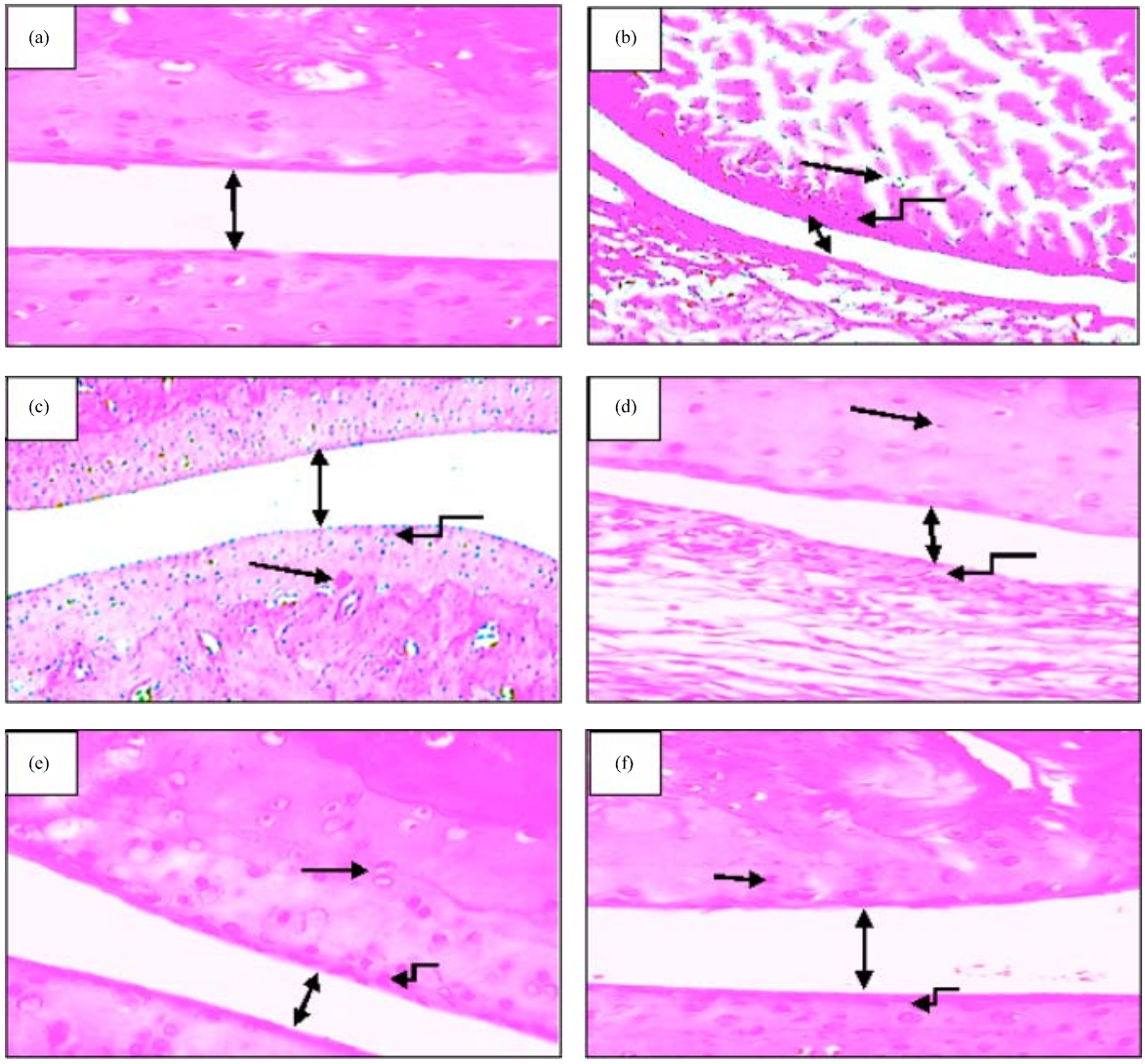

Fig. 4(a-f): Effect of PGTF on histopathology of FCA-induced arthritic rats, group showing joint space narrowing (↔), pannus formation (↩) and cell infiltration (→), (a) Normal control, (b) Arthritic control, (c) Indomethacin, (d) PGTF (50 mg kg^{-1}) (e) PGTF (100 mg kg^{-1}) and (f) PGTF (200 mg kg^{-1}), FCA: Freund's Complete Adjuvant, PGTF: *Punica granatum* L. leaves tannin fraction

swelling. Arthritic score is used for clinical assessment of joint swelling in rheumatoid arthritis (Patel *et al*., 2012a). In the present study results showed that, treatment with PGTF in arthritic rats significantly decreased arthritic score by inhibiting inflammatory response of rheumatoid arthritis. Production of inflammation in FCA induced arthritis is associated with pain and motor dysfunction (De Castro Costa *et al*., 1981). As a result of hyperalgesia induced by inflammation, the animals exhibited squeaking and leg withdrawal on flexion of the inflamed joint. Treatment of arthritic rats with PGTF showed a significant decrease in dorsal flexion pain and motility score by inhibiting pain associated with inflammatory response exhibited by FCA. Thus, PGTF may have the potential as a therapeutic agent and can be used for symptomatic treatment of rheumatoid arthritis because of its anti-inflammatory action which delays progression of disease along with pain.

Rheumatoid factor (RF) is increased in diffuse collagen disease and positively observed in 80% of rheumatoid arthritis patients (Kim, 1984). RF autoantibody such as IgM and IgA are the key pathogenic

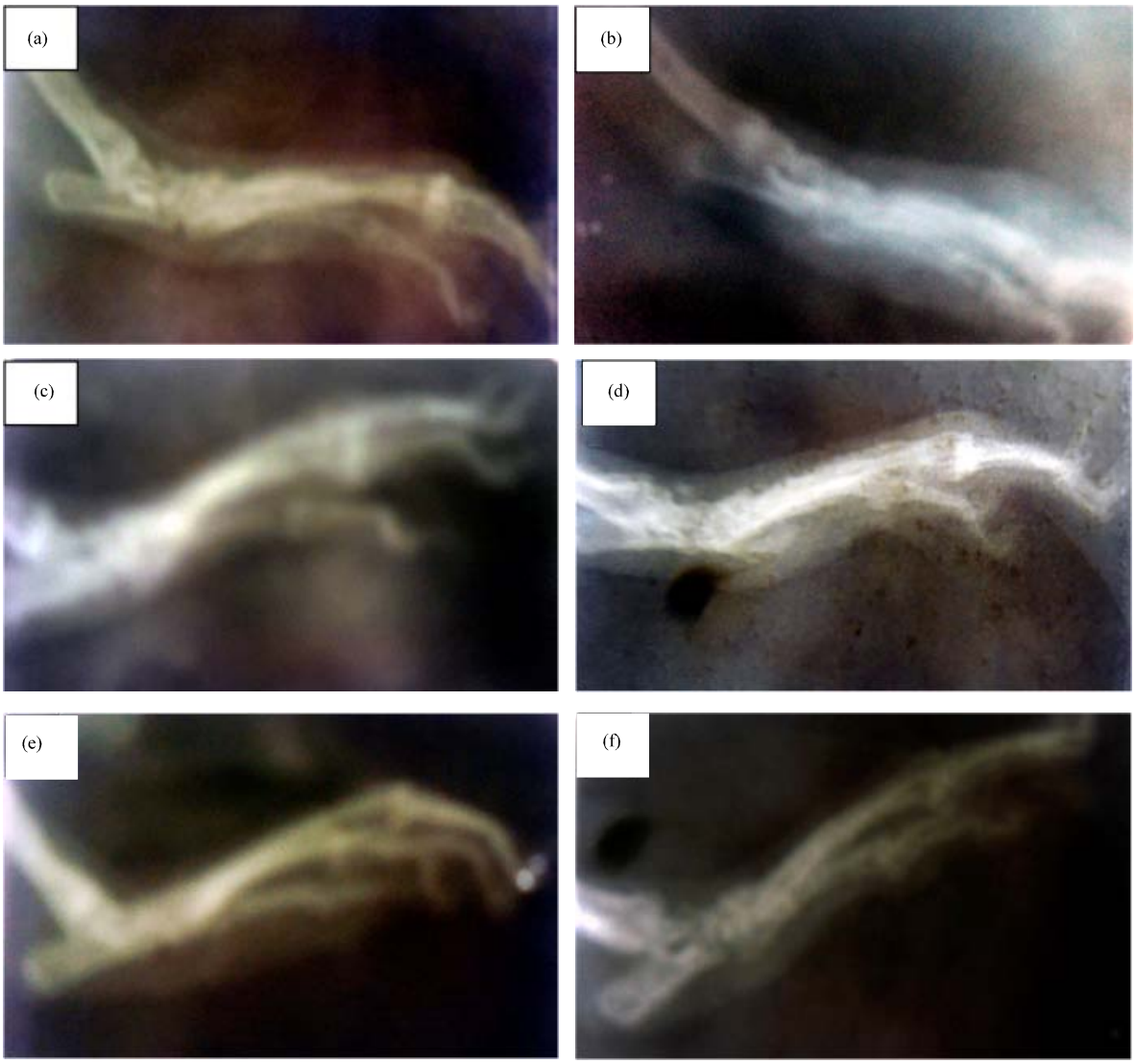

Fig. 5(a-f): Effect of PGTF on radiology of ankle joint in FCA-induced arthritic rats, (a) Normal control, (b) Arthritic control, (c) Indomethacin, (d) PGTF (50 mg kg^{-1}) (E) PGTF (100 mg kg^{-1}) and (f) PGTF (200 mg kg^{-1}). FCA: Freund's Complete Adjuvant, PGTF: *Punica granatum* L. leaves tannin fraction

markers triggered against Fc fragment of IgG and citrullinated peptides in arthritis due to progressive joint destruction (Van der Linden *et al*., 2009). In the present study, PGTF treatment in arthritic rats significantly reduced level of serum RF and exhibits anti-arthritic activity probably mediated by suppressing generation of autoantibody towards Fc fragments and protecting cartilage degradation. It is previously reported that concentration of C-reactive protein (CRP) in the blood positively correlates disease severity and progression of rheumatoid arthritis similar to rheumatoid factor (Jung *et al*., 2005). In rheumatoid arthritis, CRP can bind with various Fc receptors by forming complement activating complexes which generate antibody towards Fc fragment and causes cartilage degradation (Jones *et al*., 2012). PGTF treatment in arthritic rats reduced level of serum CRP as compared with arthritic rats which confirms that PGTF exhibits anti-arthritic activity by suppressing generation of autoantibody towards Fc fragments and protecting cartilage degradation.

It is reported that hydroxyproline is the product of excessive catabolism of collagen and cartilage matrix glycoprotein and is excreted in urine (Chakraborty *et al*.,

2010). In the present study, evaluation of urinary hydroxyproline was carried out to determine the protective role of PGTF in degradation of collagen and cartilage matrix glycoprotein. Oral treatment with PGTF in arthritic rats restored excessive secretion of urinary hydroxyproline which suggest that PGTF suppresses the progression of disease by inhibiting cartilage degradation. Oxidative stress inflicts damage to joints because of excessive generation of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) in rheumatoid arthritis (Phillips *et al*., 2010). Generation of superoxide radicals in body results in collagen degradation that may trigger other inflammatory reactions and tissue destruction through activation of neutrophils. In present study, the increase in systemic and local oxidative stress is verified by marked increase in reactive oxygen species (ROS) such as Superoxide Dismutase (SOD) and catalase along with reactive nitrogen species (RNS) such as nitric oxide in serum. In case of ROS generation, superoxide dismutase (SOD) is one of the key anti-oxidant enzymes which protect cells from toxic superoxide radicals (Sharma *et al*., 2011). Catalase is another anti-oxidant enzyme that protects cells from superoxide radicals by interacting endogenous H_2O_2 and liberating water and oxygen (Karakoc *et al*., 2007). Results of the present study demonstrated that, PGTF treatment in arthritic rats reduced serum level of SOD and catalase, which suggests that PGTF exhibits anti-arthritic activity probably mediated by inhibiting generation of ROS in FCA-induced arthritic animals. Previous reports have shown that macrophages secrete inducible nitric oxide synthase (iNOS) involved in production of large amount NO (Ignarro, 2002). Since the adhesion of neutrophils to the endothelial cells is mediated by NO and acts as a pro-inflammatory mediator in arthritic inflammation, its assessment is of importance for evaluation of anti-arthritic activity (Kubes *et al*., 1991). In the present study, significant reduction in serum NO level was observed in FCA- induced arthritic rats after treatment with PGTF. Thus, PGTF have the ability to inhibit the release of ROS and RNS associated with the oxidative stress in FCA-induced arthritic animals.

It is reported that the subplanter injection of FCA in rat paw results in generation of inflammatory arthritis (Patel *et al*., 2012b). Lysosomal membrane degradation by inflammatory stimuli causes secretion of lysosomal contents such as Serum Glutamate Oxaloacetate Transaminase (SGOT), Serum Glutamate Pyruvate Transaminase (SGPT) and Alkaline Phosphatase (ALP) in rheumatoid arthritis (Chayen and Bitensky, 1971). Results of the present study demonstrated that, PGTF treatment in arthritic rats significantly reduced level of serum lysosomal enzymes in dose dependent manner and exhibits anti-arthritic activity probably mediated by stabilizing lysosomal membrane associated with arthritic inflammation.

To confirm the involvement of immune-suppressant mechanism in the anti-arthritic activity of PGTF, hematological parameters such as hemoglobin (Hb), red blood cells (RBC), white blood cells (WBC) and erythrocyte sedimentation rate (ESR) were evaluated. Anemia is most common extra cellular manifestation formed due to decrease in Hb and RBC level in rheumatoid arthritis. The most important cause of anemia might be the sequestering of iron in the reticuloendothelial system and synovial tissue which results in decrease in level of plasma iron that leads to failure of bone marrow to respond to anemia (Mowat, 1971). Results demonstrated that PGTF treatment in arthritic rats significantly, increased Hb level and RBC count in arthritic rats. The increase in total WBC count in arthritic rats might be due to the stimulation of immune system against the pathogenic microorganisms which is evidenced by the infiltration of inflammatory mononuclear cells in the joint of arthritic rats (Maria *et al*., 1983). ESR is an indirect measurement used for determining the severity of rheumatoid arthritis which is influenced by plasma concentration of fibrinogen, immunoglobulins, RF and Hb (Skogh *et al*., 2003). In the present study PGTF treatment in arthritic rats significantly decreased WBC count and ESR level in arthritic rats. Treatment with PGTF increased level of Hb, RBC and decreased level of WBC, ESR suggest that anti-arthritic activity of PGTF probably mediated by immune-suppressant mechanism.

Spleen is a vital organ involved in immune responses and serves as the reservoir for the cells and antibody formation. It is also reported that, increase in spleen weight in FCA-induced arthritis is associated with the splenomegaly, generalized lymphadenopathy and alteration of hepatic function (Patil *et al*., 2012). PGTF treatment in arthritic rats reduced spleen weight and increased liver weight as compared with non-treated arthritic rats which confirmed that PGTF exhibits anti-arthritic activity by immune-suppressant mechanism. Further thymus atrophy is reported in the FCA-induced arthritis via the pituitary-adrenal axis suppression which is well known side effect of steroidal anti-inflammatory drugs (Dujovne and Azarnoff, 1975). In the present study treatment of arthritic rats with PGTF ameliorates thymus atrophy which suggested that PGTF act by different inhibitory mechanisms than steroidal anti-inflammatory drugs.

Moreover, protective effect of PGTF in progression of joint damage was further confirmed by the histopathological and radiological study of ankle joint. In

the present study, ankle joint histopathological sections of untreated arthritic rats showed dense cellular infiltration, synovial hyperplasia along with pannus formation. Treatment with PGTF in arthritic rats showed reduced cellular infiltration, synovial hyperplasia and pannus formation in ankle joint, which suggest that PGTF can effectively block the disease progression in arthritic rats. In this study, for evaluation of joint damage in arthritic rats, radiographic examination of ankle joint was carried out. Ankle joint X-rays of untreated arthritic rats was showed excessive soft tissue swelling and bone erosion. Treatment with PGTF in arthritic rats showed reduced soft tissue swelling and bone erosion in X-rays of ankle joint, which confirmed that PGTF can effectively block the disease progression in arthritic rats.

It is clear from the results that PGTF possess significant anti-arthritic activity in experimental animals, we also carried out qualitative, quantitative, thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC) analysis of PGTF to identify the phytoconstituents responsible for the proposed activities. Qualitative phytochemical analysis illustrated that there is presence of hydrolysable tannins in PGTF fraction. Total tannin assay was employed for quantitative analysis of PGTF, which showed 80% (w/w) of tannins. It is previously reported that *P. granatum* Linn. (Punicaceae) contains hydrolysable tannins including ellagitannins and gallotannins. Ellagitannins are mainly found in the pericarp, bark, seeds and flowers however practically undetected in the leaves (Wang *et al*., 2006). Gallotannins, which are mostly found in the leaves of pomegranate, consist of a couple of galloyl groups and therefore can be considered as the derivatives of gallic acid (Wang *et al*., 2010). Therefore gallic acid was selected as co-chromatographic standard for standardization of fractionation protocol. TLC and HPTLC analysis of PGTF in the present study showed the presence of gallic acid which suggests presence of hydrolysable gallotannins in PGTF fraction.

Thus the present study suggests that PGTF has a good therapeutic action on symptoms of arthritis in FCA-induced arthritic rats. The anti-arthritic potential of PGTF is mediated through multiple mechanisms viz., immune-suppressant, anti-inflammatory and anti-oxidant activity. Treatment of PGTF in arthritic rats also demonstrates improvement in structural and functional integrity of ankle joint. Thus, it can be concluded that PGTF possess significant anti-arthritic activity which is due to its hydrolysable tannin component gallotannins.

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