

## Original Research Article

# Effect of ethanol extract of *Punica granatum* L against Freund's complete adjuvant-induced arthritis in rats

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### Abstract

**Purpose:** To investigate the protective effect of ethanol extract of *P. granatum* against arthritis in rat model.

**Methods:** Twenty-six adult male Wistar rats (120 - 150 g) were separated into four groups (n = 6): normal control, arthritic control and two treatment groups. With the exception of normal control group, arthritis was induced by intraplantar administration of Freund's complete adjuvant (FCA) on the 1st day of drug administration. The arthritic control group was not treated, while the treatment groups received extract orally at 500 or 750 mg/kg for the period of 4 weeks and at the end of each week, paw volume, thermal hyperalgesia, arthritic score and mechanical nociceptive threshold were performed to assess arthritis. Biochemical indicators and inflammatory cytokines in serum were determined using standard procedures.

**Results:** There was significant decrease in paw volume and arthritic score; paw withdrawal latency was enhanced in extract-treated groups, compared to arthritic control group ( $p < 0.05$ ). Furthermore, ALT, AST and ALP levels, as well as RF and MDA activities decreased significantly with extract treatment, compared with arthritic control group ( $p < 0.05$ ). Treatment with the extract attenuated the altered level of interleukin 1 $\beta$  (IL-1 $\beta$ ) and TNF- $\alpha$  levels in arthritic rats. Histological examination showed that treatment with the extract significantly reversed histological changes induced by arthritis.

**Conclusion:** The results reveal that the beneficial effect of ethanol extract of *P. granatum* against FCA-induced arthritis is due to its ability to reduce the levels of inflammatory cytokines.

**Keywords:** *Punica granatum*, Rheumatoid arthritis, Cytokines, Enzymes, Inflammation

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## INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disorder which affects about 1 % of the world's population [1]. It is a systemic, inflammatory, symmetric and chronic disorder characterized by rheumatoid pannus and articular inflammation which leads to destruction of bones and

cartilages [2,3]. Although the pathogenesis of RA has not been fully elucidated, it is believed to be caused by immune reaction in the synovial joint due to persistent infection of the joint [4]. Early symptoms of RA include pain and swelling around the joint and joint dysfunction, while the late stage of the disease is accompanied by disability and bone damage as a result of joint

deformity and stiffness [5]. Anti-inflammatory and analgesic drugs used in the treatment of RA serve as mere palliatives. Anti-CD20 and anti-TNF- $\alpha$  therapies are used to control immune responses in RA [6,7]. At present, drugs used for treating RA produce serious side effects. Therefore, the use of alternative medicine for long-term management of RA has a great prospect.

*Punica granatum* L, also known as pomegranate, is a fruit-bearing deciduous shrub in the family *Punicaceae*, and it is used traditionally for the management of several diseases [8]. Extracts of *P. granatum* have been shown to possess immunomodulatory, antioxidant, antifungal, bactericidal and estrogenic activities [9-12]. However, the effect of *P. granatum* extract on RA has not been reported.

The aim of present investigation was to determine the beneficial effect of extract of *P. granatum* against arthritis.

## EXPERIMENTAL

### Experimental rats

Male albino wistar rats weighing (120 - 150 g) were purchased from Shanghai Animal House, China. Animals were stored as per the guideline (Humidity: 48%; Temperature: 25°C and 12 h light/dark cycles). Study on the animal performed in the study was approved by the Institutional Animal Ethical Committee of Dezhou People Hospital, China (approval no. DPH/IAEC/2017/02) and followed the guidelines of Association for the Assessment and Accreditation of Laboratory Animal Care International [13].

### Preparation of plant extract

The peels of *P. granatum* were obtained from a local vendor, shade-dried and then pulverized using an electric blender. A portion of the powder (150 g) was exhaustively extracted with 400 ml of absolute ethanol in a soxhlet apparatus. Vacuum rotatory evaporator was used to concentrate the extract, and the resultant concentrate was freeze-dried by lyophilization.

### Experimental design

Twenty-six adult male Wistar rats were separated into four groups (n=6): normal control, arthritic control group and two treatment groups. With the exception of normal control group, arthritis was induced in the rats using Freund's complete adjuvant (0.1 ml) which was injected on

the first day of treatment in the intraplantar region of the left hind paws of the rats. Inactivated *Mycobacterium tuberculosis* (10 mg) was dried in 1 ml of paraffin oil for the preparation of Freund's complete adjuvant. The arthritic control group was not treated, while the treatment groups received 500 or 750 mg/kg bwt extract/day p.o. for 28 days.

### Assessment of the development of arthritis

Development of arthritis was assessed in the rats by paw volume, thermal hyperalgesia, arthritic score, mechanical nociceptive threshold on days 0, 7, 14, 21 and 28 of treatment using a plethysmometer. Arthritis score was determined as per the reported method [14].

### Collection of blood and tissue samples

After 4 weeks of treatment, rats were anesthetized and collection of blood was performed from retro-orbital plexuses. Serum was separated out by centrifuging the blood for 10 min at 3000 rpm for biochemical analyses. The rats were euthanized by cervical dislocation and their ankle joints were excised for histopathological examination.

### Biochemical analysis

Serum activity of alanine amino transaminase (ALT), Aspartate aminotransferase (AST), alkaline phosphatase (ALP), malondialdehyde (MDA) and arthritis factor (RF) were estimated using their respective assay kits, while the levels of TNF- $\alpha$  and IL-1 $\beta$  were assessed by using ELISA kits.

### Tissue histology

Histological examination of the right hind limb was performed using hematoxylin and eosin (H & E) staining. Histopathological changes were assessed based on the degree of inflammatory cell infiltration, underlying bone destruction and articular cartilage damage. The extent of cartilage damage was also determined using Mankin score.

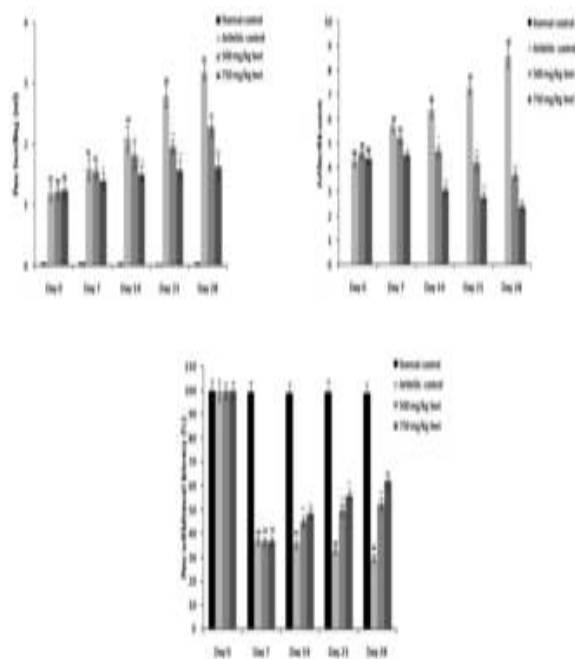
### Statistical analysis

Data are represented as mean  $\pm$  SD, and the Graph Pad Prism (6.1) software was used for the statistical analysis. Groups were compared using Dunnett's post hoc multiple test range. Statistically significance considered for the value having  $p < 0.05$ .

## RESULTS

### *P. granatum* extract ameliorates the development of RA

There were significant increases ( $p < 0.05$ ) in paw swelling and arthritic score, and significant decrease in paw withdrawal latency in arthritic control group than normal control group. However, after treatment with the extract, changes in these parameters were significantly and dose-dependently reversed (Figure 1).



**Figure 1:** Extract of *P. granatum* attenuates paw swelling, paw withdrawal latency and arthritic score in the rats.  $^{##}P < 0.05$ , than normal control group;  $^{**}p < 0.05$ , than arthritic control group

### Effect of *P. granatum* extract on the levels of some marker enzymes, RF and MDA

The activity of marker enzymes and values of RF and MDA were enhanced in arthritic control group than to normal control group. However, treatment with the drug attenuates the altered levels of these parameters in arthritis rats (Table 1).

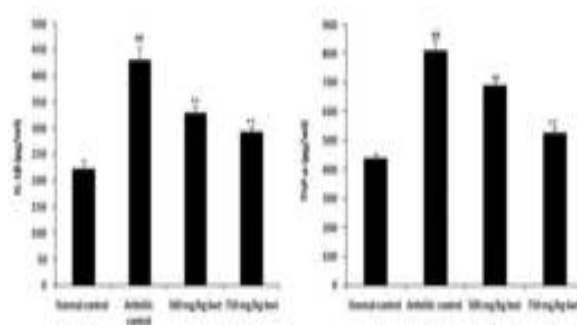
**Table 1:** Extract of *P. granatum* attenuates some marker enzymes and MDA level in arthritis rats

Group	ALT(U/L)	AST (U/L)	ALP (U/L)	MDA (nmol/ml)	RF (IU/ ml)
Normal control	43.72±3.16	41.48±2.17	79.23±3.61	3.16±0.21	0.00 ± 0.00
Arthritic control	181.40 ±11.83 <sup>a</sup>	147.33±12.29 <sup>a</sup>	437.3±23.10 <sup>a</sup>	7.59±0.37 <sup>a</sup>	86.94±3.72 <sup>a</sup>
500 mg/kg extract	139.60 ±6.26 <sup>ab</sup>	113.27±9.12 <sup>ab</sup>	329.1±14.70 <sup>ab</sup>	6.28±0.27 <sup>ab</sup>	47.31±2.42 <sup>ab</sup>
750 mg/kg extract	93.71±7.29 <sup>abc</sup>	89.38±4.91 <sup>abc</sup>	186.4±11.30 <sup>abc</sup>	4.49±0.23 <sup>abc</sup>	29.73±1.33 <sup>abc</sup>

<sup>a</sup>  $P < 0.05$  than normal control group; <sup>b</sup>  $p < 0.05$  than arthritic control group; <sup>c</sup>  $p < 0.05$  than 500 mg/kg group

### Effect of ethanol extract of *P. granatum* on levels of inflammatory cytokines

Figure 2 shows that the levels of TNF- $\alpha$  and IL-1 $\beta$  were significantly enhanced up to 431 and 810 pg/ml respectively in arthritic control group, but were significantly and dose-dependently reduced ( $p < 0.05$ ) after treatment with extract.

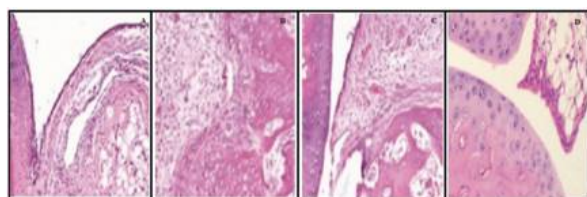


**Figure 2:** Effect of ethanol extract of *P. granatum* on serum levels of inflammatory cytokines.  $^{##}P < 0.05$  than normal control;  $^{**}p < 0.05$  than arthritic control

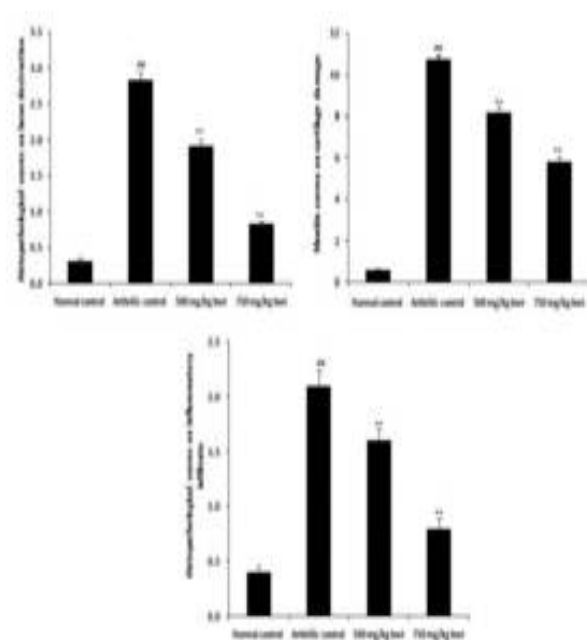
### Effect of ethanol extract of *P. granatum* on histology of knee joint

Tissue section of knee joint of normal control rats revealed clearly visible articular cavity and smooth articular cartilage surface without pathological alteration or infiltration of inflammatory cells in the synovium. However, histopathological changes such as hyperplasia of capillary, infiltration of inflammatory cells, changes in the structure of sub-synovial collagen fiber and thickening of extracellular matrix were observed in the arthritic control group.

The pathological changes induced by RA were markedly mitigated on treatment of the arthritic rats with ethanol extract of *P. granatum*. Results of Mankin score showed that the extract significantly mitigated cartilage damage caused by arthritis. Bone destruction and infiltration of inflammatory cells were significantly alleviated in extract-treated groups than arthritic control group (Figure 3).



(I)



(II)

**Figure 3:** Effect of ethanol extract of *P. granatum* on the histology of knee joints of the rats. I: Photomicrographs of tissue sections. **A:** Normal control group; **B:** Arthritic control; **C:** 500 mg/kg bwt group; and **D:** 750 mg/kg bwt group. II. Histopathological scores for bone destruction, cartilage damage and inflammatory infiltrate.  $^{###}P < 0.05$  than normal control;  $^{**}p < 0.05$  than arthritic control

## DISCUSSION

Rheumatoid arthritis is an autoimmune disorder of joint with the symptom of cartilage deterioration & inflammation of synovium [2]. Symptoms of RA include dyskinesia and arthralgia of limb joint.

Autoimmunity may occur as a result of imbalance between humoral immunity and cellular immunity [15]. The levels of inflammatory cytokines are significantly elevated in cellular immunity when compared with humoral immunity [16]. Circulatory cytokines in the synovium are one of the parameter contribute in the development of RA [17].

The present study investigated the protective activity of ethanol extract of *P. granatum* in FCA induced arthritis in rats. The results showed that

there were significant increases in swelling in paw & arthritic score, and significant decreases in paw withdrawal latency in arthritic control group, when compared with the normal control group. However, after treatment with the extract, changes in these parameters were significantly and dose-dependently reversed.

Arthritis is characterized mainly by swelling of the synovium due to the proliferation of synovial cells, and it has been reported that serum activities of ALP, ALT and AST are elevated in arthritis due to hepatic lesions [18]. These enzymes are used as markers for the localization of bone loss in bone resorption and formation [19]. Malondialdehyde (MDA) is an important index of lipid peroxidation since most diseases are accompanied by elevated MDA levels. In this study, the activity of ALP, AST & ALT, and values of RF and MDA were significantly higher in arthritic control group than in normal control group. However, treatment with the extract significantly and dose-dependently reduced the activities and levels of these parameters.

Cytokines are involved in the pathogenesis of RA [20]. They increase erosion of bone, degradation and destruction of articular cartilage, and inflammation. Anti-inflammatory drugs used in the management of arthritis reduce the production of cytokines. It has been reported that the expression of TNF- $\alpha$  is significantly upregulated in arthritic mice [21]. In RA, inflammatory cytokines inhibit the synthesis of bone, stimulate osteoclastic action and destroy cartilage collagen. In this study, the levels of IL-1 $\beta$  and TNF- $\alpha$  were significantly increased in the arthritic control group, relative to normal control group, but were significantly and dose-dependently reduced after treatment with the extract. Histopathological examination also showed that treatment with the extract significantly reversed histological changes induced by arthritis.

## CONCLUSION

The findings of this study that the protective effect of the ethanol extract of *P. granatum* against arthritis is due to its ability to reduce the levels of inflammatory cytokines.

## DECLARATIONS

### Acknowledgement

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### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yanming Wang and Tao He has done the experimental work and contributed equally for the presented work. Zhiming Li designed and supervises the work and writes the manuscript, Shujun Gai performed statistical analysis for this work.

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