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Research Article

The Prophylactic Effects of Pomegranate Peel in a Rat Model of Rheumatoid Arthritis: Study on Arthritis Score and Expression of Inflammatory Markers'

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ABSTRACT

Rheumatoid arthritis (RA) is a joint disease, chronically cause permanent joint impairment leading to inability of daily life activities. Early diagnosis and preventive managements of RA are recommended to overcome the disease. The current drugs provide benefits for RA patients, unfortunately could not be used for long period and as prevention agents, due to the adverse effects. Even though clinical and laboratory studies of pomegranate for osteoarthritis and RA had been done, the effects of the pomegranate peel on MMP-9, TNF-α, and IL-6 of RA are still unrevealed. TNF-α promotes inflammation process in RA and collaborates with osteoclasts to trigger osteoporosis. IL-6 shows negative effect on osteoblasts differentiation and MMP-9 stimulates cartilage degradation and inflammation mediated by synovial fibroblast. This study measured TNF-α, IL-6, MMP-9, and arthritis score (AS) of RA rats treated with ethanolic extract of pomegranate peel (EPP) to evaluate its potency as a RA prevention agent. Male Lewis rats (three groups, five each), 200 g, received 80 mg, 160 mg, and 320 mg of EPP/rat respectively, in alternate day, within 60 days. On the 30th day, the rats were subcutaneously injected with 0,1 ml mycobacterium-complete Freund's adjuvant (1 mg/mL) on plantar of the right hind paws to induce RA. Serum IL-6 and TNF-α were determined by ELISA. Immunohistochemistry processed-synovial MMP-9 slices of ankle joints were evaluated by light microscope (400× magnification). Arthritis score of Smit was used to determined AS. Data were analyzed by Kruskall Wallis, Mann Whitney U, and Pearson correlation test. $p < 0.05$ was significant. The EPP of 320 mg corrected serum TNF-α and IL-6, and synovial MMP-9 of RA rats ($p < 0.05$). No significant change was observed in arthritic score following the EPP treatment ($p > 0.05$). In conclusion, the results indicate the EPP may potential to be developed as preventive agent of rheumatoid arthritis.

Keywords: Arthritic score, IL-6; MMP-9, Pomegranate, TNF-α

Introduction

Rheumatoid arthritis (RA) is a disease characterized by chronic inflammation of the synovial of small joints including hands and feets. Pathogenesis of the disease was based on autoimmune process linked to MHC class II-HLA-DR4 and protein tyrosine phosphatase, non-receptor type (PTPN) protein of T-cell [1]. Cartilage damages of the joints are reported to be a main cause of permanent joints destruction leading to physical impairment [2]. Concerning prevention efforts of progressive disability and permanent destruction of the joint early diagnosis and effective treatment are needed [3, 4]. Accordingly, it is urgent to find a prophylactic agent for RA. To date the therapeutic agents of the disease are corticosteroids, disease modifying antirheumatoid drugs (DMARD), and nonsteroidal antiinflammation drugs (NSAID). There are two types of DMARD namely methotrexate and biological drugs. The latter are

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reported to improve clinical signs and radiographic images of RA better than the methotrexate [5]. Nine biological drugs have been approved by Food and Drug Administration of USA and Medicines Agency of European as therapeutic agents of RA. Biological drugs were administered for patients with moderate to severe RA or for them with no improvement of illness after being treated with conventional drugs [6]. Nevertheless, systematic and meta-analysis studies reported the prevalence of serious infection risk in RA patients is associated with the biological drugs medication [7]. Interleukin-6 and TNF-α show dominant hierarchy in the pathogenesis of RA during acute as wells chronic phases [8]. A study on human synovial fibroblast showed MMP-9 stimulates cartilage degradation and inflammation mediated by synovial fibroblast [9]. Serum MMP-9 elevations were detected in patients with RA [10]. A systematic review on studies of RA patients reported oxidative stress involves in pathogenesis of RA [11]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor maintaining the defense system of the cells against oxidative stress. Nrf2 of the joints was activated in the arthritic mice and RA patients. Nrf2-knockout mice showed massive cartilage injuries and oxidative damage during AIA [12]. Living systems generates ROS, oxygen-derived radicals including hydroxyl radicals (·OH), superoxide radical ($O_2^{-}\cdot$), perhydroxyl radical ($HO_2\cdot$), peroxyl radical (ROO·), hydrogen peroxide (H_2O_2), and singlet oxygen (O_2), the latter two are non-free radical species easily converted into free radicals. Reactive nitrogen species (RNS) is another radical produced by living system, such as Peroxynitrite ($OONO^-$), Nitric oxide (NO·), and nitrogen dioxide ($NO_2\cdot$) [13]. Excessive production of ROS and RNS during any conditions including inflammation process in RA results in oxidative stress, a condition that the amount of ROS and RNS exceed that of antioxidants neutralizing the radicals. This condition leads to cartilage and bone destruction [14].

Pomegranate (*Punica granatum* L) possesses therapeutic effects on chronic inflammatory diseases including rheumatoid arthritis (RA) and is rich in polyphenol leading to antioxidative properties of the plant parts [15]. The pomegranate peel is approximately 60% of the fruit weight and contains caffeic acid, gallic acid, ellagic acid, kaempferol, querceine, punicalin, punicalagin, luteoline, ellagitannins, pelletierine alkaloids, and minerals such as Na, Mg, Ca, Mg, P, and K [16]. The peels have been reported to have pharmaceutical activities as antioxidative, hepatoprotective, nephrotoxicity protective, antidiarrheal, antibacterial, antifungal, antimalarial, and hypolipidemic agent [17].

The present study evaluated the potency of 80% ethanolic extract of pomegranate peel (EPP) as prevention agent of RA by measuring TNF-α and IL-6 of serum, synovial MMP-9 of ankle joints, and arthritis score of RA rat model. As far as we concern the simultaneous effects of EPP on these four parameters in RA rats have not been revealed yet. This study used mycobacterium-complete Freund adjuvant (m-CFA) to induce rheumatoid arthritis in rats) [18].

Material and Methods

Ethanolic extract of pomegranate peel (EPP) preparation

Pomegranates were obtained from The Materia Medica of the East Java provincial Health Office, certificate No. 074/705/101.8/2015. Pomegranate peel powder was soaked in 80% ethanol for 48 hours. The filtrate was put in a rotary evaporator (at 60°C) to evaporate the ethanol. The alcohol evaporation was facultatively continued using water bath (at 50°C) for 12 hours. Finally, the filtrate (EPP) was stored at room temperature for another 12 hours. The EPP was mixed with 0,5 % of sodium carboxymethylcellulose (Na-CMC) suspension (in water). The present study prepared 3 kinds of doses namely; 320 mg, 160 mg, and 80 mg of the EPP (each dose in 1 ml of 0.5 % Na-CMC). The doses were adopted and modified from Sari *et al.* (2010) [19], she and her colleagues used EPP doses of 80 mg, 40 mg and 20 mg/200 g BW for rats.

Rheumatoid-arthritic rats preparation and arthritis score (AS) measurement.

Five groups of male Lewis rats (five each), age 8 weeks, weigh 180-200g were bred at the animal house of Medical Faculty-Brawijaya University. Each rat was housed in a polyethylene cage (40 × 45 × 20 cm) equipped with wood shaving-bottom and wire-removable cover. The room temperature was at range of 22 – 25°C, humidity range was 50 – 60%, and light was set on for 12 hours. Research procedure passed the ethical clearance

from Research Ethic Committee of Brawijaya University, certificate No. 47/EC/KEPK-S1/02/2016. Three groups of rats were administered with 320 mg, 160 mg, and 80 mg/rat of EPP respectively by gastric feeding tube, 30 times EPP administrations, in alternate day (within 60 days). The plantar of right hind paws on the day 30th were subcutaneously injected with 0.1 mL m-CFA (1 mg/1 mL) to induce RA. Arthritic score was used as a presentation of typical inflammation signs of the hind paws as response to immune process toward m-CFA induction. The signs were found on day 10-13th post the induction. The assessments of the AS were based on the arthritis assessment score [20] (Table 1).

Table 1. Scoring of arthritis assessment [20]

Signs	Score
Oedema and hyperemia of 1 toe	0.25
Oedema of hyperemia of at least 2 toes	0.50
Oedema of foot pad	0.75
Oedema and hyperemia of toes and oedemic foot pad	1.00
Oedema and hyperemia of toes and foot pad	1.25
Oedema and hyperemia of toes and minor oedema of foot pad and ankle	1.50
Oedema and hyperemia of toes and major oedema of foot pad and ankle	1.75
Oedema and hyperemia of toes, foot pad, and ankle	2.00

Serum TNF-α and IL-6, and synovial MMP-9 measurements

At the end of the study (on the 60th day), a deep anesthesia was done by putting the rats in transparent glass box (20 × 20 × 20 cm) containing ether saturated- air. The box was used for only a rat. Pain responses of unconscious rats were tested prior to thoracotomy. Blood were intracardially aspirated by 10 mL syringes with 19G needle, then were collected in a vacutainer without EDTA, subsequently were immediately centrifuged at 4500 rpm for 15 minutes to obtain serum. Finally, the serum was stored under -20°C. ELISA technique was applied to measure serum TNF-α and IL-6, biotinylated monoclonal antibody specific for rat TNF-α and IL-6 were respectively used. The existing colours were read by Elisa reader at λ 450 nm. The readings were duplicated. This study used ELISA Kit of rat IL-6 (Thermo Scientific^{TM}) and TNF-α (Invitrogen^{TM}) both of the Thermo Fisher Scientific. The results were reported in pg/mL.

As soon as intracardiac blood aspiration was completely done, the right hind leg was collected by cutting ligaments and tendons of the knee joints. The clean leg (without fur) was put in an organ container filled with buffered neutral formalin 10%, and stored at room temperature for 48 hours. Decalcification process were done by dipping the legs in 10% EDTA (pH 7.4) solution at 4°C for 3 weeks, during which the EDTA solutions were renewed every 4 days. Completed decalcification process was tested by a needle penetration. The 4 µm-thick bone slices of ankle joints were prepared by cutting bone-paraffine blocks using microtome, subsequently the immunohistochemistry process was conducted, followed by 3,3′-Diaminobenzidine (DAB) staining and hematoxylin (as counterstain) to detect MMP-9 existence. The staining process made the complexes of MMP-9 and its antibody exhibited brown colour. The complexes with brown colour were observed by light microscope (magnification 400×) to calculate the synovial MMP-9 densities of ankle joints by ImmunoRatio software (Figure 1).

Statistical analysis

Data were analyzed by Krusskal Wallis test continued by Mann Whitney U test. The value of $p < 0.05$ was considered to be significant. Correlation test of Pearson was done among the parameters (MMP-9, TNF-α, and IL-6).

Results and Discussion

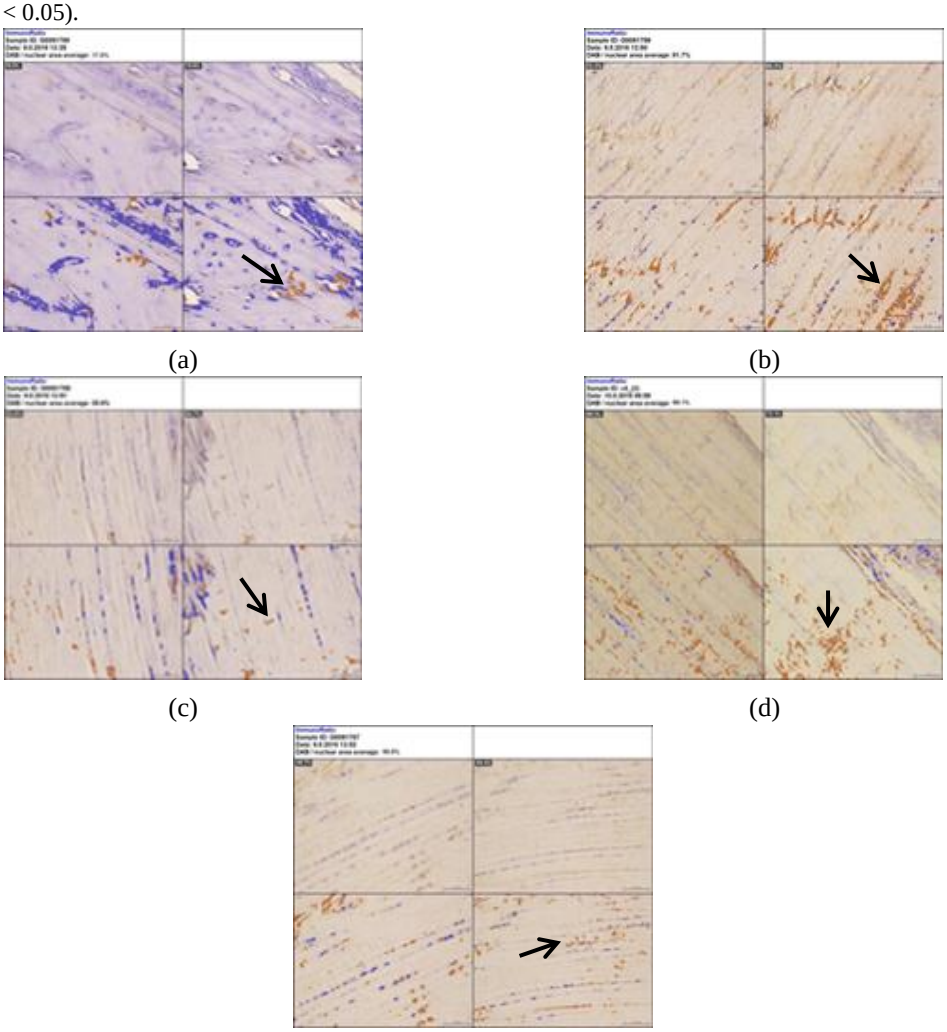
The Effects of EPP on serum IL-6 and TNF-α

Table 2 shows the EPP doses of 160 mg and 320 mg/rat significantly reduce serum TNF-α of arthritic rats ($p < 0.05$). Only the EPP dose of 320 mg/rat significantly corrected both serum IL-6 and TNF-α ($p < 0.05$). This dose prevents the percentage increase of serum IL-6 and TNF-α to be only 8. 28 % and 11. 12 % respectively ($p < 0.05$) (Table 2). The EPP was given for 30 times, in alternate day (within 60 days). It was reported that allegic acid (an active component of pomegranate peel) lower serum TNF-α of arthritic mice due to AIA induction at the plantars of the right hind paws [21]. The fruit peel contains 10–50 mg/100 g of ellagic acid, the value is much higher than that of the fruit juice [22]. NF-κB have a pivotal role, as a mediator for inducing the pro-inflammatory

Table 2. The Level of IL-6 and TNF-α in the serum of healthy and rheumatoid arthritis rats, and rheumatoid arthritis rats receiving EPP

Group	IL-6 (pg/ml) Mean ± SD (n = 5)	IL-6 (% increase) (n = 5)	TNF-α (pg/ml) Mean ± SD (n = 5)	TNF-α (% increase) (n = 5)
Healthy rats	868. 67 ± 89. 87 [a]	-	249. 60 ± 72. 11 [a]	-
Arthritic rats	1311. 33 ± 223.46 [b]	50.20 ± 13.31 [a]	390. 40 ± 42. 69 [b]	62. 43 ± 26.32 [a]
Arthritic rats + EPP 320 mg	941.33 ± 93. 164 [a ,3]	8.48 ± 4.93 [b, 1]	256. 80 ± 47. 64 [a, 2]	11.12 ± 4.40 [b, 1]
Arthritic rats + EPP 160 mg	1142.00 ± 71.13 [b, 2]	31.96 ± 6.75 [c, 2]	299. 00 ± 65. 55 [a, 1, 2]	35. 52 ± 10.80 [c, 2]
Arthritic rats + EPP 80 mg	1294.67 ± 79.95 [b,1]	49.57 ± 6.90 [a, 2]	357. 40 ± 30. 26 [b, 1]	49. 08 ± 25.83 [c, 2]

Note: The serum TNF-α, IL-6, and the percentages of increase of both parameters (the parameter values of healthy rats correspond to 100%) were presented as mean ± SD. Data were statistically analyzed by Kruskall Wallis continued by Mann Whitney U test. ([a, b]) Different alphabet notifies the difference is significant ($p < 0.05$). ([1, 2]) Comparison of values were done between the groups of arthritic rats receiving EPP and those receiving no EPP. Different Arabic number notifies a significant difference ($p < 0.05$).



(a) (b) (c) (d) (e)

Figure 1. The synovial MMP-9 densities of ankle joints. Synovial MMP-9 are identified as brown figure (black arrows) using light binocular microscope (400×). The densities of MMP-9 were determined by ImmunoRatio. Healthy rats (a), rheumatoid arthritis rats (RA) (b), RA rats receiving 320 mg of EPP/day (d), RA rats receiving 160 mg/day of EPP (d), and RA rats receiving 80 mg/day of EPP (e).

gene to transcript pro-inflammatory cytokines including TNF-α and IL-6 [23].

In vitro study on cell line of macrophages-RAW264.7 induced by lipopolysaccharide showed polyphenols of pomegranate peel (punicalagin and ellagic acid) suppressed the activation of NF-κB [24]. Polyphenol is a compound possessing high anti-inflammatory capacity and antioxidant activity [25].

A meta-analysis and systematic study on the research of the effects of antirheumatoid agents on CFA-induced arthritic rats categorized the research into 3 namely; prophylactic (the administration of antirheumatoid prior to generation of autoantibody response), periarthritis (anti rheumatoid drugs were given as soon as after induction of autoantibody response) and therapeutic intervention (antirheumatoids were applied after arthritis development) [26]. Disease prevention and early therapy of RA were recommended by The European society (EULAR) to minimize permanent joints destruction [27]. Rats of the present study had been receiving EPP for 15 times, in alternate day (within 30 days) when the animals were plantary induced with m-CFA to generate antibody response. Subsequently the EPP administrations were continued for the next 30 days (15 times administrations in alternate day). The EPP dose of 360 mg/rat have maintained the normal level of serum TNF-α and IL-6 in m-CFA-induced rats, the serum levels of the both parameters approximate to those of the healthy rats ($p > 0.05$) (Table 2).

Anti-TNF and anti-IL-6 drugs were recommended by EULAR for treating RA to be significant namely: infliximab (INF), adalimumab (ADA) and golimumab (GLM) which are monoclonal antibodies; etanercept (ETN) and certolizumab (CTZ), both are the recombinant TNF receptor. The anti-interleukin-6 was tocilizumab [3, 28].

A study on fibroblast-like synoviocytes (FLS) stimulated with synovial fluid of RA patients showed that IL-6 inhibition reduced MMP-9 activities. The study used tocilizumab (TCZ), an anti-IL-6 receptor antagonist at dose 200 µg/ml to blockade IL-6 [29]. Our study revealed EPP significantly reduced serum IL-6 and synovial MMP-9 of RA model rats (Table 2 and 3), these results supported the in vitro study of Blas and colleagues 2017, since the reduction of serum IL-6 correlates with the reduction of serum MMP-9 ($r = 0.885$, $p < 0.05$). In addition, high concentration of serum IL-6 was detected in the serum and synovial fluid of patients with RA [30]. The present study shows synovial MMP-9 do not significantly correlates with serum TNF-α ($r = 0.841$, $p > 0.05$), and serum TNF-α shows a strong correlation with serum IL-6 ($r = 0.899$, $p < 0.05$).

The effects of EPP on synovial MMP-9

Table 3 shows the effective dose of EPP to reduce MMP-9 is 320 mg/rat ($p < 0.05$), compared with MMP-9 of arthritic rats receiving no EPP. Two types of MMP namely MMP-2 and MMP-9 are known to have pro-homeostatic and pro-inflammatory properties respectively. It is established MMP-2 has a protective role in molecular basis etiology of RA by degrades and inactivates cytokines and chemokines [31]. MMP-9 is formed in many kinds of tissues, systemic MMP-9 derive from white blood cells especially in inflammation state. A study on patients with RA reported the synovial fluid level of MMP-9 and MMP-2 are higher than the serum level of normal persons, their physiological tissue inhibitors TIMP-1 and TIMP-2 respectively also elevate. The condition due to the elevated recruitment of neutrophils, macrophages and other inflammatory cells to the joint site [10]. Angiogenesis in inflammation state facilitate macrophages and monocytes to penetrate synovial tissues in order to release TNF-α and IL-6. The latter both induce MMP-9 expression [32]. The present study showed synovial MMP-9 of m-CFA induced - RA rats increased up to 350% (Table 3) and the EPP dose of 320 mg corrected the synovial MMP-9, the extract inhibited the increase percentage of MMP-9 to 61.57 % ($p < 0.05$) (Table 3).

Matrixmetalloprotein (MMP) inhibitors development has been an attractive therapeutic intervention unfortunately clinical study reported broad-spectrum MMP inhibitors show limited advantages, encouraging to develop selective inhibitor for MMPs. A compound named JNJ0966 was reported to inhibit the conversion of pro MMP-9 to MMP-9 active without any inhibition of the enzyme activities [33]. We used 80% ethanolic extract of EPP in the present study and did not conduct bone examination, but previous study demonstrated that hydro-ethanolic (30 : 70) extract of pomegranate peel prevent the mineral density reduction of and the microarchitecture impairment

Table 3. The Synovial MMP-9 density and arthritic score of healthy and rheumatoid arthritis rats, and rheumatoid arthritis rats receiving EPP

Group	Synovial MMP-9 density Mean ± SD (n = 5)	Synovial MMP-9 Density (% increase) Mean ± SD (n =5)	Arthritic score Mean ± SD (n = 5)
Healthy rats	17. 52 ± 6. 07 [a]	0	0.00 ± 0.00
Arthritic rats	79. 42 ± 5. 82 [b]	425.32 ± 270.65[a]	1. 65 ± 0.22 [a]
Arthritic rats + EPP 320 mg	26. 34 ± 8. 61 [a,1]	61.57 ± 10.48 [b]	1.4 ± 0. 14 [a]
Arthritic rats + EPP 160 mg	68. 98 ± 11. 10 [b,2]	342.04 ± 186.53 [c]	1. 4 ± 0.14 [a]
Arthritic rats + EPP 80 mg	53. 70 ± 10. 12 [c,2]	246.17 ± 158.98 [d]	1. 45 ± 0. 14[a]

The synovial MMP-9 density, arthritic score, and increase precentages of the values of MMP-9 (MMP-9 values of healthy rats correspond to 100%) were showed as Mean values ± SD.

Data were statistically analyzed using Kruskall Wallis continued by Mann Whitney U test.

([a,b,c]) The values are compared with those of healthy and/or arthritic rats, different alphabets notify significant value differrences ($p < 0.05$). ([1, 2]) Comparison of values were done between the groups of arthritic rats receiving EPP, different arabic number presents a significant difference ($p < 0.05$).

of the bone in ovariectomized (OVX) C57BL/6J mice. The latter study also reported RAW264.7 cells exposed to serum of mice received diet-enriched with the extract, reduced osteoclast differentiation and bone resorption [34]. Studies reported IFN-α, IL-6, and MMP-9 correlates with atherosclerosis pathogenesis involving inflammation process [35].

A clinical study found carotid artery intima media thickness and number of carotid plaques of RA patients were increased, without classic CV risk factor or CV events. This indicates that inflammation process of chronic RA increases the CVD risk [36]. In respect with the present study results the EPP may potential in preventing or attenuating atherosclerosis in chronic RA patients by minimizing the serum level of the cytokines-induced risk factors. In addition, EPP contain polyphenols, substances reported to attenuate arterial stiffness by stabilizing serum paraoxonnase-1 (PON-1) [37]. Nevertheless, to achieve the goal, need sustainable research on EPP.

A clinical study reported serum IL-6 and TNF-α levels of obese persons significantly higher than those of nonobese subjects [38]. Another study found body mass index increase a risk in suffering rheumatoid arthritis [39].

Higher plasma level of these cytokines is observed in healthy older and associated with reduction of muscle mass and strength linked to aging [40]. Pathogenesis of elderly onset of rheumatoid arthritis (EORA) is thought to link with immune aging process [41]. Understanding how risk factors including immune aging is involved in RA pathogenies motivate development of new prophylactic agent for RA.

The Effect of EPP on arthritis score (AS)

Even though the three biomarkers (synovial MMP-9 and serum TNF-α and IL-6) were significantly corrected by the EPP treatment at dose of 320 mg/rat, this dose of EPP only slightly reduced the AS ($p > 0.05$) (Table 3). Pathophysiology of RA consists of 2 phases; the priming phase initiating immune activation and the effector phase inducing systemic inflammation. During immune activation, IL-6 induces Th17 development, the expression of anticitrullinated protein antibody (ACPA) and rheumatoid factor (RF), leading to active synovitis. Systemic inflammation of RA is characterized by CRP elevation, anemia, and fatigue mainly mediated by IL-6. Meanwhile the local inflammation signs, arthralgia, swelling, and joint destruction are mainly mediated by

TNF-α. Interleukine-6 play role in an activation of immune pathway and inflammation process [27]. There is a dynamic hierarchical role of cytokines during the development of RA, IL- 6 and TNF-α play dominant role in acute and chronic phases of the disease [7]. A study on AIA induced arthritic mice intraperitoneally injected with allegic acid showed that the acid could reduce right hind paws oedeme by nearly 20% and lower

serum TNF-α levels [21]. Accordingly, it is proposed to explore the effective doses of EPP to ameliorate the physical signs (arthritis score) in the RA rats in respect to the present study results.

An acute toxicity test of EPP on *Danio rerio* (Zebra fish) and a toxicity prediction of the extract using *in silico* study reported the compounds of EPP are safe. The EPP has no constituents to induce impairments of androgen hormone, reproductive organs, and heart. Among the constituents of EPP, Brevifolin has the lowest toxicity level [42]. These toxicity test results encourage efforts to promote EPP as prophylactic agent of RA. It is proposed to complete the clinical signs of RA rats with a parameter quantitatively measured such as diameter and or volume of the affected joints.

Conclusion

Ethanolic extract of pomegranate peel (EPP) potential to be developed as antirheumatoid agent. The presents study revealed the EPP significantly prevents the elevation of synovial MMP-9, serum IL-6 and TNF-α. The dose of EPP did not significantly improve the physical signs (determinate by AS) of rheumatoid arthritis rats. The results of the present study motivate to explore optimal doses of EPP preventing the biomarkers elevation and correcting AS values.

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