Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Juglanin Attenuated Adjuvant-Induced Arthritis via Inactivating NF-κB/IκBα and A Disintegrin-Like and Metalloproteinase Domain with Thrombospondin-1 Repeat Pathways in Experimental Rats

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Submitted: 22-Mar-2020 Revised: 20-Apr-2020 Accepted: 28-Aug-2020 Published: 16-Feb-2021

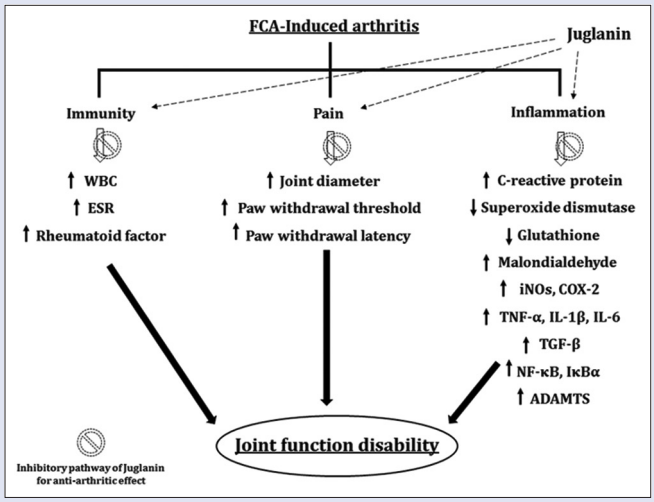
ABSTRACT

Background: Rheumatoid arthritis (RA) is an autoimmune and inflammatory disease affecting multiple joints and worsening the quality of life. Juglanin, a natural aldose reductase inhibitor, has shown to exhibit anti-inflammatory, anti-nociceptive, and anti-oxidant properties. **Objective:** The present study was undertaken to evaluate their possible mechanism of action against adjuvant-induced arthritis (AIA), i.e., Freund's complete adjuvant (FCA)-induced arthritis, in experimental rats. **Materials and Methods:** FCA (0.1 mL) was administered into the subplantar region of female Wistar rats paw to induced AIA. The rats were treated with vehicle (distilled water, 10 mL/kg) or leflunomide (10 mg/kg), or juglanin (10, 20, and 40 mg/kg, respectively) orally for the next 16 days. Various biochemical, molecular, and histological parameters were evaluated to determine the potential of juglanin. **Results:** Subplantar administration of FCA resulted in a statistically significant ($P < 0.05$) induction of AIA reflected by alteration in paw volume, joint diameter, paw withdrawal threshold, and paw withdrawal latency, which was statistically significantly inhibited ($P < 0.05$) by juglanin (20 and 40 mg/kg). It also statistically significantly attenuated ($P < 0.05$) FCA-induced elevated hepatic oxido-nitrosative stress and mRNA expressions of tumor necrosis factor-alpha (TNF-α), interleukin (IL) IL-1β, IL-6, transforming growth factor-beta (TGF-β), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOs). Western blot analysis revealed that juglanin statistically significantly downregulated ($P < 0.05$) protein expressions of NF-κB, IκBα, a disintegrin-like and metalloproteinase domain with thrombospondin-1 repeats (ADAMTS)-4, and ADAMTS-5 in hepatic tissue. It also statistically significantly attenuated ($P < 0.05$) histopathological anomalies induced in the tibiotarsal joint. **Conclusion:** The present communication suggests that juglanin attenuated altered mechano-tactile allodynia and hyperalgesia via inhibition of elevated oxido-nitrosative stress, cytokines (TNF-α and ILs) levels, immune-inflammatory (TGF-β, NF-κB, Iκβα, COX-2, and iNOs) mediators, and ADAMTS, thus exerting its anti-arthritic potential.

Key words: A disintegrin-like and metalloproteinase domain with thrombospondin-1 repeats, arthritis, Freund's complete adjuvant, Iκβα, juglanin, NF-κB, transforming growth factor-Beta

SUMMARY

- Freund's complete adjuvant administration in subplantar paw region induces arthritis
- Juglanin at doses of 20 and 40 mg/kg displayed marked inhibition in FCA induced decrease in thermal and mechanical hyperalgesia
- Juglanin also noticeably downregulated serum oxido-nitrosative stress and mRNA expressions of hepatic tumor necrosis factor-alpha, ILs, transforming growth factor-beta, cyclooxygenase-2, and inducible nitric oxide synthase
- FCA-induced upregulated hepatic protein expressions of NF-κB, Iκβα, and a disintegrin-like and metalloproteinase domain with thrombospondin-1 repeats (4 and 5) were significantly inhibited by juglanin
- Juglanin can be measured as one of the important moieties from plant origin for the management of arthritis.



Abbreviations used: ADAMTS: A disintegrin-like and metalloproteinase domain with thrombospondin-1 repeats; AIA: Adjuvant-induced arthritis; ALT: Alanine transaminase; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; CRP: C-reactive protein; COX-2: Cyclooxygenase-2; ESR: Erythrocyte sedimentation rate; FCA: Freund's complete adjuvant; HDL: High-density lipoprotein; iNOS: Inducible nitric oxide synthase; IL1β: Interleukin-1beta; LDL: Low-density lipoprotein; MDA: Malondialdehyde; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; Iκβα: Nuclear factor of kappa light polypeptide gene enhancer in B cell inhibitor α; ROS: Reactive oxygen species; GSH: Reduced glutathione; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction; SOD: Superoxide dismutase; TC: Total cholesterol; TGF-β: Transforming growth factor-beta; TG: Triglyceride; TNF-α: Tumor necrosis factor-alpha; VLDL: Very low-density lipoprotein.

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DOI: 10.4103/pm.pm_106_20

Access this article online
Website: www.phcog.com
Quick Response Code:

Cite this article as: Ning C, Ning S, Luo H, Yan L. Juglanin attenuated adjuvant-induced arthritis via inactivating NF-κB/IκBα and A disintegrin-like and metalloproteinase domain with thrombospondin-1 repeats pathways in experimental rats. Phcog Mag 2020;16:803-11.

INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic, relapsing autoimmune disorder and one of the most common forms of arthritis, mainly affecting joints.[1,2] It is described by severe pain, inflammation, deformity, stiffness, and tissue damages that lead to limited of movements.[3] It has been well documented that autoantibodies infiltration in the synovial joint origins articular cartilage erosion, which resulted deformities in bone, premature death, and joint disability.[1-3] Around 20 million people, i.e., 1%–2% of the world population, shows RA.[3] RA is an age-related autoimmune disorder in women between the age of 40 and 50 years, which has been appeared to be at a greater risk of arthritis occurrence as compared to men.[4]

Despite wide research, the etiopathogenesis of arthritis is poorly progressed. It has been reported that pro-inflammatory influx is a vital pathway during the induction of arthritis.[5] Tumor necrosis factor (TNF-α) and interleukin-1 (IL-1) have suggested as important pathogenic molecules during RA, thus management of arthritis is oriented toward the amelioration of elevated levels of pro-inflammatory cytokines.[2,3,5] Besides, eicosanoid mediators such as prostaglandins (including cyclooxygenases [COX], lipoxygenases [LOX]) and leukotrienes are known to activate inflammatory cytokines and immune cell, which cause inflammation and joint degradation. In addition, elevated production of reactive oxygen species (ROS) such as superoxide anions, peroxynitrite, and hydrogen peroxide is also thought to contribute a vital role in the induction and maintenance of RA.[1,2,6]

RA is the most frequent immune-inflammatory diseases, thus existing therapies usually focused on their anti-inflammatory and immunosuppressive properties, which help to check the process of inflammation, and therefore may also help in the repair process.[3] Treatment regimen comprising disease-modifying anti-rheumatic drugs is used to inhibit the immune-inflammatory response, whereas non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are employed for symptomatic relief.[7] However, these treatment regimens provide symptom relief in fractions of patients and associated with well-known side effects.[3] Now, most of the modern pharmaceutical industries serge the development of new treatment regimen from the herbal sources. Thus, an array of experimental models play noteworthy role in the evaluation of the efficacy of these therapeutic moieties.[8] Documented studies by a number of researchers have well recognized adjuvant-induced arthritis (AIA) as one of the important experimental models to evaluate the efficacy of various molecules.[1,2,6,9] The Freund's complete adjuvant (FCA)-induced AIA mimics most of the clinico-pathological characteristics of RA including pain, swollen and stiffen joints, elevated C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) levels, etc.

Juglanin (kaempferol 3-O-arabinofuranoside), a natural aldose reductase inhibitor, widely present in *Polygonum aviculare,* has been shown to exhibit an array of pharmacologic activity including anti-inflammatory, anticancer, antioxidant, and hepatoprotective properties.[10-12] Juglanin inhibits lipopolysaccharide-induced release of inflammatory markers, including TNF-α, ILs COX-2, and inducible nitric oxide synthase (iNOS) protein expressions.[13] It exerts its hepatoprotective effect against fructose-induced hepatitis via inhibition of the TLR4/NF-κB signaling pathway.[12] Juglanin inhibits NF-κB phosphorylation through p65 inactivation to reduce cellular senescence in human dermal fibroblasts.[10] However, the potential of juglanin against AIA has not yet reported in available literature. Thus, the present study was undertaken with the aimed to determine the efficacy of juglanin against FCA-induced arthritis in experimental rats.

MATERIALS AND METHODS

Animals

Female Wistar rats (6–8 weeks, 150–180 g) were obtained from the Laboratory Animal Center of Chongqing Medical University, China. The rats were housed in polypropylene cages at a temperature of 24°C ± 1°C with a normal dark-light cycle, with free access to standard food and water. All experiments were carried out between 08:00 h and 17:00 h in a quiet laboratory. The experimental protocol number (XJU-E201911-09, dated 23/06/2019) was approved by the animal care and use committee of the Chongqing Medical University, China.

Chemicals, and kits

Juglanin (purity ≥97%) was purchased from ChemScene (LLC, NJ, USA) and FCA was purchased from Sigma Chemical Co. (St Louis, MO, USA). Total RNA extraction kit and one-step quantitative reverse transcription-polymerase chain reaction (RT-PCR) kit were procured from MP Biomedicals India Private Limited (Mumbai, Maharashtra, India). Primary antibodies of p-NF-κB (nuclear factor kappa-light-chain-enhancer of activated B Cells), p-IkBα (nuclear factor of kappa light polypeptide gene enhancer in B cell inhibitor α), a disintegrin-like and metalloproteinase domain with thrombospondin-1 repeats (ADAMTS)-4 and ADAMTS-5 were procured from Abcam, Cambridge, MA, USA.

Experimental design

The animals were separated into various groups (n = 12 each) where they received either distilled water (10 mL/kg, p. o. as a normal and AIA, i.e., Group I and II) or leflunomide (10 mg/kg, as a standard, i.e., Group III) or juglanin (5, 10, and 20 mg/kg, i.e., group IV to VI). The dose selection of juglanin was based on the previously reported studies.[12-14]

Adjuvant-induced arthritis

On day 0 of study, FCA (0.1 mL with 0.6 mg heat-inactivated *Mycobacterium tuberculosis* H37Ra) was injected intradermaly into the sub-plantar region of the rat paw to induce AIA in the five groups, i. e., Groups II to VI.[9] The animals were allowed to grow AIA for 12 days, and then they were treated with respective treatment for the next 16 days (once a day). A plethysmometer (UGO Basile, Italy) was used to determined the paw volume.[9] Percent inhibition of paw volumes was calculated according to the equation reported earlier.[9] Pain latency against mechanical hyperalgesia (paw withdrawal latency) was determined by using Randall-Selitto (Ugo Basile Model 7200, Gemonio, Italy).[9]

On day 28, retro-orbital puncture technique was used to withdraw a blood, which was further applied for various biochemical estimations (ESR, CRP, red blood cell [RBC], Hb, white blood cell [WBC], PLT, alkaline phosphatase [ALP], aspartate aminotransferase [AST], alanine transaminase [ALT], total cholesterol [TC], high-density lipoprotein [HDL], low-density lipoprotein [LDL], and very LDL [VLDL]). After blood withdrawal, the animals were sacrificed to collect the ankle joint for histology assessment and liver for antioxidant parameters (superoxide dismutase [SOD], glutathione [GSH], malondialdehyde [MDA], and NO),[15] mRNA expressions of TNF-α, IL-1β, IL-6, transforming growth factor-beta (TGF-β), COX-2, and iNOS by using quantitative real-time polymerase chain reaction (qRT-PCR)[15] and protein expressions of NF-κB, IκBα, ADAMTS-4, and ADAMTS-5 by using Western blot assay.[15] Graph Pad Prism 5.0 software (Graph Pad, San Diego, CA, USA) was used to accomplish data analysis. Data were expressed as mean ± standard error mean and analyzed by using one-way ANOVA followed by Tukey's multiple range *post hoc* analysis for parametric tests. $P < 0.05$ was considered statistically significant.

RESULTS

Body, spleen, and liver weights

The body and liver weight had noticeably reduced ($P < 0.05$), whereas spleen weight increased markedly ($P < 0.05$) in AIA group when compared to the normal group. However, variations in the body, liver, and spleen weights induced by FCA were effectively ($P < 0.05$) reduced by leflunomide (10 mg/kg) administration when compared with AIA group. Administration of juglanin at doses of 20 and 40 mg/kg markedly ($P < 0.05$) increased body and liver weight as well as increased spleen weight when compared with AIA group. However, alterations in body, liver, and spleen weights induced by FCA were more noticeably ($P < 0.05$) inhibited in leflunomide (10 mg/kg)-treated group when compared with juglanin treatment [Table 1].

Paw volume, joint diameter, paw withdrawal threshold, and latency

A marked ($P < 0.05$) increase in joint diameter and paw volume was detected in AIA group after administration of FCA when compared with normal group. Leflunomide (10 mg/kg) treatment and juglanin administration at doses of 20 and 40 mg/kg effectively ($P < 0.05$) reduced the joint diameter and paw volume as compared with AIA group. FCA administration caused marked ($P < 0.05$) decreased in paw withdrawal latency and threshold of AIA group when compared with normal group. Leflunomide (10 mg/kg) markedly ($P < 0.05$) attenuated FCA-induced decreased in paw withdrawal latency and paw withdrawal threshold when compared with AIA group. Juglanin treatment at a dose of 20 and 40 mg/kg also suggesting a marked ($P < 0.05$) increase in paw withdrawal latency and threshold as compared with AIA group. FCA-induced decreased paw withdrawal threshold was more noticeably ($P < 0.05$) attenuated by leflunomide (10 mg/kg) when compared with juglanin, whereas paw withdrawal latency was more effectively ($P < 0.05$) attenuated by juglanin (40 mg/kg) when compared with leflunomide (10 mg/kg) treatment [Figure 1 and Table 2].

Aspartate aminotransferase, alanine transaminase, alkaline phosphatase, albumin, and serum C-reactive protein

Serum AST, ALT, ALP, and CRP levels were increased noticeably ($P < 0.05$), whereas the serum albumin level was decreased prominently ($P < 0.05$) after the administration of FCA in AIA group when compared with normal group. However, the changes in levels of these enzymes were effectively ($P < 0.05$) lessened by leflunomide (10 mg/kg) when compared with AIA group. Serum AST, ALT, ALP, and CRP levels had effectively ($P < 0.05$) decreased, whereas level of albumin in serum had markedly ($P < 0.05$) increased by juglanin treatment at doses of 20 and 40 mg/kg when compared with AIA group [Table 1].

Hematological parameters and erythrocyte sedimentation rate

The WBC, platelets, and ESR levels had noticeably ($P < 0.05$) increased; in contrast, RBC and Hb levels marked ($P < 0.05$) decreased in AIA group when compared with that of the normal group. Hematological and ESR changes induced by FCA were noticeably ($P < 0.05$) attenuated by leflunomide (10 mg/kg) when compared with that of the AIA group. When compared with AIA group, the increased level of WBC, platelets, and ESR had effectively ($P < 0.05$) decreased by treatment with juglanin at doses of 20 and 40 mg/kg, whereas it noticeably ($P < 0.05$) increased levels of RBC and Hb. However, hematological and ESR alterations induced by FCA were more efficiently ($P < 0.05$) lessened by leflunomide treatment (10 mg/kg) as compared to juglanin treatment [Table 3].

Serum lipid profile

There were marked ($P < 0.05$) augmented in triglyceride (TG), TC, LDL-C, and VLDL-C, whereas there was a marked ($P < 0.05$) decrease in HDL-C levels in AIA group as compared with normal group. FCA-induced alteration in serum lipid profile was noticeably ($P < 0.05$) attenuated by leflunomide (10 mg/kg) when compared with that of the AIA group. When compared with AIA group, the augmented level of TG, LDL-C, TC, and VLDL-C had markedly ($P < 0.05$) decreased by treatment with juglanin at doses of 20 and 40 mg/kg, whereas it noticeably ($P < 0.05$) increased the levels of HDL-C. However, FCA-induced modification in TG, HDL-C, TC, LDL-C, and VLDL-C was more efficiently ($P < 0.05$) attenuated by leflunomide treatment (10 mg/kg) as compared to juglanin treatment [Table 4].

Hepatic oxido-nitrosative stress

FCA administration markedly ($P < 0.05$) decreased hepatic GSH and SOD levels, whereas markedly ($P < 0.05$) increased hepatic MDA and NO levels in AIA group as compared with normal group. Leflunomide (10 mg/kg) treatment effectively ($P < 0.05$) elevated the hepatic GSH and SOD levels, whereas markedly ($P < 0.05$) reduced the levels of hepatic MDA and NO as compared with AIA group. Further, administration of juglanin at doses of 20 and 40 mg/kg noticeably ($P < 0.05$) decreased the hepatic NO and MDA levels, whereas efficiently ($P < 0.05$) augmented the hepatic GSH and SOD levels as compared with AIA group. However, changed GSH, SOD, MDA, and NO levels were more markedly ($P < 0.05$) restored by leflunomide (10 mg/kg) when compared with juglanin treatment [Table 5].

Rheumatoid factor was prominently elevated ($P < 0.05$) in AIA control group when compared with normal group. Leflunomide (10 mg/kg) administration and juglanin treatment at doses of 20 and 40 mg/kg efficiently ($P < 0.05$) decreased rheumatoid factor when compared with AIA group [Table 5].

Table 1: Effects of juglanin on Freund's complete adjuvant-induced alterations in body weight, spleen weight, liver weight, serum aspartate aminotransferase, alanine transaminase, alkaline phosphatase, albumin, and serum C-reactive protein levels in arthritic rats

Treatment	Body weight (g)	Spleen weight (g)	Liver weight (g)	AST (U/ml)	ALT (U/ml)	Alkaline phosphatase (U/L)	Albumin (g/dl)	Serum CRP (mg/L)
Normal	240.50±3.01	1.02±0.02	10.90±0.21	61.98±1.92	44.14±3.48	59.57±6.77	4.56±0.39	0.89±0.10
AIA control	179.00±2.72$^{\#}$	2.01±0.03$^{\#}$	7.74±0.34$^{\#}$	98.21±2.19$^{\#}$	79.49±3.12$^{\#}$	180.30±6.94$^{\#}$	1.32±0.31$^{\#}$	3.98±0.21$^{\#}$
LF (10)	220.66±5.20$^{*,\$}$	1.29±0.04$^{*,\$}$	10.61±0.22$^{*,\$}$	75.54±2.96$^{*,\$}$	49.98±2.61$^{*,\$}$	85.86±5.72$^{*,\$}$	4.12±0.20$^{*,\$}$	1.90±0.20$^{*,\$}$
J (10)	195.66±6.36	1.84±0.05	8.13±0.27	93.71±2.92	78.82±1.50	174.40±5.77	2.29±0.22	3.59±0.20
J (20)	208.83±4.28$^{*,\$}$	1.64±0.03$^{*,\$}$	9.12±0.17$^{*,\$}$	78.02±3.81$^{*,\$}$	63.06±1.70$^{*,\$}$	138.90±5.08$^{*,\$}$	2.72±0.39$^{*,\$}$	2.54±0.11$^{*,\$}$
J (40)	218.66±4.22$^{*,\$}$	1.45±0.04$^{*,\$}$	9.52±0.23$^{*,\$}$	71.87±3.15$^{*,\$}$	58.04±2.21$^{*,\$}$	98.47±5.51$^{*,\$}$	4.01±0.31$^{*,\$}$	1.97±0.16$^{*,\$}$

Figures in the parenthesis indicate a dose in mg/kg. n=6, data was analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. $^{\#}P<0.05$ as compared to normal group; $^{*}P<0.05$ as compared to AIA control group and $^{\$}P<0.05$ as compared to each other. AIA: Adjuvant-induced arthritis; LF: Leflunomide; J: Juglanin; AST: Aspartate aminotransferase; ALT: Alanine transaminase; CRP: C-reactive protein

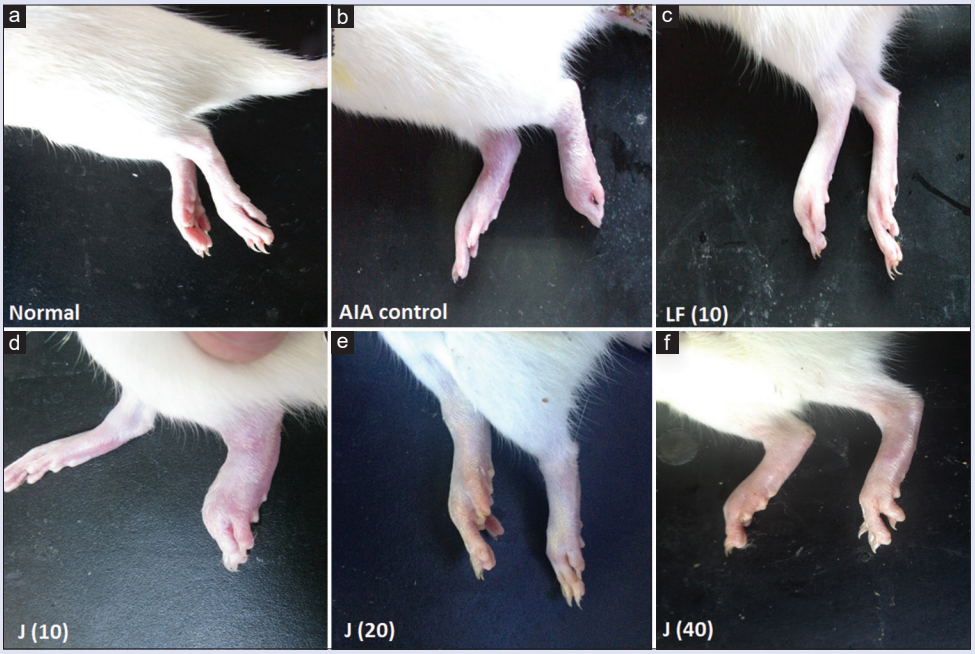

Figure 1: Effects of juglanin on morphological representations of rat paw after administration of FCA. Representative images of paw from (a) normal, (b) AIA control, (c) leflunomide (10 mg/kg), (d) juglanin (10 mg/kg), (e) juglanin (20 mg/kg) and (f) juglanin (40 mg/kg) treated rat

Table 2: Effects of juglanin on Freund's complete adjuvant-induced alterations in the change in paw volume, change in joint diameter, paw withdrawal threshold, and paw withdrawal latency in arthritic rats

Treatment	Area under the curve				
	Change in paw volume (ml)	Change in joint diameter (mm)	Paw withdrawal latency (s)	Von Frey-Paw withdrawal threshold (gr)	Randall Sillito-Paw withdrawal threshold (gm)
Normal	0.00±0.00	0.00±0.00	241.60±6.92	1785.00±41.74	8290.00±72.92
AIA control	91.85±1.62$^{\#}$	86.46±0.44$^{\#}$	119.70±7.28$^{\#}$	774.10±24.85$^{\#}$	4859.00±130.20$^{\#}$
LF (10)	70.64±0.84$^{*,\$}$	70.78±0.99$^{*,\$}$	153.70±7.94$^{*,\$}$	1020.00±23.53$^{*,\$}$	5953.00±160.60$^{*,\$}$
J (10)	90.73±0.58	85.58±1.02	126.90±8.55	815.00±12.94	5085.00±106.30
J (20)	81.51±0.62$^{*,\$}$	79.04±1.06$^{*,\$}$	142.00±9.78$^{*,\$}$	879.60±18.42$^{*,\$}$	5478.00±45.96$^{*,\$}$
J (40)	70.33±0.96$^{*,\$}$	71.15±0.85$^{*,\$}$	155.60±8.66$^{*,\$}$	1017.00±26.61$^{*,\$}$	5899.00±91.91$^{*,\$}$

Figures in the parenthesis indicate a dose in mg/kg (n=6), data was analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. $^{\#}P$<0.05 as compared to normal group; $^{*}P$<0.05 as compared to AIA control group and $^{\$}P$<0.05 as compared to each other. AIA: Adjuvant induced arthritis; LF: Leflunomide; J: Juglanin; AUC: Area under the curve

Table 3: Effects of juglanin on Freund's complete adjuvant-induced alterations in hematological parameters and erythrocyte sedimentation rate in arthritic rats

Treatment	RBC (×10^6/μL)	WBC (×10^3/μL)	Platelets (×10^9/μL)	Hb (g/dL)	ESR (mm)
Normal	9.33±0.21	12.33±0.33	915.20±28.75	15.33±0.42	3.23±0.27
AIA control	4.67±0.33$^{\#}$	19.83±0.48$^{\#}$	1630.00±23.19$^{\#}$	10.00±0.52$^{\#}$	8.57±0.31$^{\#}$
LF (10)	8.17±0.48$^{*,\$}$	13.50±0.43$^{*,\$}$	970.20±23.70$^{*,\$}$	15.50±0.34$^{*,\$}$	3.87±0.34$^{*,\$}$
J (10)	5.17±0.48	18.67±0.56	1579.00±21.95	11.00±0.37	8.21±0.22
J (20)	7.00±0.37$^{*,\$}$	16.67±0.33$^{*,\$}$	1372.00±26.94$^{*,\$}$	12.67±0.42$^{*,\$}$	6.09±0.23$^{*,\$}$
J (40)	7.67±0.33$^{*,\$}$	13.83±0.48$^{*,\$}$	1122.00±24.84$^{*,\$}$	13.33±0.42$^{*,\$}$	4.50±0.31$^{*,\$}$

Figures in the parenthesis indicate a dose in mg/kg (n=6), data was analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. $^{\#}P$<0.05 as compared to normal group; $^{*}P$<0.05 as compared to AIA control group and $^{\$}P$<0.05 as compared to each other. AIA: Adjuvant induced arthritis; LF: Leflunomide; J: Juglanin; RBC: Red blood cells; WBC: White blood cells; ESR: Erythrocyte sedimentation rate; BSL: Blood sugar level

Hepatic tumor necrosis factor-alpha, interleukin-1 beta, interleukin-6, transforming growth factor-beta, cyclooxygenase-2, and inducible nitric oxide synthase mRNA expressions

Administration of FCA marked (P < 0.05) upregulated levels of hepatic TNF-α, ILs, TGF-β, COX-2, and iNOS mRNA expressions of AIA control group when compared with normal group. Leflunomide (10 mg/kg) treatment noticeably (P < 0.05) downregulated the levels of hepatic TNF-α, ILs, TGF-β, COX-2, and iNOS mRNA expressions when compared with that of the AIA group. Juglanin at doses of 20 and 40 mg/kg suggesting marked (P < 0.05) inhibition in the FCA-induced upregulated hepatic TNF-α, ILs, TGF-β, COX-2, and iNOS mRNA expressions when compared with AIA group.

When compared with juglanin treatment, leflunomide (10 mg/kg) treatment more efficiently ($P < 0.05$) downregulated the level of hepatic TNF-α, TGF-β, and COX-2 mRNA expressions, whereas juglanin (40 mg/kg) markedly ($P < 0.05$) downregulated ILs and iNOS mRNA expressions when compared to leflunomide (10 mg/kg) treatment [Figure 2].

Table 4: Effects of juglanin on Freund's complete adjuvant-induced alterations in serum lipid profile in arthritic rats

Treatment	Triglyceride (mg %)	Total cholesterol (mg %)	HDL-C (mg %)	LDL-C (mg %)	VLDL-C (mg %)
Normal	63.57±5.13	15.24±1.29	26.14±0.96	3.86±0.64	12.71±1.03
AIA control	141.20±3.92$^{\#}$	41.35±1.50$^{\#}$	3.96±1.27$^{\#}$	17.15±0.79$^{\#}$	28.24±0.78$^{\#}$
LF (10)	77.13±5.76$^{*,\$}$	22.60±1.44$^{*,\$}$	20.99±1.64$^{*,\$}$	8.78±0.79$^{*,\$}$	15.43±1.15$^{*,\$}$
J (10)	132.10±4.72	37.63±1.54	7.40±1.33	15.06±1.02	26.42±0.94
J (20)	118.40±5.98$^{*,\$}$	29.50±0.99$^{*,\$}$	14.61±0.91$^{*,\$}$	14.00±1.09$^{*,\$}$	23.68±1.20$^{*,\$}$
J (40)	88.24±5.04$^{*,\$}$	24.52±1.19$^{*,\$}$	16.66±1.33$^{*,\$}$	9.94±0.88$^{*,\$}$	17.65±1.01$^{*,\$}$

Figures in the parenthesis indicate a dose in mg/kg (n=6), data was analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. $^{\#}P$<0.05 as compared to normal group; $^{*}P$<0.05 as compared to AIA control group and $^{\$}P$<0.05 as compared to each other. AIA: Adjuvant induced arthritis; LF: Leflunomide; J: Juglanin; HDL-C: High-density lipoprotein-cholesterol; LDL-C: Low-density lipoproteins-cholesterol; VLDL-C: Very low-density lipoprotein-cholesterol

Table 5: Effects of juglanin on Freund's complete adjuvant-induced alterations in liver antioxidant parameters and rheumatoid factor in arthritic rats

Treatment	SOD (U/mg of protein)	GSH (µg/mg of protein)	MDA (nM/mg of protein)	NO (µg/mL)	RF (IU/mL)
Normal	3.22±0.15	2.49±0.05	0.33±0.04	107.50±11.87	0.00±0.00
AIA control	0.53±0.20$^{\#}$	0.34±0.13$^{\#}$	3.15±0.04$^{\#}$	482.30±12.91$^{\#}$	56.91±3.91$^{\#}$
LF (10)	2.74±0.17$^{*,\$}$	2.05±0.10$^{*,\$}$	1.07±0.02$^{*,\$}$	152.90±14.48$^{*,\$}$	30.23±6.06$^{*,\$}$
J (10)	1.31±0.14	0.71±0.09	2.83±0.05	448.60±15.03	50.73±3.09
J (20)	1.86±0.14$^{*,\$}$	1.07±0.10$^{*,\$}$	1.71±0.06$^{*,\$}$	353.60±14.32$^{*,\$}$	34.91±5.21$^{*,\$}$
J (40)	2.27±0.16$^{*,\$}$	1.86±0.09$^{*,\$}$	1.06±0.05$^{*,\$}$	197.00±10.71$^{*,\$}$	29.58±2.25$^{*,\$}$

Figures in the parenthesis indicate a dose in mg/kg (n=6), data was analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. $^{\#}P$<0.05 as compared to normal group; $^{*}P$<0.05 as compared to AIA control group and $^{\$}P$<0.05 as compared to each other. SOD: Superoxide dismutase; GSH: Glutathione peroxidase; MDA: Malondialdehyde; NO: Nitric oxide; RF: Rheumatoid factor

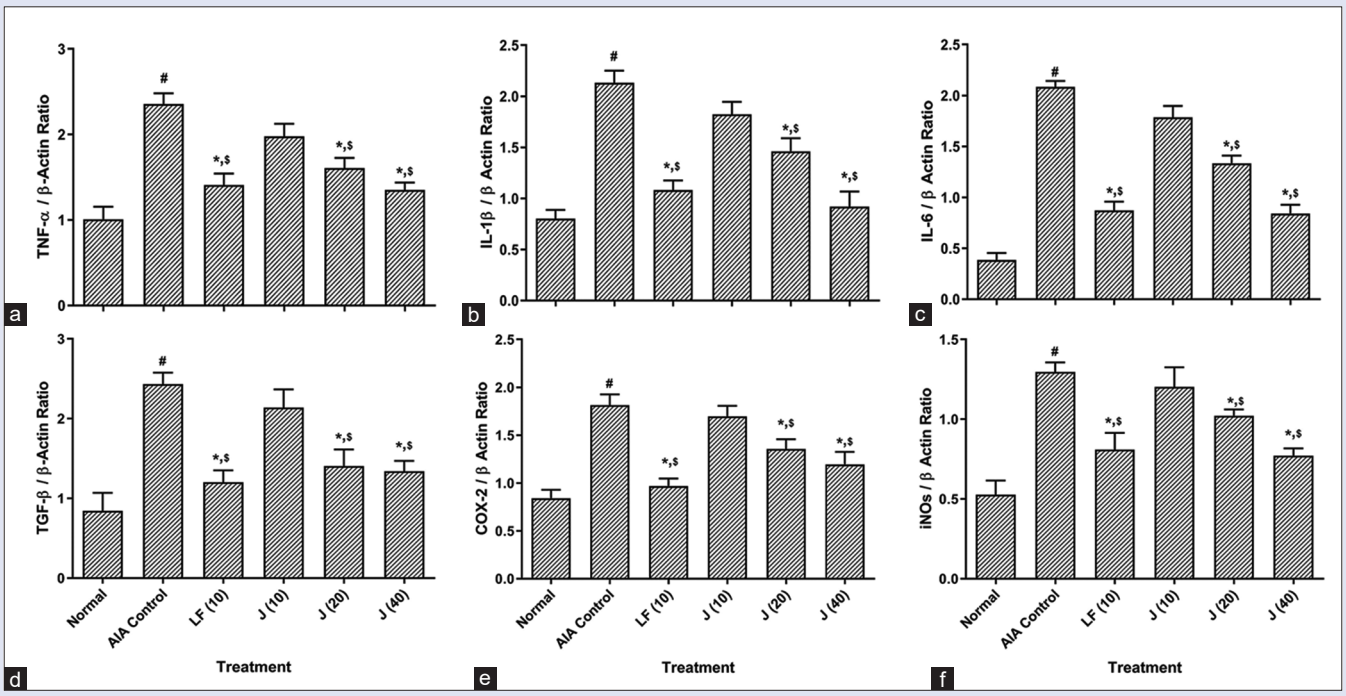

Figure 2: Effects of juglanin on FCA induced alterations in hepatic TNF-α (a), IL-1 β (b), IL-6 (c), TGF-β (d), COX-2 (e) and iNOs (f) mRNA expression in arthritic rats. Data are expressed as mean ± standard error mean (n = 4) and analyzed by one-way ANOVA followed by Tukey's multiple range test. $^{\#}P < 0.05$ as compared to normal group, $^{*}P < 0.05$ as compared to the AIA-control group, and $^{\$}p < 0.05$ as compared to one another. AIA: Adjuvant-induced arthritis, LF: Leflunomide, J: Juglanin, TNF-α: Tumor necrosis factor-alpha, IL-1 β: Interleukin-1 beta, Nrf2: Nuclear factor-like 2, HO-1: Heme Oxygenase-1, TGF-β: Transforming growth factor-Beta

Hepatic NF-κB, IκBα, a disintegrin-like and metalloproteinase domain with thrombospondin-1 repeats-4 and a disintegrin-like and metalloproteinase domain with thrombospondin-1 repeats-5 protein expressions

The FCA administration marked ($P < 0.05$) regulation of hepatic NF-κB, IκBα, ADAMTS-4, and ADAMTS-5 protein expressions in the AIA control group when compared with that of the normal group. Leflunomide (10 mg/kg) treatment efficiently ($P < 0.05$) inhibited and upregulated hepatic NF-κB, IκBα, ADAMTS-4, and ADAMTS-5 protein expressions when compared with that of the AIA control group. Juglanin at doses of 20 and 40 mg/kg suggested marked ($P < 0.05$) downregulation of hepatic NF-κB, IκBα, ADAMTS-4, and ADAMTS-5 protein expressions when compared with AIA group. Leflunomide (10 mg/kg) noticeably ($P < 0.05$) downregulated the hepatic NF-κB, IκBα, ADAMTS-4, and ADAMTS-5 protein expressions when compared to juglanin treatment [Figure 3].

Tibiotarsal joint histopathology

Histopathological analysis of the tibiotarsal joint of the normal group is found to be devoid of inflammatory cell infiltration, synovial proliferation and destruction, pannus formation, and joint destruction [Figure 4a]. FCA administration marked ($P < 0.05$) increased inflammatory infiltration, with cartilage and synovial destruction, necrosis, and pannus formation in AIA group, when compared with normal group [Figure 4b]. Leflunomide (10 mg/kg) treatment noticeably ($P < 0.05$) decreased inflammatory infiltration, necrosis, and cartilage destruction [Figure 4c]. Juglanin (10 mg/kg) suggested the presence of inflammatory cells, cartilage, and synovial destruction [Figure 4d]. However, juglanin at doses of 20 and 40 mg/kg treatment showed marked ($P < 0.05$) attenuation in FCA-induced high inflammatory cells, cartilage, and synovial destruction when compared with AIA group [Figure 4e-g].

DISCUSSION

RA is considered an autoimmune inflammatory disease affecting multiple joints and causes progressive destruction of articular and

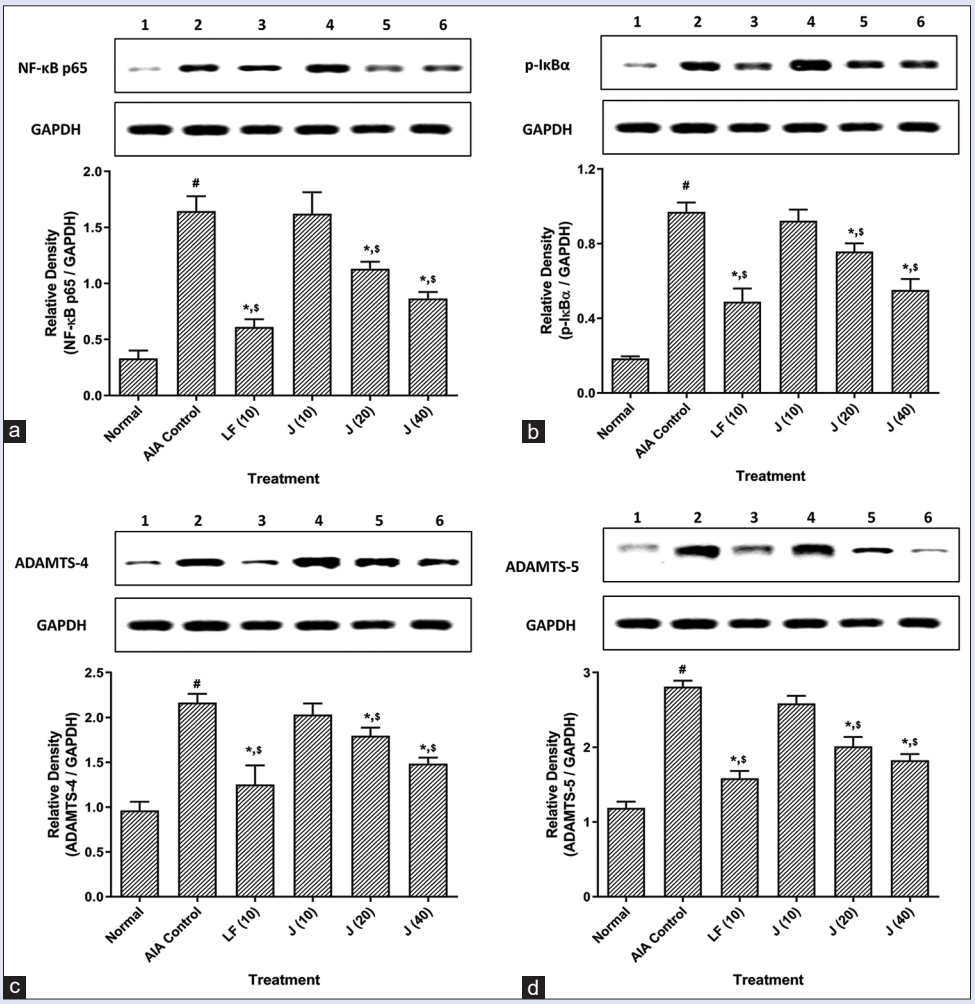

Figure 3: Effects of juglanin on FCA induced alterations in hepatic NF-κB (a), IκBα (b), ADAMTS-4 (c), and ADAMTS-5 (d) protein expressions in arthritic rats. Data are expressed as mean ± standard error mean ($n = 4$) and analyzed by one-way ANOVA followed by Tukey's multiple range test. $^{\#}P < 0.05$ as compared to normal group, $^{*}P < 0.05$ as compared to the AIA-control group, and $^{\$}P < 0.05$ as compared to one another. Lane 1: Protein expression of normal rats; Lane 2: Protein expression of AIA control rats; Lane 3: Protein expression of Leflunomide (10 mg/kg, p. o.) treated rats; Lane 4: Protein expression of juglanin (10 mg/kg, p. o.) treated rats; Lane 5: Protein expression of juglanin (20 mg/kg, p. o.) treated rats and Lane 6: Protein expression of juglanin (40 mg/kg, p. o.) treated rats. AIA: Adjuvant-induced arthritis, LF: Leflunomide, J: Juglanin, NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells, IκBα: Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha, ADAMTS: A disintegrin and metalloproteinase with thrombospondin motifs

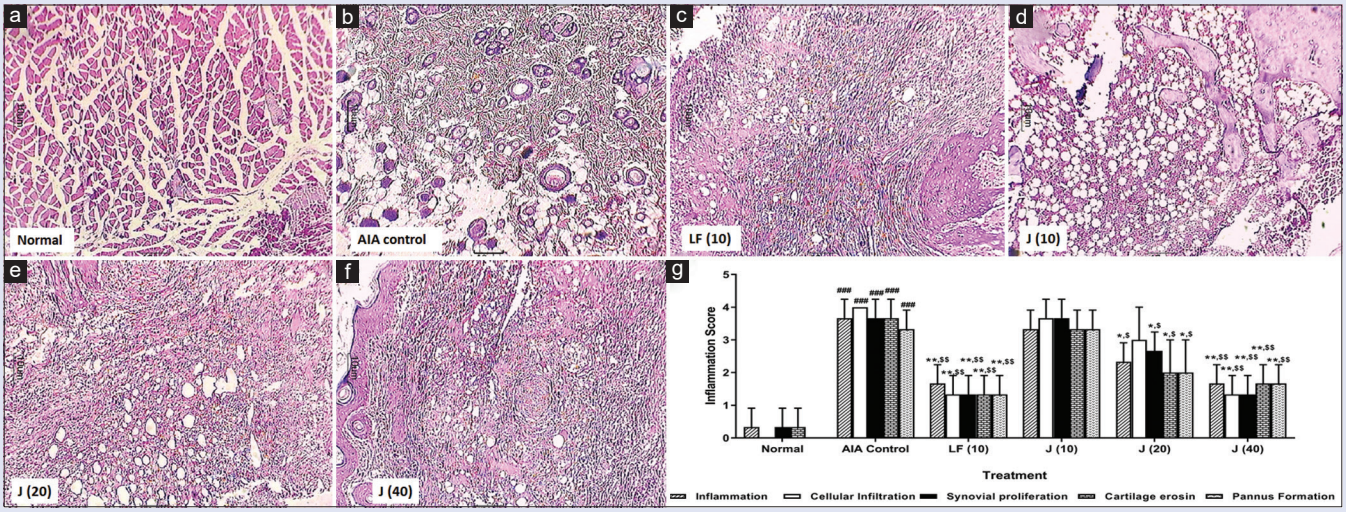

Figure 4: Effects of juglanin on the histopathology of tibiotarsal joints after administration of FCA. Representative histological images from (a) normal, (b) AIA control, (c) leflunomide (10 mg/kg), (d) juglanin (10 mg/kg), (e) juglanin (20 mg/kg), and (f) juglanin (40 mg/kg) treated rat. Images stained with H and E (×100). The quantitative representation of histological score (g). Data were expressed as mean ± standard error mean ($n = 3$), and one-way ANOVA followed by the Kruskal-Wallis test was applied for *post hoc* analysis. $^{\#}P < 0.05$ as compared to normal group, $^{*}P < 0.05$ as compared to the AIA-control group, and $^{\$}P < 0.05$ as compared to one another. AIA: Adjuvant-induced arthritis, LF: Leflunomide, J: Juglanin

periarticular structures, thus worsening the quality of life. Despite progressive research in pharmacological mediations toward the management of RA, this disease is still highly inactivating. Therefore, therapeutic moieties from natural origin with a novel approach deliver greater promise in the clinical management of RA. FCA-induced arthritis is an extensively used animal model used in an array of studies for recognizing potential therapeutic targets. Adjuvant-induced arthritis (AIA) have has a clinicopathological semblance with RA along with elevated inflammatory influx, which plays an imperative role in the etiology of RA.[1,2] In the present study, intradermal FCA administration resulted in marked arthritis induction that characterized by signs of hyperalgesia, lack of mobility, and increased joint diameter. However, administration of juglanin attenuated changed hyperalgesia and mechano-tactile allodynia through downregulation of oxido-nitrosative stress, cytokines (TNF-α and ILs) levels, immune-inflammatory (TGF-β, NF-κB, Ikβα, COX-2, and iNOS) mediators, and ADAMTS, thus employing its anti-arthritic potential.

Administration of intoxicants such as FCA in laboratory animals over limited period results in the induction of adverse events that can be evaluated by a number of parameters such as body weight, food intake, relative organ weights, essential organs pathology, biochemistry, and hematology. The previous researcher also documented that modification in body weight during AIA induced arthritis to provide insight into disease state.[1,2] Additionally, the release of TNF-α and ILs during the acute inflammation caused inhibition in leptin activity that features to decreased body weight. Besides, the spleen is a vital lymphoid organ which plays a critical role in immune responses, and during the immune-inflammatory disease such as arthritis, spleen acts as cells and antibody storage that linked with elevated immune inflammatory response. During the FCA-induced arthritis, the spleen was inflamed with altered hepatic function, which was reflected by increased spleen weight and liver dysfunction, which is in line with the suggestion of the previous investigator.[16] The inhibition in the splenic enlargement and cachexia by the juglanin treatment provides an innuendo to the ability of juglanin to modulate the immune system.

Changes in complete cell count is an assurance of hematological alteration clinically.[1] It has been suggested that administration of test moiety releases macrophages, which may cause inhibition in the production of various elements in the bone marrow such as erythropoietin, colony-stimulating factors, and thrombopoietin[17] that may lead to an alteration in the hematological count. A noteworthy increase in the platelet and WBC count in the AIA has been reported by various researchers,[1,17] which may be due to the activation of the immune system against invading stress. An elevated IL-1β inflammatory response in AIA caused a subsequent rise in colony-stimulating factors that caused an increase in WBC count. Besides, premature destruction of RBC and decreased levels of erythropoietin caused reduction of Hb level during arthritis. Additionally, inflammatory response caused elevated concentration of acute-phase reactant proteins such as ESR in plasma. In this study, the alteration in hematological count in AIA rats after administration of FCA falls in accordance with the reports of the previous researcher.[1] Administration of juglanin noticeably diminished the elevated levels of WBCs, platelet, and ESR as well as increased levels of Hb and RBCs in AIA-induced arthritis, which might be due to inhibition of the release of an inflammatory mediator.

Determination of levels of an array of biomarkers such as ALT, AST, and ALP can be considered an important tool for the evaluation of anti-arthritic potential of the moiety.[1,16] It was well documented that elevated activities of ALT, AST, and ALP were measured as indices of hepatic and renal dysfunction, which is also an essential feature of AIA.[1] Elevated levels of serum ALT and AST reproduce the formation of bradykinins that is a hallmark of inflammatory response.[2,9] Findings of this study recommend that FCA administration resulted in marked elevated serum ALT, AST, and ALP levels; however, juglanin treatment expressively attenuated FCA-induced alteration in these biomarkers.

The high amount of circulating lipids within the bloodstream delivers insight into the disease state of various pathological diseases, including rheumatic arthritis.[1] Hyperlipidemia is a disease depicted by elevated levels of serum lipoprotein or cholesterol. Previous researchers also established that lipid-related disorders owe their origin to high serum cholesterol.[1] The significant transporters of cholesterol tissues having atherogenic potential are the LDLs, while the HDL transports excess cholesterol from peripheral tissues to the liver. Thus, HDL defends against numerous health issues. Also, cholesterol played a significant

role in membrane stabilization due to its rigid planar structure. Thus, changes in the levels of the lipid profile are linked with an increased risk of oxidative stress. A study proposed that RA patients displays increased levels of serum lipid via altering hepatic LDL activity or changing the LDL production rate.[18] The present study suggesting that FCA-induced arthritic rats showed increased serum lipid profile; in contrast, administration of juglanin attenuated these elevated levels. Research carried out by previous researchers also showed that the administration of juglanin reduced plasma TG and TC in rats,[12] and the findings of a current investigator is accordance with proposals of earlier researchers.[12]

Studies have documented the participation of ROS in RA pathophysiology.[1] Generation of RO species has been suggested to change the joint cavity followed by formation of edema. Lipid peroxidation is energetic pathophysiological mechanism of injury exist during RA, which can be determined by tissue MDA levels.[1] Whereas, SOD is an essential first-line antioxidant enzyme that plays a significant role in the conversion of superoxide anion to water and oxygen via formation of hydrogen peroxide. It is also reported that arthritis patients linked with a decreased level of SOD.[1,2] GSH is an intracellular antioxidant that suggesting a significant role during protecting the cells from oxidative injury. In this study, the elevated levels of MDA in the AIA group are associated with free radicals medicated damage, which in turn diminished the levels SOD and GSH, which consistent with results of earlier researcher.[1] However, juglanin treatment noticeably attenuated elevated oxidative stress by virtue of its scavenging property against free radicals.

Leukocytes induced release of TNF-α and ILs display a significant role in the induction and progression of RA.[3] It was suggested that antigen-primed helper T cells and activated macrophages are the originators for these pro-inflammatory cytokines.[3] These cytokines have been documented as critical biomarkers in clinical and experimental settings during RA.[5] Release of these pro-inflammatory cytokines revealed by various features of inflammatory RA, including inflammation in synovial tissue, proliferation of synovium, destruction of bone and cartilage. Also, CRP is another important marker of inflammation that has been dramatically raised during inflammation reaction in RA. The previous researcher documented that increased TNF-α, and ILs levels in arthritic rats after administration of FCA,[1] and the present result study is in line with suggestion of the earlier researcher. Juglanin expressively inhibited FCA induced elevated levels of TNF-α, and ILs reflecting it's inhibitory potential against the inflammatory mediators that might inhibit pathogenesis of RA.

The researcher has documented that iNOS and COX-2 are inducible inflammatory enzymes plays a vital role in the pathogenesis of inflammatory responses.[1] Cytokine including IL's has an ability to modulate the expression of nitric oxide (NO) that in turn cause bone resorption and cartilage destruction.[3] Also, COX-2 is a bifunctional enzyme catalyzing the formation of prostaglandins from arachidonic acid. Studies have reported that COX-2 expression after inflammatory infiltration that sensitizes peripheral terminals of sensory fibers indirectly.[11] Furthermore, several researchers reported a significant increase in hepatic COX-2 expression.[11] Thus, inhibition of these inducible inflammatory enzymes measured an important strategy for the amelioration of RA. Findings of present study showed that FCA administration results in marked up-regulation in mRNA expression of hepatic iNOS and COX-2, whereas administration of juglanin attenuated these upregulations. This inhibitory potential of juglanin is in accordance with suggestion of previous research that reported the anti-inflammatory mechanisms of juglanin via inhibition of COX-2 and iNOS expression.[13]

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) has been documented to perform a central dogma role during the release of inflammatory markers (COX-2) and cytokines (TNF-α and IL's).[15] In the normal cell, NF-κB absorbed by the IκB (Inhibitor kappa B) protein and persisted inactivated state in the cytoplasm. IκB Kinase (IKK)-α and IKKβ are two subunits of the IKK complex that regulates and control the IκB production[15] However, phosphorylation followed by degradation of IκB results in activation of NF-κB and its transfer to the nucleus where it drives the pro-inflammatory cytokines and inflammatory mediators.[13] The current study displayed the up regulated protein expression of p-IκBα along with NF-κB. Furthermore, the subunit p65 is one of the predominant forms of NF-κB, has been implicated during inflammation and hyperalgesia.[1] The elevated expressions of NF-κB p65 in AIA control rats recommended the induction of hyperalgesia reflected by altered paw withdrawal latency and threshold. The previous researcher showed that juglanin attenuated elevated IκBα and NF-κB expressions,[14] and which is in rationale with findings of present study, which might be responsible for its antinociceptive potential.

Aggrecan (ACAN), also known as cartilage-specific proteoglycan core protein, and ADAMTS (A Disintegrin-like and Metalloproteinase domain with Thrombospondin-1 repeats) is a member from aggrecan family.[19] It is well reported that ADAMTS-4 and ADAMTS-5 activity significantly expressed in synovial fluid and cartilage of patients with RA, and these are the primary enzymes that play vital roles during the degradation of aggrecan and collagens in cartilage.[19] Recent studies established the effect of TNF-α, IL's, and TGF-β on the expression of ADAMTS-4 and ADAMTS-5, which results in formation of osteoclast and thus resulted in joint destruction.[19,20] Data of the present study proposed that AIA administration associated with elevated expressions of ADAMTS-4 and ADAMTS-5, which might, in turn, caused cartilage and joint destruction. However, the administration of juglanin inhibits the AIA-induced up regulated ADAMTS-4 and ADAMTS-5 expression via inhibition of pro-inflammatory cytokines, thus employs its protective potential against joint destruction. Recently, Chen *et al.* documented the protective effect of juglanin in human chondrocytes via modulation of response of TNF-α, IL-1 β, iNOS, COX-2, NF-κB, ADAMTS-4 and ADAMTS-5 *in vitro*[20] and findings of the present study are in accordance with proposal of earlier researcher.[20]

Nowadays, many medical practitioners have frequently been prescribing NSAIDs and immunosuppressants for treating inflammatory and arthritic diseases. However, few are associated with serious adverse including retention of water and salt, gastric mucosal damage, etc., Thus, alternative agents from the herbal origin with fewer severe side effects have been broadly implicated clinically for the management of RA.[21,22] Researchers have studied various isolated moieties from plant origin, including pycnogenol, curcumin, gingerols, bromelain for the treatment, and prevention of arthritis.[23] Thus, the current study investigated that juglanin can be considered one of the important moieties from plant origin for the management of arthritis.

CONCLUSION

The results of the present investigation proposed that juglanin possesses anti-arthritic potential against adjuvant (FCA)-induced arthritis via attenuation of changed mechano-tactile allodynia and hyperalgesia in experimental rats. This anti-arthritic activity composed via downregulation of elevated oxido-nitrosative stress, cytokines (TNF-α and ILs) levels, immune-inflammatory (TGF-β, NF-κB, Ikβα, COX-2, and iNOS) mediators and ADAMTS.

Financial support and sponsorship

Basic research project of Natural Science of Shaanxi Province (No. 2020JQ-998).

Conflicts of interest

There are no conflicts of interest.

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