Pentahydroxy flavonoid isolated from *Madhuca indica* ameliorated adjuvant-induced arthritis: Possible role of TNF-α, IL's, NF-kβ, Ikβα, COX-2, P2X7

Yongliang Tang
Xian Jiaotong University: Xi'an Jiaotong University
Daotao Xie
Xian Jiaotong University: Xi'an Jiaotong University
Wenqing Gong
Xian Jiaotong University: Xi'an Jiaotong University
Hongtao Wu
Xian Jiaotong University: Xi'an Jiaotong University
Yi Qiang (coke44pjy@sina.com)
Northwest Hospital https://orcid.org/0000-0001-9476-5677

Research Article

Keywords: ,5,7,3',4'- Pentahydroxy flavone, Freund's Complete Adjuvant, Rheumatoid Arthritis, TNF-α, IL-1β, IL-6, NF-kβ, Ikβα, COX-2, P2X7

Posted Date: April 12th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-413694/v1

Abstract

Background

Rheumatoid arthritis (RA) is a chronic autoimmune disorder associated with progressive joint disability. *Madhuca indica* J. F. Gmel (family Sapotaceae) is an Indian medicinal plant reported to have an array of pharmacological properties.

Objective

To evaluate the anti-arthritic activity of isolated phytoconstituent from methanolic extract of *Madhuca indica* Leaves (MI-ALC) and its possible mechanism of action in FCA induced experimental arthritis.

Materials and methods

Polyarthritis was induced in female Wistar rats by intradermal administration of FCA (0.1 ml) into the tail. Polyarthritis was allowed to develop for the next 32 days. Then rats were treated with isolated phytoconstituent from MI-ALC (5, 10, and 20 mg/kg, p.o.)

Results

HPTLC, FTIR, and LC-MS spectral analysis of phytoconstituent isolated from MI-ALC confirmed the structure as 3,5,7,3',4'- Pentahydroxy flavone (i.e., QTN). Treatment with QTN (10 and 20 mg/kg) showed significant inhibition ($p < 0.05$) in FCA-induced increased paw volume, joint diameter, paw withdrawal threshold, and latency. The elevated synovial oxido-nitrosative stress and protein levels of TNF-α, IL-1β, and IL-6 were significantly reduced ($p < 0.05$)by QTN. Western blot analysis revealed QNT significantly ameliorated ($p < 0.05$) up-regulated NF-kβ, Ikβα, COX-2, and P2X7 protein expressions.

Conclusion

QTN ameliorates FCA-induced hyperalgesia via inhibition of elevated oxido-nitrosative stress, inflammatory mediators (NF-kβ, Ikβα, COX-2, and P2X7), and pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in experimental rats.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, autoimmune, and inflammatory disease mainly characterized by inflammation in the joint and synovial membrane, deformity, destruction of cartilage, and pain, leading to loss of joint function restricted limbs motions [1, 2]. RA shows symmetrical progression affecting

metacarpophalangeal and metatarsophalangeal joints, proximal interphalangeal, ankle, and wrist [3]. This immune-inflammatory arthritis affects almost 1–2%, i.e., 20 million of the general population worldwide, and the incidence is three times higher in women between the age of 40 and 50 years than men [4, 5]. It is associated with significantly higher lifetime costs along with a poor quality of life.

Researchers documented that the influx of immune-inflammatory cells and the release of pro-nociceptive and pro-inflammatory mediators into joints leads to synovial inflammation [2, 4]. The release of inflammatory mediators such as prostaglandins and leukotrienes, also activates primary nociceptive fibers, which results in joint hyperalgesia and nociception [2]. Moreover, cyclooxygenases (COX) enzymes play a vital role in activating immune cells and the induction of inflammatory cytokines [6, 7]. Furthermore, elevated generation of free radicals, including hydroxyl radicals, reactive oxygen species (ROS), and reactive nitrogen species (RNS), have an ability to modulate the structure of membrane lipids, DNA, proteins, and cartilage [2]. Thus, the generation of free radicals is suggested as one of the important mechanisms during the pathogenesis of RA [2]. Based on such underlying mechanisms, significant effort has been taken during the last few decades to develop new therapeutic moieties for RA management.

The use of therapies such as Non-steroidal anti-inflammatory drugs (NSAIDs) (such as aceclofenac, ibuprofen), disease-modifying anti-rheumatic drugs (DMARDs) (such as Cyclosporin A, methotrexate), anti-CD20 and anti-tumor necrosis factor (anti-TNFα) (such as adalimumab, infliximab) are currently available remedies for the management of RA [2]. These treatments halt the progression of disease via suppression of the immunological response. However, these therapies provide symptomatic relief in the fraction of patients, as well as are associated with several side effects such as gastrointestinal discomfort, an increase in cardiovascular risk, and nephrotoxicity [2]. Thus, there is a need for conventional and alternative therapy with significant safety and efficacy.

Natural products, especially those derived from herbs, have contributed to the development of modern therapeutic drugs since ancient times. Herbal medicinal products target specifically defined mediators of inflammation and pain. The major benefit of using herbs and other natural products lies in their limited or no undesirable side effects [8]. Therefore, the interdisciplinary efforts of researchers are aimed at identifying new herbal products, and defining their mechanisms of action has been reinforced. This has facilitated the discovery and development of safe and effective natural products to treat chronic pain [1]. Various animal models of Adjuvant induced arthritis (AIA) have been utilized extensively to analyze the potential of such herbal moieties. Freund's complete adjuvant (FCA)-induced arthritis is one of the most employed AIA animal models of arthritis that possesses many common features with human rheumatoid arthritis [1, 2, 9, 10].

Madhuca indica J. F. Gmel (family Sapotaceae) is an Indian medicinal plant found in moist habitats and reported to have an array of pharmacological properties, including anti-inflammatory, hepatoprotective, cardioprotective, antiulcer, analgesic, antidiabetic, and wound healing [11–17]. The literature revealed that *Madhuca indica* contains chemical constituents such as quercitrin, quercetin, myricitrin, myricetin,

erythritol, β-carotene xanthophylls, n-octacosanol, n-hexacosanol, palmitic acid, oleanolic acid, etc. [18]. A recent study on Pentahydroxy flavone, an isolated phytoconstituents from methanol extract of *Madhuca indica*, showed antiulcer activity via inhibition of cyclooxygenase (COX)-II, TNF-α, IL-1β, and iNOs [16]. The researcher showed that Pentahydroxy flavone isolated from *Madhuca indica* modulated expression of Nrf2 (nuclear factor-like 2) and PPAR-γ (peroxisome proliferator-activated receptor-gamma) as well as attenuated elevated oxidative stress to exert its cardioprotective activity [17]. However, its anti-arthritic activity has not yet been evaluated. Thus, the objective of the present investigation was to evaluate the anti-arthritic activity of phytoconstituent isolated from the methanolic extract of *Madhuca indica* Leaves and its possible mechanism of action in FCA induced experimental arthritis.

2. Material And Methods

2.1. Animals

Female Wistar rats (150–180 g) were obtained from the animal house of Xi'an Central Hospital. The rats were housed in polypropylene cages at 24 ± 1°C with a 12h:12h dark-light cycle, free access to standard pellet feed, and filtered water. All experiments were carried out between 08:00 h and 17:00 h in a quiet laboratory. The research protocol (no. XCH202013771) was approved by the Institutional animal ethics committee (IAEC) of Xi'an Central Hospital.

2.2. Chemicals and reagents

Rat specific TNF-α, IL-1β, and IL-6 enzyme-linked immunosorbent assay (ELISA) Kit were obtained from Bethyl Laboratories Inc., Montgomery, TX, USA. The primary antibodies of NF-kβ, Ikβα, COX-2, P2X7, and GAPDH were purchased from Abcam, Cambridge, MA, USA. Microliter syringe (Hamilton, Bonaduz, Switzerland) was obtained from Anchrome Enterprises (I) Pvt. Ltd (Mumbai, India). Methanol, ethyl acetate, toluene, formic acid, precoated thin layer chromatographic (TLC) silica gel plates (Kieselgel 60, F-254, 0.2 mm) was purchased from Merck Life Science Pvt Ltd, India. High-performance thin-layer chromatography (HPTLC) spectra were recorded on a Linomat V (Camag, Muttenz, Switzerland).

2.3. Preparation, isolation, and characterization of an isolated molecule from methanolic extract of *Madhuca indica*

It was carried out according to a previously reported method [17]. Briefly, weighed quantity (500 g) of air-dried powder (Mesh size-16) of the leaves of *Madhuca indica* J. F. Gmel. was macerated with distilled methanol at room temperature by soaking it with eventual stirring for 7 days and filtered. The filtrate was dried in a tray dryer maintained at 60°C. Semisolid methanolic extract of *Madhuca indica* J. F. Gmel. (MI-ALC) was obtained. The preliminary phytochemical screening of MI-ALC was carried out according to the earlier reported methods [17]. Phytochemical analysis of MI-ALC was performed for the identification of phytochemicals like alkaloids, flavonoids, steroid & phenols, etc.

MI-ALC further fractionates with chloroform as per the previously reported method [17]. Briefly, the parent methanolic extract (MI-ALC) was fractionated with chloroform. A weighed quantity (20 g) of the chloroform fraction was mixed with 70 mL of acetone and subjected to column chromatography (height, 60 cm; diameter, 3 cm) eluting with a mobile phase containing acetone: n-hexane (0.5:9.5). Elution was carried at the flow rate of 2 mL/min. Their polarity order increased the polarity of the mobile phase, and fractions (50 mL each) were collected in the amber-colored bottle. The remaining loaded material in the column that cannot be eluted with the mobile phase was eluted with methanol and collected as methanol fraction and completed column chromatography. All fractions were analyzed by HPTLC, and fractions showing similar compounds were pooled together and labeled alphabetically as A to H. The pooled fractions were fraction A (1–12), B (13–22), C (23–38), D (39–50), E (51–62), F (63–70), G (71–85) and H (86–112). Fractions A-G was obtained by the eluting column with the mobile phase (n-hexane: acetone 9.5:0.5).

A standard stock solution of quercetin was prepared by dissolving 10 mg of the drug in 10 ml of methanol to get a concentration of 1000 µg/ml. With the help of microsyringe (100 µL capacity), 5 µL of the solution was diluted to 100 µL to get a final concentration of 50 µg/mL (50 ng/µL). Different mobile phases containing various ratios of Benzene, Methanol, Acetone, Toluene, Formic Acid, and Ethyl acetate were examined. Finally, the mobile phase containing Toluene: Ethyl Acetate: Formic Acid (6: 3.5: 0.5 v/v/v) was selected as optimal for obtaining well-defined and resolved peaks. Other chromatographic conditions like chamber saturation time, run length, sample application rate, and volume, the distance between tracks, detection wavelength were optimized to give reproducible Rf value for the drug. The standard stock solution was scanned over 200–400 nm, and the spectra were obtained. It was observed that the drug showed considerable absorbance at 370 nm, as shown in **Supplementary File 1**.

A powdered extract (20 mg) was weighed and transferred to a 10 ml volumetric flask containing about 5 ml of methanol, ultrasonicated for 5 min. The solution was filtered using Whatman paper No. 41 and further diluted to 10 ml with methanol. 1 µl volume of sample solution was applied on the HPTLC plate. After chromatographic development, the peak area of the QTN spot in the sample chromatograph was measured, and the concentration of QTN in the sample was estimated from the calibration curve. The standard stock solution of quercetin (50 ng/µl) was applied on the HPTLC plate in a range of 1–10 µl with the help of CAMAG 100 µL sample syringe using Linomat 5 sample applicator to obtain a final concentration 50–500 ng/band for quercetin. The plate was developed and scanned under the above established chromatographic conditions. The calibration curve of quercetin was plotted of peak area vs. concentration.

The final drying of the test sample was carried out on a Rotavac under reduced pressure. Out of all the fractions, fraction D was further processed for isolation of active compound(s) by preparative TLC. From this fraction D, 5 compounds were isolated, labeled as D1, D2, D3, D4, and D5, respectively. It was further subjected to preparative HPTLC for the final purification of the compound. Isolation of active constituent was carried out by preparative HPTLC. The dried component was further analyzed by HPTLC for the

determination of R*f* and λmax. The chemical structure of the isolated compound was elucidated by FT-IR and LC-MS spectroscopy.

2.4. Adjuvant-induced polyarthritis (AIA)

On day 0 of the study, AIA was induced in female rats (150–200 g) (5 groups, i.e., group II to VI, n = 18) by a single intradermal injection of 0.1 ml FCA (Sigma Aldrich, St. Louis, USA) into the tail of the rats [19]. FCA contains 0.6 mg heat-inactivated Mycobacterium tuberculosis H37Ra emulsified in a sterile mixture of paraffin oil, saline, and Tween 80. The 32 days were allowed to develop arthritis. A separate group of rats (group I, n = 18) was maintained as normal and did not receive FCA. After the development of AIA (after 32 days), animals were received either distilled water (10 ml/kg, p.o. i.e., group I and II) or leflunomide (10 mg/Kg, as a standard, i.e., group III) or QTN (5, 10 and 20 mg/kg, i.e., group IV to VI) for next 28 days [17].

Paw volume was determined by using a plethysmometer (UGO Basile Italy) [20]. Pain latency against mechanical hyperalgesia (paw withdrawal threshold) was determined by using Randall-Selitto, i.e., paw pressure test (Ugo Basile Model 7200) as well as von Frey hair application [20]. Paw withdrawal latency, i.e., Hargreaves test, was performed according to the method described elsewhere using a standard apparatus (UGO Basile, SRL Biological Research Apparatus, Italy) [21, 22].

2.5. Blood withdrawal and biochemical analysis

On the last day of study (on day 60), rats were sacrificed by cervical dislocation, and blood was withdrawn for Erythrocyte Sedimentation Rate (ESR), C-reactive protein (CRP)[20] and hematological measurements (Red blood cell (RBC), hemoglobin (Hb), White blood cell (WBC), and platelets (PLT)). The serum was separated by centrifugation using an Eppendorf cryocentrifuge and used for serum turbidity measurement [23]. The levels of serum albumin, alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), and total cholesterol (TC) were measured by a spectrophotometer (UV-visible spectrophotometer, Jasco V-530, Tokyo, Japan) using a commercially available reagent kits according to the procedure provided by the manufacturer (Accurex Biomedical Pvt. Ltd., Mumbai, India).

2.6. Tissue preparation and biochemical analysis

All animals were sacrificed at the end of the study; the synovial tissues were immediately isolated. Tissue homogenates were prepared with 0.1 M Tris-HCl buffer (pH 7.4), and supernatant of homogenates was employed to estimate superoxide dismutase (SOD), reduced glutathione (GSH), lipid peroxidation (MDA), and nitric oxide (NO) as described previously [24]. The levels of TNF-α, IL-1β, and IL-6 were determined by Enzyme-linked immunosorbent assay (ELISA) using commercial kits (Thermo Fisher Scientific, USA) as per manufacturers' instructions.

2.7. Determination of NF-kβ, Ikβα, COX-2, and P2X7 protein expressions

Synovial tissue was sonicated in Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Inc.). The lysates were centrifuged at 10,000 X g for 10 min at 4°C. Protein concentration was determined using a Bicinchoninic Acid (BCA) assay kit (Beyotime Shanghai, China) on ice for 30 min. Equal amounts of extracted protein samples (50 µg) were separated by 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat dry milk at 37°C for 1 h and incubated overnight at 4°C with the primary antibodies that recognized NF-kβ, Ikβα, COX-2, P2X7, and GAPDH. Then they were incubated with secondary antibody at 37°C for 2 h. Protein bands were visualized using the Chemiluminescent kit (Bio-Rad Laboratories, Inc.), GAPDH served as the loading control.

2.8. Histopathology of tibiotarsal joint

Ankle joints from the three rats of each group were separated, cleaned, washed in cold physiological saline, and preserved in 10% formaldehyde solution until histopathological studies. At the time of staining, sections of tibiotarsal joints were cut (5 µm thickness) with the help of microtome, deparaffinated, and stained using hematoxylin and eosin (H and E) stain. The specimens were mounted on slides by the use of Distrene Phthalate Xylene (DPX). Sections were examined under a light microscope (Olympus DP71, DP-BSW Ver.03.03, Olympus Medical Systems India Private Limited, India) to obtain a general impression of the histopathology features specimen and infiltration of cells in epithelium and sub-epithelium. The intensity of histological aberrations in the tibiotarsal joint was graded as Grade 0 (not present or very slight)l; Grade 1 (mild); Grade 2 (moderate); and Grade 3 (severe) as described in the literature.

2.9. Statistical analysis

Data were expressed as Means ± standard error of the mean (SEM). All statistical tests were performed using Prism 5.0 (Graph Pad, San Diego CA, USA) statistical software. The data were considered significant at $p < 0.05$.

3. Results

3.1. Isolation, fractionation, and characterization of D3 (i.e., QTN) from MI-ALC

The percent yield of methanolic extract of leaves of *Madhuca indica* J. F. Gmel. (MI-ALC) was 26.8 %, and it showed the presence of glycosides, flavonoids, tannins, steroids, and phenolic compounds. The yield fraction D of MI-ALC (yellowish-green) was 5.42 g, and that of D3 (i.e., QTN) was 30.8 mg.

The HPTLC linearity profile of marker compound (quercetin) and QTN from 50 to 500 ng/µl at 370 nm shown in Supplementary File 1A and B. The regression analysis of the calibration curves showed that the correlation coefficient, intercept, and slope was 0.980, -550.1, and 40.50, respectively (Supplementary File 1C). The measurement of the peak area at three different concentration levels showed low values of %

R.S.D. (< 2%) for inter-and intra-day variation, which suggested an excellent precision of the method. The UV spectrum of the test sample is superimposable with that of standard quercetin, indicating the purity of the peak (Supplementary File 1D).

The chromatograph of marker compound (i.e., quercetin) was shown in Fig. 1A, whereas the chromatograph of isolated fraction QTN was shown in Fig. 1B. The compound QTN was obtained as an amorphous powder. It had a melting point of 307–309°C. FTIR spectrum of compound QTN showed in Supplementary File 2A. The LC-MS spectrum of QTN showed the molecular ion peak at m/z 302, indicating the molecular formula $C_{15}H_{10}O_7$ (Supplementary File 2B). The compound turned dark green with $FeCl_3$ indicated the presence of the phenyl group. The chemical structure was confirmed by comparing the melting point, FTIR, and LC-MS spectral data with those reported in the literature for 3,5,7,3',4'- Pentahydroxy flavone (i.e., QTN) (Supplementary File 2B)[17].

3.2. Effects of QTN on FCA-induced alteration in body weight, joint diameter, paw volume, paw withdrawal latency, and paw withdrawal threshold in arthritic rats

There was a significant decrease ($p < 0.05$) in body weight, whereas a significant increased ($p < 0.05$) in joint diameter, paw volume, paw withdrawal latency, and paw withdrawal threshold in AIA control rats as compared to normal rats. Administration of Leflunomide (10 mg/kg) significantly inhibited ($p < 0.05$) FCA-induced alterations in body weight, joint diameter, paw volume, paw withdrawal latency, and paw withdrawal threshold as compared to AIA control rats. Whereas administration of QTN (10 and 20 mg/kg) also significantly increase ($p < 0.05$) body weight and significantly decreased ($p < 0.05$) joint diameter, paw volume, paw withdrawal latency, and paw withdrawal threshold as compared to AIA control rats. (Table 1 and Fig. 2)

Table 1
Effects of QTN on FCA-induced alterations in body weight, joint diameter, paw volume, paw withdrawal latency, and paw withdrawal threshold (Von-Frey hair and paw pressure test) in arthritic rats

Treatment	AUC (Body weight (gm))	AUC (Joint diameter (mm))	AUC (Paw volume (ml))	AUC (Paw withdrawal latency (sec))	AUC (Paw withdrawal threshold (g))	AUC (Paw withdrawal threshold (g))
Normal	6299.00 ± 56.77	0.18 ± 0.05	0.18 ± 0.05	242.50 ± 6.92	1803.00 ± 41.74	8315.00 ± 72.92
AIA Control	5468.00 ± 37.06$^{\#}$	87.06 ± 0.44$^{\#}$	91.84 ± 1.62$^{\#}$	120.60 ± 7.28$^{\#}$	791.70 ± 24.85$^{\#}$	4884.00 ± 130.20$^{\#}$
LF (10)	5773.00 ± 96.24$^{*,\$}$	71.37 ± 0.99$^{*,\$}$	70.64 ± 0.84$^{*,\$}$	154.60 ± 7.94$^{*,\$}$	1037.00 ± 23.53$^{*,\$}$	5978.00 ± 160.60$^{*,\$}$
QTN (5)	5552.00 ± 103.6	86.18 ± 1.02	90.72 ± 0.58	127.80 ± 8.55	832.60 ± 12.94	5110.00 ± 106.30
QTN (10)	5681.00 ± 75.02$^{*,\$}$	79.64 ± 1.06$^{*,\$}$	81.50 ± 0.62$^{*,\$}$	142.90 ± 9.78$^{*,\$}$	897.20 ± 18.42$^{*,\$}$	5503.00 ± 45.96$^{*,\$}$
QTN (20)	5771.00 ± 72.76$^{*,\$}$	71.74 ± 0.85$^{*,\$}$	70.33 ± 0.96$^{*,\$}$	156.40 ± 8.66$^{*,\$}$	1034.00 ± 26.61$^{*,\$}$	5924.00 ± 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, QTN: 3,5,7,3',4'-Pentahydroxy flavone, AUC: Area Under Curve.

3.3. Effects of QTN on FCA-induced alteration in AST, ALT, ALP, albumin, CRP, and rheumatoid factor in arthritic rats

The levels of serum AST, ALT, ALP, and CRP were significantly ($p < 0.05$) increased, whereas serum albumin level significantly ($p < 0.05$) decreased in the AIA control rats as compared to normal rats. Leflunomide (10 mg/kg) treatment significantly ($p < 0.05$) increased serum albumin level whereas it significantly ($p < 0.05$) decreased serum AST, ALT, ALP and CRP levels as compared to AIA control rats. The FCA-induced alterations in serum AST, ALT, ALP, albumin, and CRP levels were significantly ($p < 0.05$) attenuated by QTN (10 and 20 mg/kg) as compared to AIA control. These alterations in levels of serum

AST, ALT, ALP, albumin, and CRP were more significantly ($p < 0.05$) attenuated by leflunomide (10 mg/kg) as compared to QTN treatment. (Table 2)

Table 2
Effects of QTN on FCA-induced alterations in serum AST, ALT, ALP, albumin, CRP, and rheumatoid factor levels in arthritic rats

Treatment	AST (U/ml)	ALT (U/ml)	Alkaline phosphatase (U/L)	Albumin (g/dl)	CRP (mg/L)	Rheumatoid factor (IU/mL)
Normal	39.95 ± 3.02	33.78 ± 1.83	69.24 ± 5.42	6.52 ± 0.38	0.99 ± 0.04	0.00 ± 0.00
AIA Control	122.90 ± 2.84$^{\#}$	165.80 ± 7.45$^{\#}$	435.00 ± 11.81$^{\#}$	2.00 ± 0.22$^{\#}$	7.65 ± 0.34$^{\#}$	60.25 ± 1.55$^{\#}$
LF (10)	59.56 ± 3.74$^{*,\$}$	51.11 ± 5.14$^{*,\$}$	136.7 ± 5.59$^{*,\$}$	6.25 ± 0.31$^{*,\$}$	2.60 ± 0.24$^{*,\$}$	38.58 ± 1.47$^{*,\$}$
QTN (5)	118.70 ± 4.26	146.60 ± 3.80	423.20 ± 6.50	2.25 ± 0.26	7.02 ± 0.18	56.96 ± 1.61
QTN (10)	88.02 ± 3.71$^{*,\$}$	90.45 ± 3.86$^{*,\$}$	276.40 ± 12.82$^{*,\$}$	4.05 ± 0.21$^{*,\$}$	4.74 ± 0.27$^{*,\$}$	47.86 ± 1.74$^{*,\$}$
QTN (20)	62.75 ± 1.42$^{*,\$}$	52.28 ± 3.82$^{*,\$}$	139.30 ± 7.38$^{*,\$}$	5.78 ± 0.22$^{*,\$}$	2.67 ± 0.13$^{*,\$}$	38.88 ± 0.97$^{*,\$}$

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, QTN: 3,5,7,3',4'-Pentahydroxy flavone, AST: Aspartate Aminotransferase, ALT: Alanine transaminase, CRP: C-reactive protein.

The subplantar administration of FCA resulted in a significant increase ($p < 0.05$) in the rheumatoid factor in AIA control rats compared to normal rats. When compared with as compared to AIA control rats, leflunomide (10 mg/kg) and QTN (10 and 20 mg/kg) treatment significant decreased ($p < 0.05$) rheumatoid factor. (Table 2)

3.4. Effects of QTN on FCA-induced alteration in hematological parameters and ESR levels in arthritic rats

There was a significantly decreased ($p < 0.05$) in RBCs and Hb levels, whereas a significant increase ($p < 0.05$) in WBCs, platelet, and ESR levels in AIA control rats as compared to normal rats. Administration of Leflunomide (10 mg/kg) significantly ameliorated ($p < 0.05$) FCA-induced alteration in hematological parameters and ESR levels as compared to AIA control rats. QTN (10 and 20 mg/kg) treatment significant increase ($p < 0.05$) RBCs and Hb levels whereas significant decreased ($p < 0.05$) WBCs, platelet and ESR levels as compared to AIA control rats. However, administration of Leflunomide (10 mg/kg) more

significantly ($p < 0.05$) attenuated FCA-induced alteration in hematological parameters and ESR levels as compared to QTN treatment. (Table 3)

Table 3
Effects of QTN on FCA-induced alterations in hematological parameters and ESR level in arthritic rats

Treatment	RBC (X 10^6/ µL)	WBC (X 10^3/ µL)	Hb (g/dL)	Platelets (X 10^9/L)	ESR (mm)
Normal	5.55 ± 0.23	5.78 ± 0.30	15.83 ± 0.52	912.80 ± 40.47	7.64 ± 0.23
AIA Control	1.95 ± 0.17$^{\#}$	12.45 ± 0.41$^{\#}$	9.81 ± 0.55$^{\#}$	1658.00 ± 35.94$^{\#}$	14.69 ± 0.27$^{\#}$
LF (10)	4.91 ± 0.26$^{*,\$}$	6.84 ± 0.29$^{*,\$}$	15.41 ± 0.73$^{*,\$}$	1013.00 ± 37.21$^{*,\$}$	9.97 ± 0.44$^{*,\$}$
QTN (5)	2.02 ± 0.21	11.92 ± 0.25	10.10 ± 0.43	1633.00 ± 38.45	14.30 ± 0.44
QTN (10)	2.94 ± 0.24$^{*,\$}$	9.55 ± 0.25$^{*,\$}$	12.16 ± 0.60$^{*,\$}$	1453.00 ± 37.97$^{*,\$}$	12.63 ± 0.42$^{*,\$}$
QTN (20)	4.45 ± 0.20$^{*,\$}$	6.96 ± 0.28$^{*,\$}$	15.39 ± 0.33$^{*,\$}$	1048.00 ± 27.22$^{*,\$}$	10.19 ± 0.43$^{*,\$}$

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, QTN: 3,5,7,3',4'-Pentahydroxy flavone, RBC: Red blood cells, WBC: White blood cells, ESR: Erythrocyte Sedimentation Rate.

3.5. Effects of QTN on FCA-induced alteration in synovial oxido-nitrosative stress in arthritic rats

The levels of synovial SOD and GSH were significantly decreased ($p < 0.05$), whereas levels of synovial MDA and NO was increased significantly ($p < 0.05$) in AIA control rats as compared to normal rats. Leflunomide (10 mg/kg) treatment significantly increased ($p < 0.05$) synovial SOD and GSH levels, whereas significant decreased ($p < 0.05$) synovial MDA and NO levels as compared to AIA control rats. Similarly, administration of QTN (10 and 20 mg/kg) significantly attenuated ($p < 0.05$) FCA-induced increased synovial oxido-nitrosative stress as compared to AIA control rats. However, elevated MDA and decreased GSH levels were more significantly ($p < 0.05$) attenuated by QTN (20 mg/kg) treatment as compared to Leflunomide (10 mg/kg). (Table 4)

Table 4
Effects of QTN on FCA-induced alterations antioxidant parameters and cytokines levels in synovial tissues of arthritic rats

Treatment	SOD (U/mg of protein)	GSH (µg/mg of protein)	MDA (nM/mg of protein)	NO (µg/mL)	TNF-α (pg/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)
Normal	5.75 ± 0.27	81.76 ± 2.57	0.97 ± 0.11	116.50 ± 13.63	34.91 ± 2.06	71.04 ± 4.01	79.24 ± 8.05
AIA Control	3.24 ± 0.26$^{\#}$	52.20 ± 3.35$^{\#}$	2.46 ± 0.09$^{\#}$	519.00 ± 11.21$^{\#}$	121.90 ± 6.66$^{\#}$	462.40 ± 11.04$^{\#}$	413.30 ± 12.58$^{\#}$
LF (10)	4.55 ± 0.25*,$^{\$}$	68.51 ± 2.09*,$^{\$}$	1.67 ± 0.13*,$^{\$}$	168.80 ± 14.86*,$^{\$}$	75.02 ± 4.57*,$^{\$}$	260.60 ± 11.06*,$^{\$}$	308.40 ± 14.17*,$^{\$}$
QTN (5)	3.31 ± 0.24	55.80 ± 1.67	2.39 ± 0.09	486.10 ± 13.86	115.90 ± 3.98	438.90 ± 5.96	391.10 ± 15.82
QTN (10)	3.99 ± 0.27*,$^{\$}$	63.13 ± 2.21*,$^{\$}$	1.95 ± 0.12*,$^{\$}$	400.40 ± 13.97*,$^{\$}$	97.11 ± 3.14*,$^{\$}$	349.20 ± 9.23*,$^{\$}$	346.50 ± 12.66*,$^{\$}$
QTN (20)	4.55 ± 0.24*,$^{\$}$	69.32 ± 1.52*,$^{\$}$	1.62 ± 0.13*,$^{\$}$	284.00 ± 14.63*,$^{\$}$	79.21 ± 3.03*,$^{\$}$	259.30 ± 5.91*,$^{\$}$	309.90 ± 13.19*,$^{\$}$

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, TNF-α: Tumor necrosis factor-alpha, IL's: Interleukins.

3.6. Effects of QTN on FCA-induced alteration in synovial tissue TNF-α, IL-1β, and IL-6 protein levels in arthritic rats

Intraperitoneal administration of FCA results in a significant increase ($p < 0.05$) in TNF-α, IL-1β, and IL-6 protein levels in the synovial tissue of AIA control rats as compared to normal rats. Leflunomide (10 mg/kg) treatment significantly inhibited ($p < 0.05$) FCA-induced elevated synovial TNF-α, IL-1β, and IL-6 protein levels as compared to AIA control rats. QTN (10 and 20 mg/kg) treatment also significantly decreased ($p < 0.05$) elevated synovial TNF-α, IL-1β, and IL-6 protein levels as compared to AIA control rats. The elevated synovial TNF-α and IL-6 protein levels were more significantly ($p < 0.05$) attenuated by leflunomide (10 mg/kg) as compared to QTN treatment. (Table 4)

3.7. Effects of QTN on FCA-induced alteration in NF-kβ, Ikβα, COX-2 and P2X7 protein expressions in synovial tissues in arthritic rats

The protein expression of NF-kβ, Ikβα, COX-2, and P2X7 in synovial tissues of AIA control rats was up-regulated significantly ($p < 0.05$) after FCA administration as compared with normal rats. Administration of Leflunomide (10 mg/kg) significantly ($p < 0.05$) down-regulated protein expression of NF-kβ, Ikβα, COX-2, and P2X7 in synovial tissues when compared to AIA control rats. However, leflunomide (10 mg/kg) treatment showed more significant ($p < 0.05$) down-regulation in protein expression of NF-kβ, Ikβα, and COX-2 in synovial tissues as compared with QTN treatment. Moreover, QTN (10 and 20 mg/kg) treatment also significantly ($p < 0.05$) inhibited FCA induced up-regulated protein expression of NF-kβ, Ikβα, COX-2, and P2X7 in synovial tissues as compared to AIA control rats. QTN (20 mg/kg) treatment showed more significant ($p < 0.05$) down-regulation in the protein expression of P2X7 in synovial tissues as compared to leflunomide (10 mg/kg) treatment. (Fig. 3)

3.8. Histopathology of the tibiotarsal joint in FCA-induced AIA in arthritic rats

Intraperitoneal administration of FCA resulted in significant histological aberrations in the tibiotarsal joint of AIA control rats reflected by significant increase ($p < 0.05$) in inflammatory infiltration, synovial proliferation, cartilage erosion, and pannus formation (Fig. 4B) as compared to normal rats. Whereas, tibiotarsal joint of normal rats showed the presence of minimal inflammatory infiltration, synovial proliferation, and pannus formation (Fig. 4A). Leflunomide (10 mg/kg) treatment significantly inhibited ($p < 0.05$) FCA-induced histological aberrations in tibiotarsal joint (Fig. 4C) as compared to AIA control rats. However, when compared with AIA control rats, QTN (5 mg/kg) treatment failed to produce any significant protection against FCA-induced histological aberrations in the tibiotarsal joint (Fig. 4D). Whereas, QTN (10 and 20 mg/kg) significantly decrease ($p < 0.05$) tibiotarsal joint of normal rats showed the presence of minimal inflammatory infiltration, synovial proliferation, and pannus formation (Fig. 4E and 4F) as compared to AIA control rats. (Fig. 4G)

4. Discussion

Rheumatoid arthritis (RA) is a chronic autoimmune illness mainly associated with progressive joint disability and cartilage damage due to the release of multiple inflammatory mediators. It has been well reported that females between the age of 40 and 50 have a significant risk for the development of RA than males [5]. Current treatment therapies such as NSAIDs, DMARDs, etc., have their limitations of well-known side effects, variations in efficacy, and high cost [2]. Thus, the various researcher has explored the safety and effectiveness of various therapeutic moieties from plant origin for RA management. *Madhuca indica* is a traditional medicine rich in various phytoconstituents dominant with the presence of flavonoids. It has been widely used to manage various inflammatory disorders due to its inhibitory

potential against histamine, serotonin, prostaglandin, and COX-2 [25]. Thus, in the present investigation, we have evaluated the anti-arthritic potential of isolated phytoconstituent (3,5,7,3',4'- Pentahydroxy flavone, i.e., QTN) from methanolic extract of *Madhuca indica* Leaves in female Wistar rats after subplantar administration of FCA. QTN exerts its antiarthritic potential via inhibition of oxido-nitrosative stress, pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), and NF-kβ, Ikβα, COX-2, and P2X7 expressions.

It has been reported that cachexia, i.e., marked decrease in body weight, is a characteristic feature of many chronic diseases, including cancer, heart or renal failure, diabetes, and Crohn's disease [26–31]. Clinically it has been shown that rheumatoid arthritis (RA) exhibited hypermetabolism and accelerated protein breakdown, which is a major reason for increased morbidity and premature mortality in those patients [32]. In the present study, there was a significant reduction in the body weight recorded in AIA control rats even before the manifestation of external signs of the illness, such as destruction of joint integrity and function disability. The result of the present study is in line with the findings of the previous researcher [33]. It has been reported that decreased body weight affected by immune inflammation and elevated levels of pro-inflammatory cytokines (such as TNF-α and IL's) are thought to play a vital role in the regulation of leptin activity [34]. In the present study, rats administered with FCA showed a significant reduction of body weight, which might be via a decrease in leptin levels, whereas administration of QTN showed the significant attenuation in FCA induced decreased body weight which might be due to inhibition of the release of inflammatory mediators.

During the inflammatory insult, the release of pro-inflammatory mediators (such as prostaglandin E2) and pro-inflammatory cytokines are responsible for the initiation of pain promotes nociceptor sensitization resulted in a decrease threshold [35, 36]. Most anti-inflammatory agents possess analgesic, i.e., reduction of allodynia and hyperalgesia as an essential ancillary property, which is widely utilized to increase pain threshold in various animal models [37–39]. It has been well established that AIA-induced arthritis is associated with altered allodynia and hyperalgesia [2, 10, 40], and in the present investigation also intradermal administration of FCA resulted in a significant reduction of allodynia and hyperalgesia evaluated by an array of behavioral assessment using Randall-Selitto, von Frey hair, and Hargreaves test. However, the administration of QTN showed significant attenuation in FCA induced alteration of allodynia and hyperalgesia by virtue of its anti-inflammatory potential.

The researcher showed that elevated paw thickness is evidence of arthritis induction [2, 10, 40, 41]. Determination of paw thickness using a plethysmometer is the well-established and standardized method during AIA-induced arthritis [2, 10, 40]. In the present study, the inflamed rat's edematous hind paw was estimated using a plethysmometer. It was further subjected to a constant force to assess the pain threshold that was examined by the Randall-Selitto assay method. Treatment with QTN significantly decreased paw thickness, which might be due to inhibition of the release of inflammatory mediators, indicating its anti-inflammatory potential in FCA-induced arthritis. This potential of cytokine blockage in pain nervous fibers by QTN might be responsible for increased pain threshold to exert its basis of analgesic effect. The presence of flavonoid moiety in the methanolic extract of *Madhuca indica* could be

responsible for its anti-inflammatory, analgesic, and anti-nociceptive activities. The present investigation results corroborate with the findings of the previous investigator where phytoconstituents isolated from methanolic extract of *Madhuca indica* showed anti-inflammatory potential via inhibition of TNF-α and IL-1β [16].

It has been reported that AIA is associated with the diminished Hb, and RBC levels represent the anemic condition of arthritic rats, which is a common diagnostic feature in patients with chronic arthritis [20, 42]. This decreased Hb, and RBC levels in AIA rats may be due to either sequestering or insufficient iron in the reticuloendothelial system and synovial or failure of bone marrow response erythropoietin along with the destruction of premature red blood cells [42]. It has been well documented that the release of inflammatory cytokines such as IL-1β played a vital role in this vicious cycle to bring about decreased Hb and RBC levels during acute phase response [26, 43–46]. Furthermore, a moderate rise in WBC count occurred during arthritic conditions due to the release of IL-1β and mediated increase in the colony-stimulating factors. Spleen played a major causative role in the shortened half-life of RBCs and subsequently anemia in AIA rats, which might cause splenic atrophy [2, 40]. Erythrocyte sedimentation rate (ESR) serves as an index of suspension stability of RBCs in plasma, and it's an indirect measurement of acute phase response for determining the disease activity in RA [40, 42]. In the present investigation, AIA rats exhibited decreased Hb and RBC levels along with increased ESR that is in line with the findings of the previous researcher [40, 42]. Thus, the reduction in the ESR and improvement in RBC and Hb count brought about by QTN treatment indicate the significant recovery from the anemic condition and further support its anti-arthritic effect.

In the present investigation, a battery of serum chemistry tests was assessed to determine the functionality of the vital organs like the liver after administration of FCA. There were significant alterations in liver functions after chronic oral administration of FCA, reflected by an increase in albumin, ALT, AST, and ALP levels. It has been reported that albumin corresponds to 50% of the total protein [47]. An elevated level of serum albumin after the FCA administration corroborated with an increased level of complete protein in synovial tissue. AST and ALT are the two cytoplasmic enzymes present in abundance in the liver [48], representing the liver function, and alteration in their levels reflects hepatic toxicity [49]. Administration of FCA caused a significant elevation in the AST and ALT level, thus produces hepatotoxicity. A recent study has documented that arthritis patients are associated with primary liver disease clinically [50]. Findings of the present investigation also suggest that FCA induced arthritis is associated with hepatotoxicity, which was reflected by elevated AST and ALT levels. However, the administration of QTN significantly attenuated these elevated levels of hepatotoxicity markers suggesting its hepatoprotective role, which might contribute to its antiarthritic potential.

Oxidative stress plays a central role in the induction and maintenance of painful arthritis [50]. It has been documented that increased production of reactive oxygen species such as hydrogen peroxide, hydroxyl, and superoxide radicals contributes to elevated oxidative stress [4, 10, 20]. This elevated oxidative stress further depletion of protective antioxidant moieties (SOD and GSH) that resulted in the elevation of lipid peroxidation (MDA), causing damage to the macromolecules in vital biomembrane [22, 37, 51]. In the present

study, the synovial SOD and GSH were significantly decreased, whereas the MDA level increased significantly after the FCA administration. However, treatment with QTN significantly attenuated FCA-induced decreased SOD and GSH in the synovial tissue suggesting its antioxidant potential that might support its antiarthritic mechanism. The result of the present investigation is in line with the findings of the previous researcher, where QTN isolated from *Madhuca indica* exerts its potential via inhibition of elevated oxidative stress [17].

The researcher has well-identified CRP as a vital marker during various inflammatory diseases. Moreover, rheumatoid arthritis patients also exhibit increased serum CRP levels associated with inflammation and tissue destruction [40]. Furthermore, a couple of inducible inflammatory enzymes, including nitric oxide (NO) and COX-2, play a key role in the activation of an inflammatory network of mediators [43]. Numerous finings documented the linkage between elevated nitric oxide and the release of pro-inflammatory cytokines (TNF-α and IL-1β) in local synovial fluid [6, 43, 52]. Furthermore, COX-2, an isoenzyme, is abundantly present in activated macrophages responsible for the synthesis of prostaglandins that mediate various inflammatory reactions [53]. Thus, dual inhibition of these inducible inflammatory enzymes (NO and COX-2) would be important in terms of symptomatic relief from pain and inflammation. In the present investigation, the activity of NO and COX-2 significantly decreased after administration of QTN, which might, in turn, inflammation and exerts its anti-nociceptive potential in FCA induced rats to modulate the paw withdrawal latency.

Cytokines such as TNF-α and IL's play a vital role in RA's pathogenesis [2]. The release of these pro-inflammatory cytokines in response to antigen-stimulated immune response cause recruitment, activation, and deposition of polymorphonuclear neutrophils (PMNs) into the joint space [9, 54]. Further, these PMN's caused the elevated response of ROS, which damages cartilage and joint [55]. The researcher reported that differentiation and proliferation of T and B cells as well as their resorption into bone inducted by TNF-α and IL-6 whereas IL-1β responsible for modulation of immune response via production of nitric oxide (NO) and prostaglandin [7, 56]. Recent evidence demonstrated an elevated response of pro-inflammatory cytokines in RA patients [57]. Thus, measures have been oriented towards the administration of anti-TNF-α antibodies to manage RA [2]. In the present investigation, the FCA administration resulted in an elevated response of these pro-inflammatory cytokines in the synovial fluid. In contrast, treatment with QTN ameliorated this influx of cytokines. The results of the present investigation are in accordance with the findings of the previous researcher. In contrast, the administration of *Madhuca indica* significantly inhibited the elevated response of TNF-α and IL-1β [17], thus exerts its anti-inflammatory potential to modulate the pathogenesis of disease.

Numerous researchers suggested that the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kB) plays a central dogma role in the induction and maintenance of immune-inflammatory disease modulation of various biomolecules such as COX-2 and pro-inflammatory cytokines [10, 53]. During the resting state, the NF-kB remains unstimulated and retain in an inactive state in the cytoplasm. Whereas, IκB kinase, which is an enzyme complex, plays an essential role in the upstream NF-κB signal

transduction pathway, and its phosphorylated activation leads to subsequent ubiquitination and degradation of 26S proteasome [58]. This cascade leads to NF-kB translocation from cytoplasm to nucleus, where it modulates the expression of various genes, including pro-inflammatory cytokines [58]. In the present investigation, activated expression of IκBα and NF-kB significantly up-regulated in synovial of AIA control rats after administration of FCA whereas QTN treatment significantly down-regulated these expressions of pro-inflammatory cytokines via inhibition of phosphorylation of IκBα and thus inactivation of NF-kB.

Purinergic Receptor-X 7 (P2X7), a protein-coding gene from the purinoceptors family, has been implicated in the induction and maintenance of various diseases associated with bone and cartilage, including rheumatoid arthritis [59]. The researcher documented its vital role in bone remodeling via the release of various pro-inflammatory factors such as prostaglandins and IL-1β into the synovial fluid [59]. It has been suggested that activation of ectonucleotidases degrade extracellular ATP, which results in the formation of active molecules such as adenosine or pyrophosphates [60]. These active molecules further promote the activation of alternative macrophages and thus initiate the release of pro-inflammatory signaling [60]. Therefore, extracellular metabolism of ATP by P2X7 modulate the sequence of inflammatory influx and thus initiate the pathogenesis of RA [59]. In this view, inhibition of P2X7 receptor activation would be beneficial for the management of RA. In the present study, treatment with QTN significantly inhibits the activation of P2X7, which might reduce the bone and cartilage damage in RA.

Recently an array of isolated phytoconstituents from herbal origin has been implicated in the management of arthritis clinically. Studies investigated the potential of various moieties such as Pycnogenol® from *Pinus pinaster* Aiton), Curcuminoids from *Curcuma longa*, Bromelain from *Ananas comosus*, etc. for the symptomatic relief of RA [61]. Furthermore, a researcher suggested that the moiety bearing carbonyl group at C-4 and a hydroxyl group at C-3 or C-5 in their structure have a chelation ability with metal ions to exert its antioxidant potential [24]. In the present investigation, isolated moiety from methanolic extract of leaves of *Madhuca indica*, i.e., QTN (3,5,7,3',4'- Pentahydroxy flavone) also possesses such hydroxyl and carbonyl groups in its structure, holding promising antioxidant potential. Thus, QTN can be considered as a potential therapeutic moiety for the management of RA clinically.

5. Conclusion

The phytoconstituent isolated from methanolic extract of leaves of *Madhuca indica* identified as 3,5,7,3',4'- Pentahydroxy flavone (i.e., QTN) exhibit antiarthritic activity. QTN ameliorates FCA-induced hyperalgesia via inhibition of elevated oxido-nitrosative stress, inflammatory mediators (NF-kβ, Ikβα, COX-2, and P2X7), and pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in experimental rats.

Abbreviations

3,5,7,3',4'-Pentahydroxy flavone (QTN); Adjuvant induced arthritis (AIA); ATP-activated P2 purinergic receptors (P2X7); Alanine transaminase (ALT); Alkaline phosphatase (ALP); Aspartate Aminotransferase (AST); C-reactive protein (CRP); Cyclooxygenase-2 (COX-2); Erythrocyte Sedimentation Rate (ESR); Freund's Complete Adjuvant (FCA); Interleukin-1 beta (IL-1β); Leflunomide (LF); Low-density lipoprotein (LDL); Malondialdehyde (MDA); Nitric Oxide (NO); Nuclear factor kappa beta (NF-kB); Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor-α (Ikβα); Reactive Oxygen Species (ROS); Reduced Glutathione (GSH); Superoxide dismutase (SOD); Tumor necrosis factor-alpha (TNF-α)

Declarations

Conflict of Interest

The authors declare that they have no conflicts of interest concerning this article.

References

1. Dudics S, Langan D, Meka RR, Venkatesha SH, Berman BM, Che CT, Moudgil KD, *Int J Mol Sci*, 2018, 19
2. Guo Q, Wang Y, Xu D, Nossent J, Pavlos NJ, Xu J (2018) Bone research 6:15–15
3. Ghosh P, Kandhare AD, Kumar VS, Rajmane AR, Adil M, Bodhankar SL (2012) Asian Pac J Trop Dis 2:S671–S678
4. Aletaha D, Smolen JS (2018) JAMA 320:1360–1372
5. Linos A, Worthington JW, O'Fallon WM, Kurland LT (1980) Am J Epidemiol 111:87–98
6. Jin JH, Kim JS, Kang SS, Son KH, Chang HW, Kim HP (2010) J Ethnopharmacol 127:589–595
7. Wong YF, Zhou H, Wang JR, Xie Y, Xu HX, Liu L (2008) Phytomedicine 15:416–426
8. Ghasemian M, Owlia S, Owlia MB, *Advances in Pharmacological Sciences*, 2016, **2016**, 11
9. Chen Z, Bozec A, Ramming A, Schett G (2019) Nat Rev Rheumatol 15:9–17
10. Honmore VS, Kandhare AD, Kadam PP, Khedkar VM, Natu AD, Rojatkar SR, Bodhankar SL (2019) J Ethnopharmacol 229:233–245
11. Shekhawat N, Vijayvergia R (2010) Inter J Mol Med Adv Sci 6:26–30
12. Khan S, Zahan D, Das R, Nasrin D, Ahsan S, Ahmed R, Sadat A, Bashar A, Nahar N, Rahmatullah M (2011) Adv Nat Appl Sci 5:122–126
13. Prashanth S, Kumar AA, Madhu B, Kumar YP, *Inter J Pharm Sci Rev Res*, 2010, 5
14. Kumar KP, Vidyasagar G, Ramakrishna D, Reddy IM, Atyam VG, Raidu CS (2011) Int J PharmTech Res 3:1073–1077
15. Anu C, Anil B, Pandurangan A (2011) Inter J Pharm Res Dev 3:13–18
16. Mohod SM, Kandhare AD, Bodhankar SL (2016) J Ethnopharmacol 182:150–159
17. Mukherjee AA, Kandhare AD, Bodhankar SL (2017) Environ Toxicol Pharmacol 56:172–185

18. Chatterjee A, Chatterjee S, Das S, Saha A, Chattopadhyay S, Bandyopadhyay SK (2012) Acta Biochim Biophys Sin (Shanghai) 44:565–576
19. Chou MM, Vergnolle N, McDougall JJ, Wallace JL, Marty S, Teskey V, Buret AG (2005) Exp Biol Med (Maywood) 230:255–262
20. Patil MVK, Kandhare AD, Bhise SD (2012) Biomed Aging Pathol 2:6–15
21. Kandhare AD, Raygude KS, Ghosh P, Ghule AE, Bodhankar SL (2012) Fitoterapia 83:650–659
22. Kandhare AD, Raygude KS, Ghosh P, Ghule AE, Bodhankar SL (2012) Neurosci Lett 511:18–22
23. Vetal S, Bodhankar SL, Mohan V, Thakurdesai PA (2013) Food Science Human Wellness 2:59–67
24. Kandhare AD, Ghosh P, Bodhankar SL (2014) Chem Biol Interact 219:101–112
25. Patel PK, Prajapati NK, Dubey B (2012) International Journal of Pharmaceutical Sciences Research 3:1285
26. Adil M, Kandhare AD, Ghosh P, Venkata S, Raygude KS, Bodhankar SL (2016) Ren Fail 38:1007–1020
27. Adil M, Kandhare AD, Visnagri A, Bodhankar SL (2015) Ren Fail 37:1396–1407
28. Devkar ST, Kandhare AD, Zanwar AA, Jagtap SD, Katyare SS, Bodhankar SL, Hegde MV, *Pharm Biol*, 2016, 1–10
29. Honmore V, Kandhare A, Zanwar AA, Rojatkar S, Bodhankar S, Natu A (2015) Pharm Biol 53:571–581
30. Kamble H, Kandhare AD, Bodhankar S, Mohan V, Thakurdesai PA (2013) Biomed Aging Pathol 3:145–151
31. Kandhare AD, Ghosh P, Ghule AE, Zambare GN, Bodhankar SL (2013) Apollo Medicine 10:87–97
32. Walsmith J, Abad L, Kehayias J, Roubenoff R (2004) J Rheumatol 31:23–29
33. Paval J, Kaitheri SK, Kumar A, Govindan S, Mohammed CA, Kumar RS, Narayanan SN, Maloor PA (2011) J Herbal Med Toxicol 5:11–16
34. Blackler R, Syer S, Bolla M, Ongini E, Wallace JL (2012) PLoS One 7:e35196
35. Kassuya CA, Ferreira J, Claudino RF, Calixto JB (2007) Br J Pharmacol 150:727–737
36. Uceyler N, Schafers M, Sommer C (2009) Exp Brain Res 196:67–78
37. Kandhare AD, Shivakumar V, Rajmane A, Ghosh P, Bodhankar SL (2014) J Nat Med 68:586–603
38. Visnagri A, Kandhare AD, Chakravarty S, Ghosh P, Bodhankar SL (2014) Pharm Biol 52:814–828
39. Kandhare AD, Raygude KS, Kumar VS, Rajmane AR, Visnagri A, Ghule AE, Ghosh P, Badole SL, Bodhankar SL (2012) Biomed Aging Pathol 2:173–186
40. Zhang G, Kandhare AD, Mukherjee AA, Bodhankar SL, Yin H (2019) Pharmacogn Mag 15:43
41. McPhee S, Hodges LD, Wright PF, Wynne PM, Kalafatis N, Macrides TA (2010) Prostaglandins Leukot Essent Fatty Acids 82:97–103
42. Lin F, Wang X, Liang Y, Liu D, Zhang Y, Zhong R, Yang Z (2018) Ann Clin Lab Sci 48:301–307
43. Honmore VS, Kandhare AD, Kadam PP, Khedkar VM, Sarkar D, Bodhankar SL, Zanwar AA, Rojatkar SR, Natu AD (2016) Int Immunopharmacol 33:8–17
44. Kandhare A, Raygude K, Ghosh P, Bodhankar S (2011) Inter J Green Pharmacy 5:236–243

45. Kandhare AD, Alam J, Patil MV, Sinha A, Bodhankar SL (2016) Pharm Biol 54:419–432
46. Visnagri A, Kandhare AD, Bodhankar SL (2015) Ren Fail 37:482–493
47. Doweiko JP, Nompleggi DJ (1991) Journal of Parenteral Enteral Nutrition 15:207–211
48. Tennekoon KH, Jeevathayaparan S, Kurukulasooriya AP, Karunanayake EH (1991) J Ethnopharmacol 31:283–289
49. Vijayalakshmi T, Muthulakshmi V, Sachdanandam P (2000) J Ethnopharmacol 69:9–15
50. Radovanovic-Dinic B, Tesic-Rajkovic S, Zivkovic V, Grgov S (2018) Rheumatol Int 38:715–724
51. Visnagri A, Kandhare AD, Kumar VS, Rajmane AR, Mohammad A, Ghosh P, Ghule AE, Bodhankar SL (2012) Biomed Aging Pathol 2:157–172
52. Tambewagh UU, Kandhare AD, Honmore VS, Kadam PP, Khedkar VM, Bodhankar SL, Rojatkar SR (2017) Int Immunopharmacol 52:110–118
53. Chen X, Zhang C, Wang X, Huo S (2019) Artif Cells Nanomed Biotechnol 47:3614–3620
54. Kaur G, Sultana S (2012) Food Chem Toxicol 50:2689–2695
55. Wang X, He X, Zhang CF, Guo CR, Wang CZ, Yuan CS (2017) Biomed Pharmacother 89:887–893
56. Zhang RX, Fan AY, Zhou AN, Moudgil KD, Ma ZZ, Lee DY, Fong HH, Berman BM, Lao L (2009) J Ethnopharmacol 121:366–371
57. Mateen S, Moin S, Shahzad S, Khan AQ (2017) PLOS ONE 12:e0178879
58. Giridharan S, Srinivasan M (2018) Journal of inflammation research 11:407–419
59. Chang X, He H, Zhu L, Gao J, Wei T, Ma Z, Yan T (2015) Chemico-Biol Interact 236:41–46
60. De Marchi E, Orioli E, Pegoraro A, Sangaletti S, Portararo P, Curti A, Colombo MP, Di Virgilio F, Adinolfi E (2019) Oncogene 38:3636–3650
61. Maksimović Z, Samardžić S, *Osteoarthritis Biomarkers and Treatments*, 2018, 1–18

Figures

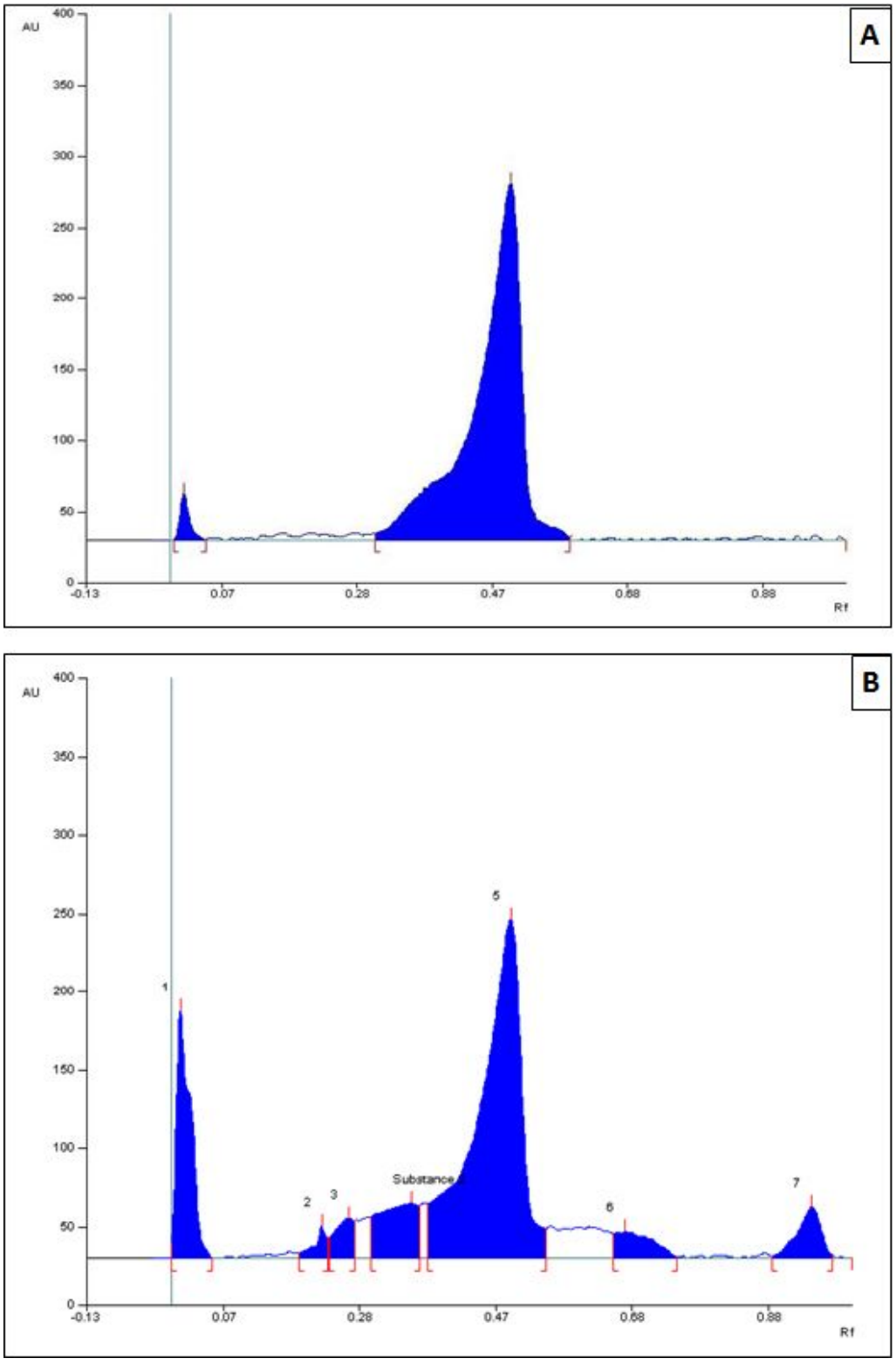

Figure 1

HPTLC profile of quercetin (A) and HPTLC profile of chloroform fraction D (QTN) of MI-ALC (B). Eluent-Toluene: Ethyl Acetate: Formic Acid (6: 3.5: 0.5) (v/v). The detection was 370 nm.

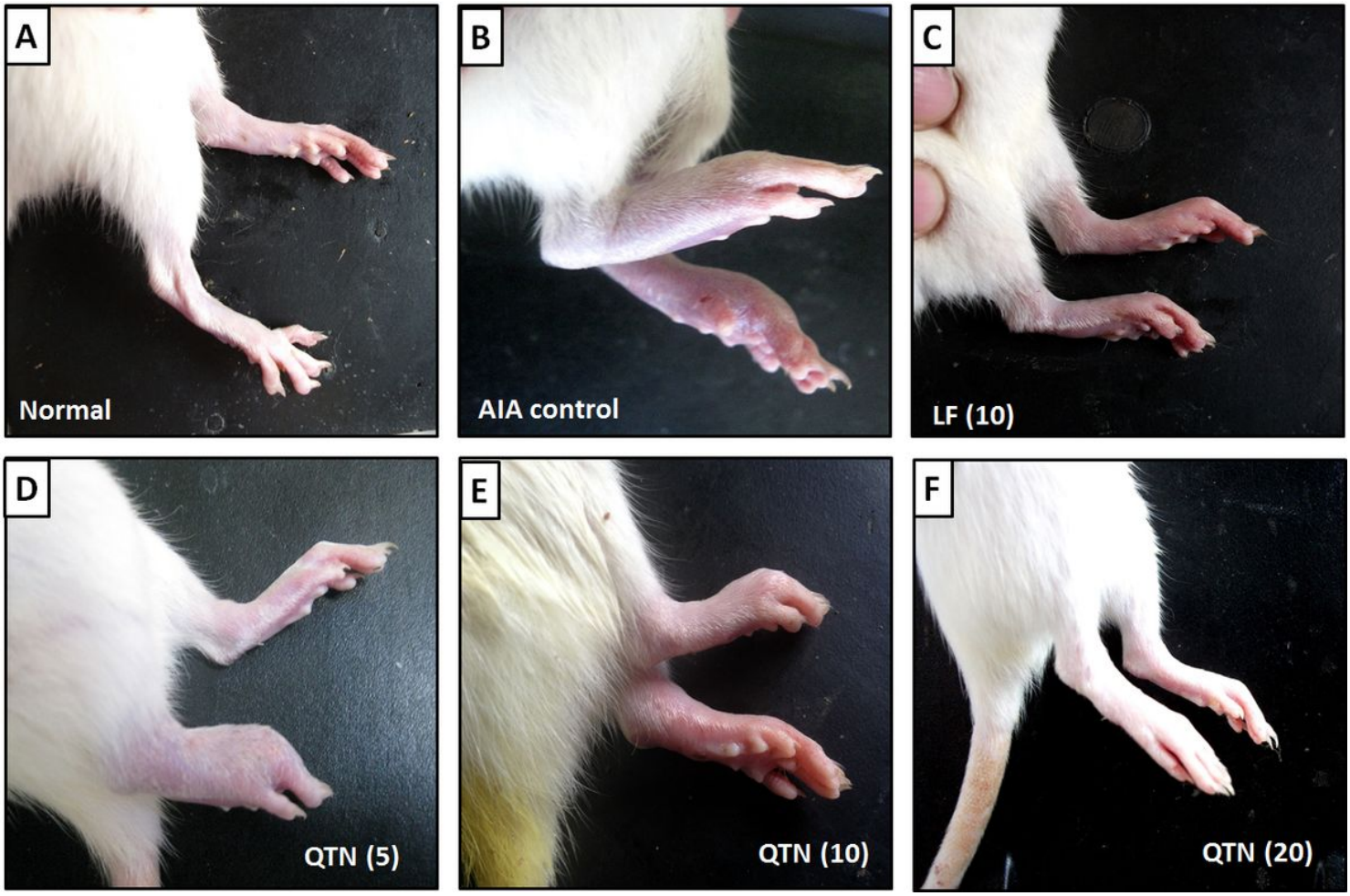

Figure 2

Effects of QTN on morphological representations of rat paw after administration of FCA arthritic rats. Representative images of paw from (A) normal, (B) AIA control, (C) Leflunomide (10 mg/kg), (D) QTN (5 mg/kg), (E) QTN (10 mg/kg) and (F) QTN (20 mg/kg) treated rat.

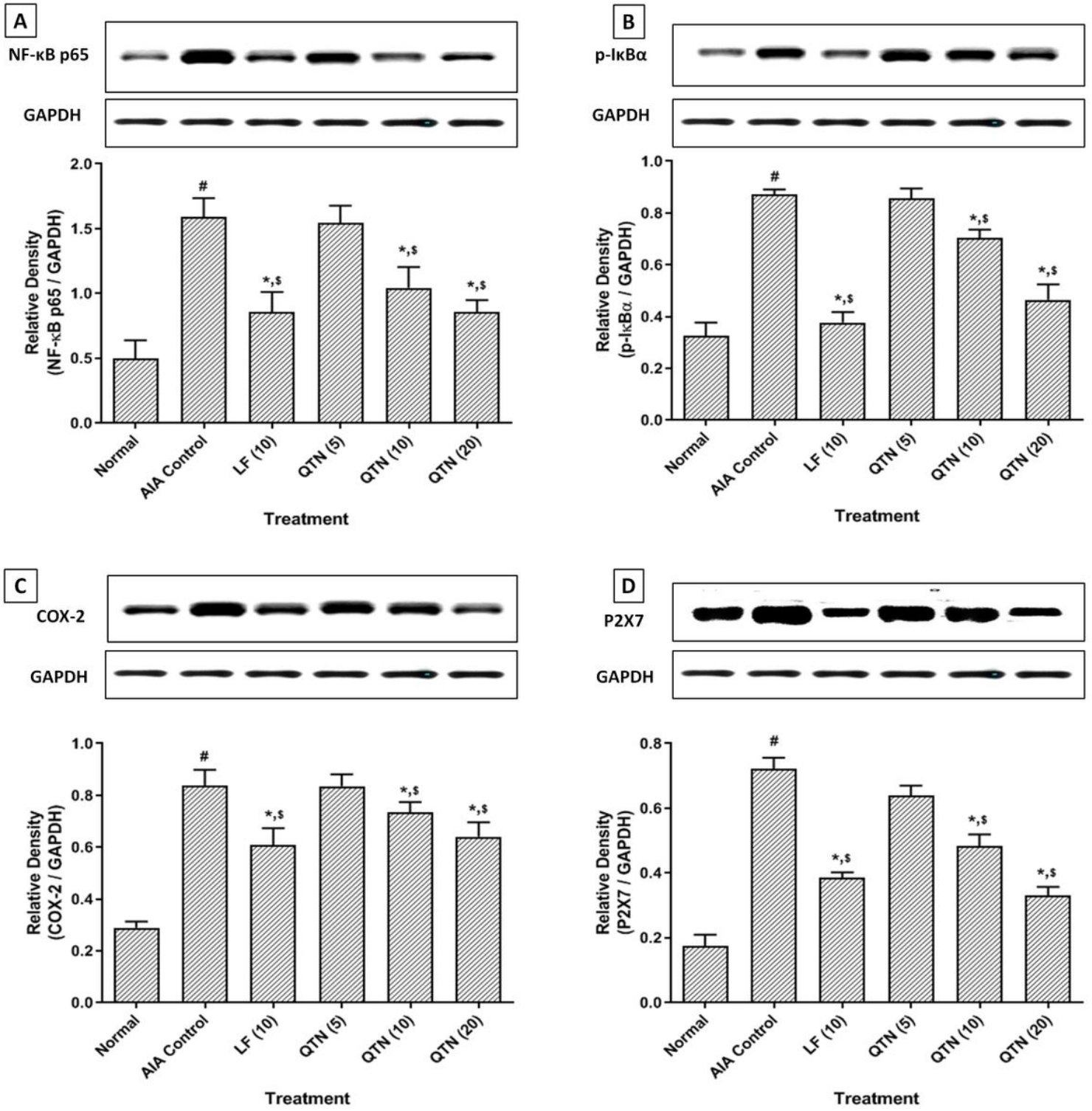

Figure 3

Effects of QTN on FCA-induced alterations in NF-kβ (A), Ikβα (B), COX-2 (C), and P2X7 (D) protein expression in synovial tissues of arthritic rats. Data are expressed as mean ± SEM (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: Ladder 1000 bp; Lane 2: mRNA expression of the normal group; Lane 3: mRNA expression of AIA control group;

Lane 4: mRNA expression of Leflunomide (10 mg/kg) treated group and Lane 5-7: mRNA expression of QTN (5, 10 and 20 mg/kg) treated group. AIA: Adjuvant-induced arthritis, LF: Leflunomide, QTN: 3,5,7,3',4'-Pentahydroxy flavone, NF-kβ: Nuclear factor kappa beta, Ikβα: nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor-α, COX-2: Cyclooxygenase-2, P2X7: ATP-activated P2 purinergic receptors.

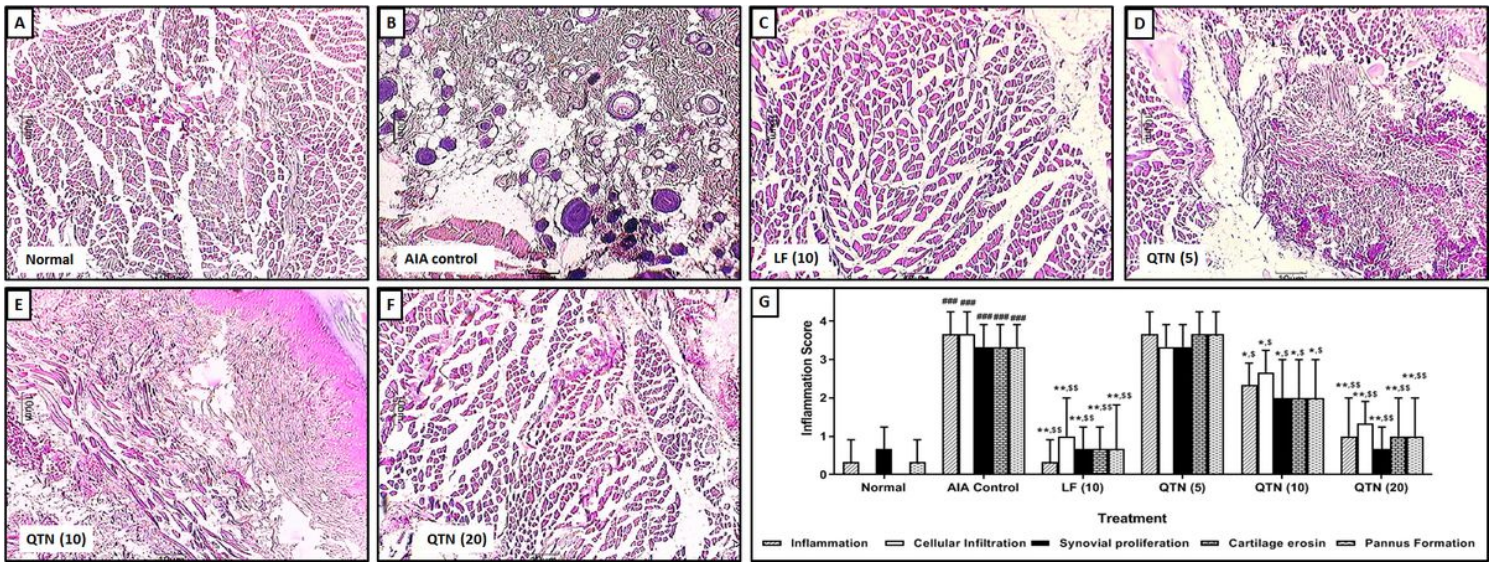

Figure 4

Effects of QTN on the histopathology of tibiotarsal joints after administration of FCA arthritic rats. Representative histological images from (A) normal, (B) AIA control, (C) Leflunomide (10 mg/kg), (D) QTN (5 mg/kg), (E) QTN (10 mg/kg) and (D) QTN (20 mg/kg) treated rat. Images stained with H&E (X 100). The quantitative representation of histological score (G). Data were expressed as mean ± S.E.M. (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.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfile1.jpg
- Supplementaryfile2.jpg