Article

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The Anti-Arthritic Role of Naringenin through Modulating Different T Helper Cells' Cytokines, Inflammatory Mediators, Oxidative Stress and Anti-Oxidant Defense System

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Background: Rheumatoid arthritis (RA) is a systemic inflammatory disorder that is usually treated with a cocktail of steroidal and nonsteroidal anti-inflammatory drugs, as well as other therapeutic modalities known as disease-modifying anti-rheumatic drugs (DMARDs), all of which have significant side effects. Our objective was to investigate the potential impact of naringenin on a rat model of RA induced by Complete Freund's Adjuvant (CFA).

Methods: This was achieved by assessing T-helper (Th) cytokines, oxidative stress, and the anti-oxidant defense system. Eighteen Wistar rats were divided into three equal (n = 6) groups: the normal group, the CFA-induced arthritic control group, and the CFA-induced arthritic group treated with naringenin at a dose of 25 mg/kg body weight daily for two weeks. The study used a variety of techniques, including spectrophotometric analysis, enzyme-linked immunosorbent assay (ELISA), and Western blot procedures, as well as ankle histological investigations and measurement of ankle circumference of the right hind leg.

Results: Naringenin treatment significantly decreased the CFA-induced elevated right hind-ankle circumference, serum rheumatoid factor (RF), anti-cyclic citrullinated peptide (ACCP), prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α), and serum interleukin (IL)-1β levels ($p < 0.05$). Naringenin significantly increased serum IL-4 levels, hepatic glutathione (GSH), and superoxide dismutase (SOD) activity when compared to the CFA-induced RA model ($p < 0.05$). Naringenin treatment decreased ankle joint protein expression of nuclear factor kappa B (NF-κB) p50, NF-κB p65, inhibitor of NF-κB (IκBα), matrix metalloproteinases (MMPs)-1, 3 and 9, and inducible nitric oxide synthase (iNOS), as well as malondialdehyde (MDA) and nitric oxide (NO) levels ($p < 0.05$). Additionally, there was a significant increase in the ankle's nuclear factor erythroid 2-related factor 2 (Nrf2) levels ($p < 0.05$). Morphological signs like leg swelling and redness were also significantly reduced. Naringenin treatment significantly improved RA's histological changes, such as pannus formation, massive inflammatory cell infiltration, synovial membrane hyperplasia, and articular cartilage erosion.

Conclusions: Naringenin has a potent anti-arthritic effect via modulating Th cells cytokines, NF-κB pathway, Nrf2, MMPs, free radical damage, and anti-oxidant defenses.

Keywords: CFA-induced rheumatoid arthritis; naringenin; inflammation; oxidative stress; anti-oxidant defense

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Background

Rheumatoid arthritis (RA) is an autoimmune joint inflammation that, if not treated, can cause joint deformity [1]. It affects roughly 1% of the world's population, with women having a higher incidence, and its prevalence rises with age [2]. Conventional treatments for RA with nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and disease-modifying anti-rheumatic drugs (DMARDs) had some success, but their toxicity, higher cost, and adverse effects limited their use [3]. As a result, researchers are constantly looking for safer and more effective anti-arthritic drugs derived from natural sources [4–6].

Citrus contains a significant number of flavonoids, which have anti-inflammatory properties [7], can reduce lipid peroxidation (LPO), and boost the anti-oxidant system [8]. Naringenin, one of these flavonoids found in orange peel, has potent anti-inflammatory and anti-oxidant properties [9]. Some citrus fruits' volatile and polar fractions (flavedo and albedo) and juice showed diphenyl picrylhydrazyl (DPPH) radical scavenging capacity and LPO inhibiting efficiency [10]. The effects of citrus peel extracts and the flavonoid hesperidin were also studied *in vivo*. They were found to have strong anti-inflammatory, anti-arthritic, and anti-oxidant properties in experimentally induced arthritic rats [11,12]. Citrus-derived flavonoids have been shown to inhibit tumor necrosis factor-α (TNF-α) activity, stimulate myeloid leukemia cell differentiation, and facilitate apoptosis. Citrus fruit compounds have demonstrated beneficial effects in inflammatory conditions such as adjuvant arthritis in rats and toxin-induced liver damage in mice. Flavonoids are active constituents with anti-oxidant properties [13]. Total flavonoids, hesperidin, naringenin, narirutin, and anti-oxidant capacity were significantly correlated [14].

Increased reactive oxygen species (ROS) production indicates an imbalance between ROS generation and clearance, which causes oxidative stress and tissue damage [15]. It was discovered that when T-cells are subjected to high levels of oxidative stress, they become insensitive to a variety of signals, including those that regulate their growth and apoptosis. This unresponsiveness has the potential to sustain the abnormal immune response that underpins disease development [16]. T cell research in RA has primarily focused on the classical $CD4^+$ T cell subsets Th1, Th2, and Th17 [17]. A Th1/Th2 cytokine imbalance with a predominance of Th1 cytokines has been proposed to be pathogenic in RA. Th1 cells produce pro-inflammatory cytokines like IFN-γ, TNF-α, and interleukin (IL)-2, which cause cartilage destruction and bone erosion [18]. IL-4 is a strong anti-inflammatory cytokine produced by $CD4^+$ Th2 lymphocytes, a subset of lymphocytes. In addition to lymphocytes, mast cells and basophils produce IL-4 [19]. The Th2 cytokine IL-4 promotes antibody-mediated immune responses [20]. Since 2005, Th17 has been recognized as a potent mediator in RA pathogenesis. IL-17 enhances the production of TNF-α, IL-1, and IL-6 by macrophages and fibroblasts [21]. Therapies that suppress pathogenic cytokines (e.g., TNF-α, IL-1β, IL-6, or IL-17) and increase anti-inflammatory cytokines (e.g., IL-4, IL-5, IL-10, and IL-13) have significantly improved RA treatment [22,23].

The anti-arthritic effects of naringenin have previously been studied [24,25], but the mechanisms of action have not been fully understood. This study aimed to determine the effects of naringenin on the adjuvant-induced arthritis rats model. We also investigated its modulatory effects on pro- and anti-inflammatory cytokines, matrix metalloproteinases (MMPs), nuclear factor erythroid 2-related factor 2 (Nrf2) levels, pro-oxidative stress, and the anti-oxidant defense system.

Materials and Methods

Experimental Animals

The study included 18 male Wistar rats (aged 6–8 weeks) weighing 115 ± 15 g. Rats were initially observed for 15 days before the experiment began to acclimatize and exclude any infections. The animals were housed in stainless steel cages (3 rats per cage) at the "Zoology Department's animal house, Faculty of Science, Beni-Suef University, Egypt", with an average temperature of 22 ± 4 °C, relative humidity of 45 ± 5%, and alternating 12-hour light/dark cycles. Animals had unlimited access to food and water, and their weights were recorded weekly throughout the study. All animal procedures adhere to the guidelines of the "Experimental Animals Ethics Committee of the Faculty of Science, Beni-Suef University, Egypt, for the use and care of animals in research (Ethical Approval Number: BSU/FS/2015/20)".

Chemicals

Complete Freund's Adjuvant (CFA) of Lot number "88H8804", a heat-killed *Mycobacterium tuberculosis* suspension in mineral oil (1 mg/mL) and naringenin (Batch code: BCBM4171V), were purchased from Sigma Chemical Company (63178, Saint Louis, MO, USA). Analytical-grade chemicals are utilized for anything else.

Animal Grouping

The animals were randomly divided into three groups (n = 6):

- **The normal control group:** Rats were given an equivalent volume of 1% carboxy-methylcellulose (CMC) orally by gavage for two weeks.
- **The RA model group:** RA was induced by administering two consecutive subcutaneous injections of 0.1 mL CFA into the rats' right hind-leg footpads over two days [26]. The rats were then given an equal amount of 1% CMC via gavage for two weeks.

• **The RA modeling + treatment intervention group:** RA was induced as previously described, and rats were treated orally with 25 mg/kg body weight of naringenin suspended in 5 mL of 1% CMC for two weeks [27].

Animal Scarification and Sample Collection

Rats were euthanized via cervical dislocation under diethyl ether inhalation anesthesia, and blood samples were collected from the jugular vein [6]. The clear non-haemolyzed supernatant sera were centrifuged at 3000 rpm for 15 minutes and frozen at –70 °C until reused to determine the levels of rheumatoid factor (RF), anti-cyclic citrullinated peptide (ACCP), prostaglandin E_2 (PGE_2) and Th1 cytokines (TNF-α & IL-1β), Th2 cytokine (IL-4), and Th17 cytokine (IL-17). Three ankles from each group's right hind legs were dissected, washed with sterile saline, and frozen at –70 °C for Western blot analysis. Other rats had their paws and ankles amputated and immersed in 10% neutral buffered formalin for histological examination. Livers were quickly removed and cut into small pieces. 0.5 g of each liver was crushed in 5 mL of normal saline. The obtained homogenates were kept at –70 °C to measure malondialdehyde (MDA), nitric oxide (NO), and glutathione (GSH) levels, as well as superoxide dismutase (SOD) enzyme activity.

Detection of Edema

The circumference of the animals' right hind paws was measured to assess edema by a white cotton thread applied just above the tarsal pad of the paw. The thread was then measured using a meter ruler [26].

Determination of Serum RF, ACCP, PGE_2 and Th1/Th2/Th17 Cytokines Levels

Enzyme-linked immunosorbent assay (ELISA) kits for PGE_2 (Catalog #: KGE004B), TNF-α (Catalog #: RTA00), IL-1β (Catalog #: RLB00), IL-4 (Catalog #: R4000), and IL-17 **(**Catalog #: M17F0) were purchased from R&D Systems Inc. (Minneapolis, MN, USA) and the methods were carried out according to the manufacturer's instructions. Serum RF was determined using an ELISA kit (Catalog No.: ER1932) purchased from Wuhan Fine Biotech Co., Ltd. B9 Bld, Wuhan, China. Serum ACCP level was detected by antibody sandwich ELISA Kit (Catalog No. LS-F67407) provided by LifeSpan Biosciences, Inc., WA, USA, according to the manufacturer's instructions.

Determination of MDA, NO, and GSH Levels and SOD Activity

Preuss *et al.*'s method [28] detected liver lipid peroxidation (LPO) expressed as MDA levels. In brief, the protein was precipitated by adding 0.15 mL of 76% trichloroacetic acid (TCA) to 1 mL of liver homogenate. The separated supernatant was then combined with 0.35 mL of thiobarbituric acid (TBA), a color developer. After 30 minutes of incubation in a water bath at 80 °C, a faint pink color was observed at 532 nm. The Montgomery and Dymock [29] method was used to measure the nitric oxide (NO) concentration in the liver. The technique is based on the Griess reaction, which converts nitrite into a purple azo compound measured at 540 nm. 1 mL of Griess reagent was added to 100 µL of homogenate supernatant. After ten minutes, the absorbance was measured at 540 nm. $NaNO_2$ was chosen as the standard. According to Beutler *et al.* [30], hepatic glutathione (GSH) was detected.

GSH content was determined by adding 0.5 mL of Ellman's reagent (40 mg 5,5′-Dithiobis 2-nitrobenzoic acid/100 mL of 1% sodium citrate) and phosphate buffer solution (pH 7) to the homogenate supernatant after protein precipitation with glacial metaphosphoric acid reagent (1.67 g/100 mL distilled water). The developed yellow color in samples and GSH standard was measured at 412 nm against a blank.

Superoxide dismutase (SOD) activity was detected using the Marklund and Marklund procedure [31]. The method relies on the presence of superoxide ions. One unit of enzyme is defined as the amount of enzyme that inhibits extinction changes by 50% in 1 minute compared to the control. To summarise, 50 µL of pyrogallol (10 mM) was combined with 1 mL of the homogenate supernatant in Tris buffer (pH 8). The initial absorbance was measured after adding pyrogallol and after 10 minutes. The inhibition of the developed yellow color at 430 nm and enzyme activity were determined.

Western Blot Analysis

Western blot analysis was performed using Shaaban *et al.*'s method [6] to detect inducible nitric oxide synthase (iNOS), nuclear factor kappa B (NF-κB) p50, NF-κB p65, inhibitor of NF-κB (IκBα), MMP-1, MMP-3, MMP-9, and Nrf2. Protein extraction and assay were performed respectively by a ReadyPrepTM kit (Catalog No. #163-2086; Bio-Rad Inc., Hercules, CA, USA) and a Bradford assay kit (SK3041; Bio basic Inc., Markham, Ontario, Canada). To separate the proteins, 20 µg of each sample was loaded into the TGX Stain-Free™ FastCast™ sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) kit (Catalogue No. 161-0181) and followed the manufacturer's instructions (Bio-Rad Laboratories Inc., Hercules, CA, USA).

The gel was constructed in a transfer sandwich configuration, with a transfer buffer positioned from the bottom (filter paper, PVDF membrane, gel, and filter paper). The transfer was carried out using a BioRad Trans-Blot Turbo at a voltage of 25 V for 7 minutes. This facilitated the migration of protein bands from the gel to the membrane. The membrane was then blocked and probed with both primary and secondary antibodies. Primary antibodies specific to the following proteins were used in the study: iNOS (Catalog No. #2982; Dilution: 1-1000, Cell Signaling and Technology, Danvers, MA, USA), IκBα (Catalog No. #9242;

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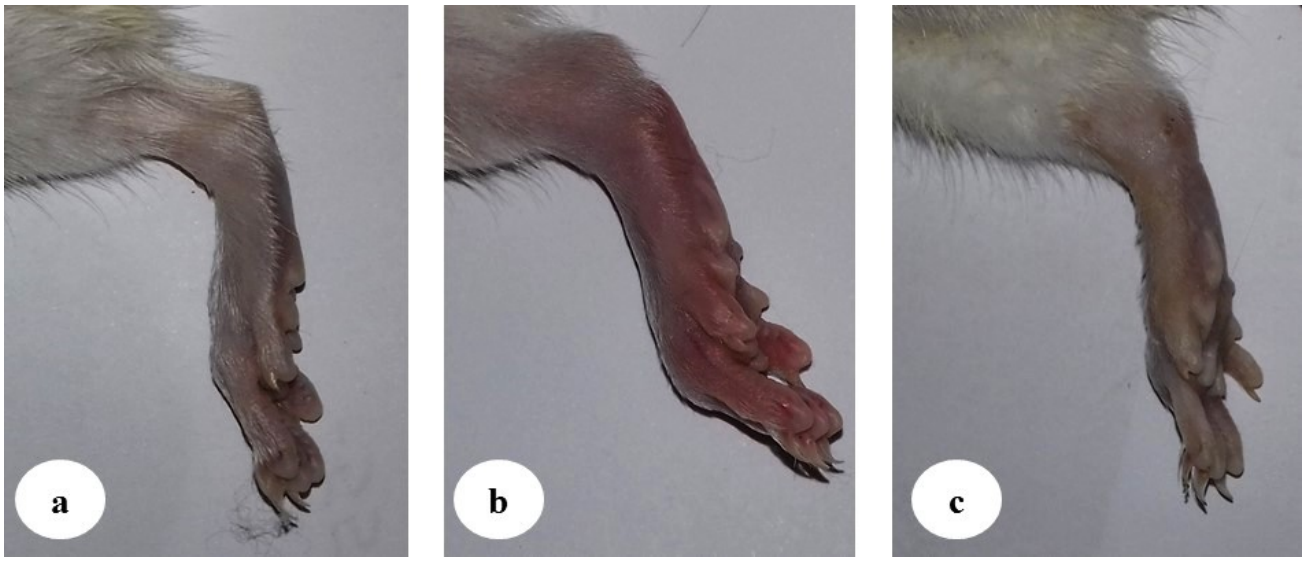

Fig. 1. Right hind legs of normal control (a), the RA model group (b), and RA modeling + treatment intervention group (c). RA, Rheumatoid arthritis.

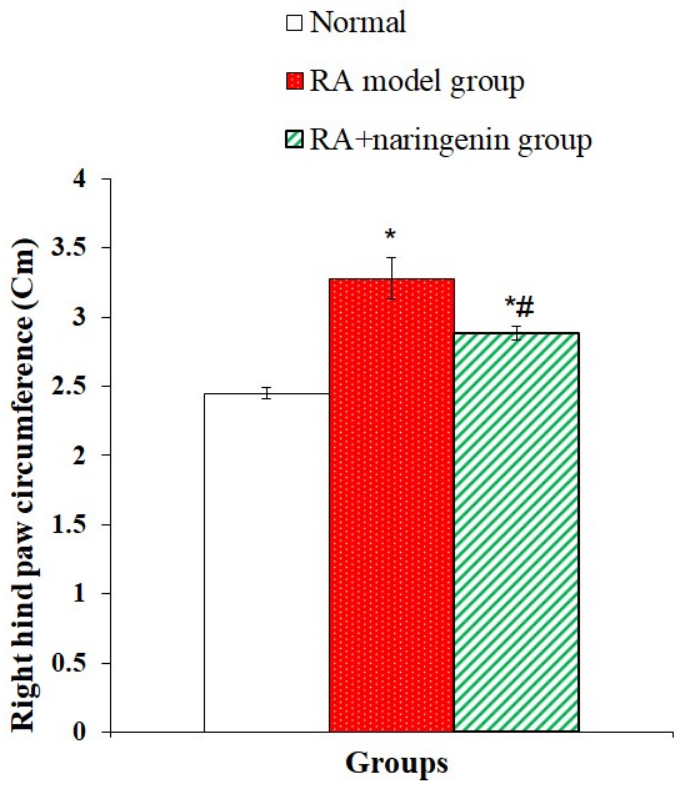

Fig. 2. Effect of naringenin on right hind paw circumference in CFA-induced arthritic rats. Statistically significant at $p < 0.05$ as compared to (*) normal group and ($^{\#}$) the RA model group. CFA, Complete Freund's Adjuvant.

Dilution: 1-1000, Cell Signaling and Technology, Danvers, MA, USA), NF-κB p50 (Catalog No. sc-8414; Dilution: 1-500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), NF-κB p65 (Catalog No. sc-8008; Dilution: 1-500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), MMP-1 (Catalog No. sc-21731; Dilution: 1-500; Santa Cruz Biotechnology, Inc.; CA, Santa Cruz, USA), MMP-3 (Catalog No. sc-21732; Dilution: 1-500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), MMP-9 (Catalog No. #3852; Dilution: 1-1000, Cell Signaling and Technology, Danvers, MA, USA), and Nrf2 (Catalog No. sc-365949; Dilution: 1-500, Santa Cruz Biotechnology, Inc.,

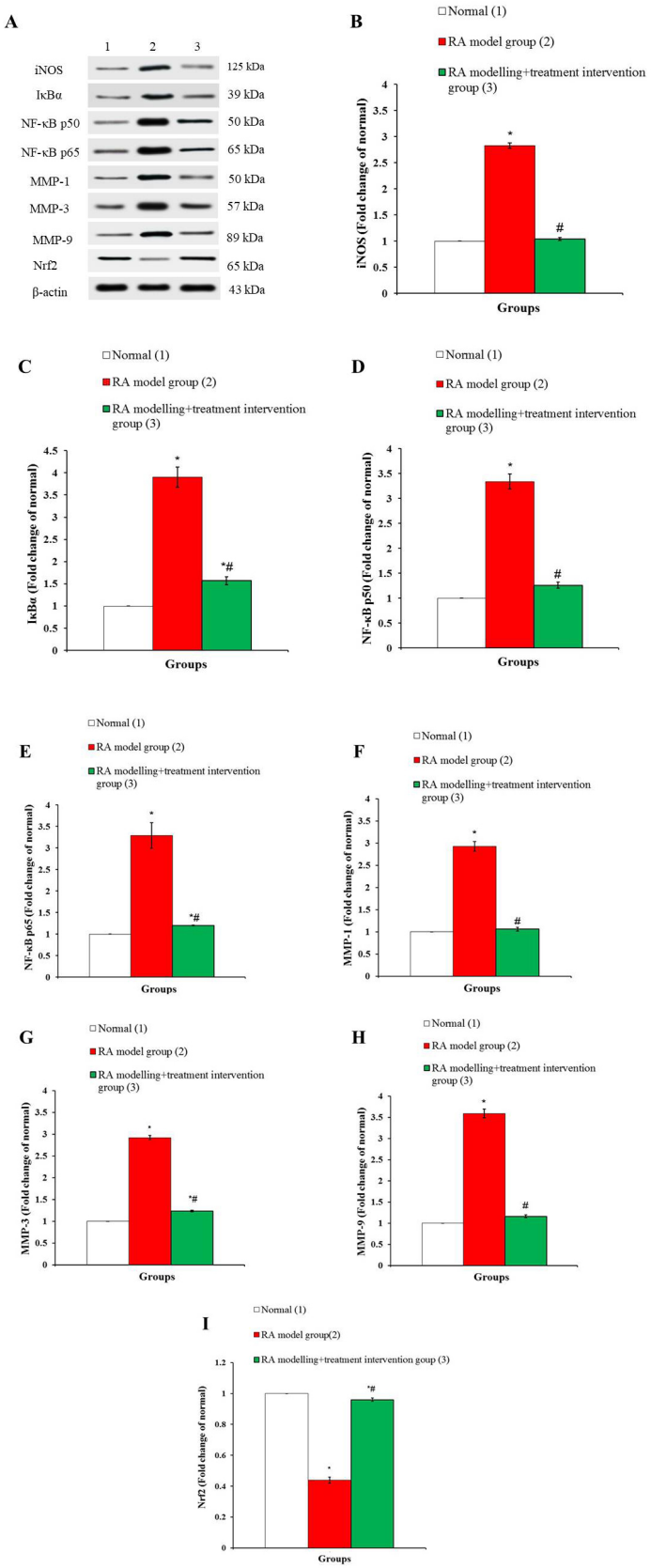

Fig. 3. Effect of naringenin administration on iNOS (B), IκBα (C), NF-κB p50 (D), NF-κB p65 (E), MMP-1 (F), MMP-3 (G), MMP-9 (H) and Nrf2 (I) protein expression in CFA-induced arthritic rats. (A) Cropped immunoblots. Statistically significant at $p < 0.05$ as compared to (*) normal group and ($^{\#}$) the RA model group. iNOS, inducible nitric oxide synthase; IκBα, inhibitor of NF-κB; NF-κB, nuclear factor kappa B; MMP, matrix metalloproteinase; Nrf2, nuclear factor erythroid 2-related factor 2.

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Table 1. Effect of naringenin on different serum parameters in CFA-induced arthritic rats.

Parameters	Normal	RA model group	RA modelling + treatment intervention group
RF (U/mL)	13.67 ± 1.26	31.32 ± 1.16* (129.11%)	20.9 ± 0.42*# (52.89%)
ACCP (ng/mL)	1.83 ± 0.09	4.17 ± 0.13* (127.87%)	1.84 ± 0.15# (0.55%)
PGE_2 (pg/mL)	132.82 ± 5.48	193.11 ± 1.83* (45.40%)	131.87 ± 1.38# (–0.71%)
TNF-α (pg/mL)	35.60 ± 2.73	85.51 ± 3.39* (140.20%)	59.50 ± 7.22*# (67.13%)
IL-1β (pg/mL)	37.57 ± 2.31	78.77 ± 5.29* (109.66%)	59.90 ± 4.55*# (59.44%)
IL-17 (pg/mL)	49.41 ± 2.97	67.52 ± 9.86* (36.65%)	63.81 ± 5.84* (29.14%)
IL-4 (pg/mL)	127.4 ± 2.33	103.51 ± 0.53* (–18.75%)	113.61 ± 6.50*# (–10.82%)

Statistically significant at $p < 0.05$ as compared to (*) normal group and (#) the RA model group. Percentage changes were calculated by comparing arthritic and arthritic-treated rats to the corresponding normal control. RF, rheumatoid factor; ACCP, anti-cyclic citrullinated peptide; PGE_2, prostaglandin E_2; TNF-α, tumor necrosis factor-α; IL, interleukin.

Santa Cruz, CA, USA). A secondary antibody conjugated with horseradish peroxidase (HRP) (Goat anti-rabbit IgG-HRP-1mg Goat monoclonal antibody, Novus Biologicals) was applied at a 1:5000 dilution after that. The blot was rinsed 3–5 times for 5 minutes each with TBST. The chemiluminescent substrate (Clarity TM Western ECL substrate, Catalogue No. 170-5060, Bio-Rad, Hercules, CA, USA) was then applied to the blot according to the manufacturer's instructions, and the resulting signals were captured using a CCD camera-based imaging system. Image analysis software was used to quantify the target proteins' band intensities, then normalized to β-actin, a housekeeping protein, using the ChemiDoc MP imager (Version 5.0, BioRad, Hercules, CA, USA).

Histopathological Investigation

The ankle joint with the articular bones was removed, washed in saline, and fixed in 10% neutral buffered formalin. The ankle joint and articular bones were removed, washed with saline, and fixed in 10% neutral buffered formalin. The fixed ankles were transferred to the Histopathology Lab for decalcification and processing for sectioning and staining with hematoxylin and eosin (H&E) using the methods of Saber *et al.* [5] and Isaac *et al.* [32]. Tissue sections were examined to detect the histological changes in the ankles using a light microscope and a digital camera (DM2500 M, Leica, Wetzlar, Germany).

Statistical Analysis

Data were coded and entered into the statistical software SPSS (Version 23.0, IBM Corp, Armonk, NY, USA) [33]. Data was presented as mean ± SD, and quantitative variables were summarised using mean and standard deviation. The Shapiro-Wilk test assessed the normality of the distribution. An analysis of variance (ANOVA) was conducted, followed by Duncan's test. Results are considered statistically significant when $p < 0.05$.

Results

Effect on Right Hind Leg Morphological Changes Right Leg Ankle Circumference

The morphological changes of the right hind leg are depicted in Fig. 1. The right hind leg of the normal rat exhibited normal morphological architecture (Fig. 1a). The right hind leg of the RA model group rats showed significant edema, redness, and ulceration (Fig. 1b). Naringenin treatment of arthritic rats resulted in a significant reduction in the morphological lesions (Fig. 1c). Fig. 2 shows a significant increase ($p < 0.05$) in right leg paw circumference in arthritic rats compared to normal. Naringenin administration in arthritic rats resulted in a significant reduction in paw circumference ($p < 0.05$) compared to the control group.

Effects on Serum RF, ACCP, PGE_2, and Various Cytokines Levels

CFA-induced arthritic rats had significantly higher serum levels of RF, ACCP, PGE_2, TNF-α, IL-β, and IL-17 compared to normal rats ($p < 0.05$). The percentages increased by 129.11%, 127.87%, 45.40%, 140.20%, 109.66%, and 36.65%, respectively. In rats with CFA-induced arthritis, serum IL-4 levels decreased by –18.75% compared to normal levels ($p < 0.05$). Naringenin therapy of arthritic rats significantly lowered elevated levels of RF, ACCP, PGE_2, TNF-α, IL-β, and IL-17, while also lowering IL-4 levels ($p < 0.05$). Naringenin treatment of arthritic rats resulted in significant variations in RF, ACCP, PGE_2, TNF-α, IL-β, and IL-4 levels compared to the healthy control group ($p < 0.05$), but not in IL-17 (Table 1).

Effects on MDA, NO, and GSH Levels and SOD Activity

Arthritic rats had significantly higher levels of MDA and NO ($p < 0.05$) than healthy rats, with increases of 53.65% and 327.66%, respectively. Naringenin therapy significantly reduced MDA and NO levels in arthritic rats ($p < 0.05$), making them lower than in the healthy control

Table 2. Effect of naringenin on MDA, NO, and GSH levels and SOD activity in CFA-induced arthritic rats.

Parameters	Normal	RA model group	RA modelling + treatment intervention group
MDA (nmol/100 mg tissue/hr)	12.60 ± 0.33	19.36 ± 0.74* (53.65%)	9.20 ± 0.11*# (–26.98%)
NO (nmol/100 mg tissue)	7.05 ± 0.20	30.15 ± 3.34* (327.66%)	6.40 ± 0.30# (–9.22%)
GSH content (nmol/100 mg tissue)	55.66 ± 1.99	28.01 ± 2.50* (–49.68%)	40.37 ± 0.54*# (–27.46%)
SOD activity (U/gm tissue)	15.15 ± 1.30	15.13 ± 0.73 (–0.13%)	18.08 ± 0.16*# (19.34%)

Statistically significant at $p < 0.05$ as compared to (*) the normal group and (#) the arthritic control group. Percentage changes were calculated by comparing arthritic and arthritic-treated rats to the corresponding normal control.
MDA, malondialdehyde; NO, nitric oxide; GSH, glutathione; SOD, superoxide dismutase.

group. Compared to the healthy control, the observed percentage changes were –26.98% and –9.22%, respectively (Table 2).

CFA significantly reduced GSH content in arthritic rats ($p < 0.05$), with a decrease of 49.68% compared to the healthy group. SOD activity in arthritic rats did not differ significantly. Naringenin administration to arthritic rats significantly improved ($p < 0.05$) GSH content and SOD activity compared to the RA model group (Table 2).

Effect on Ankle Protein Expression of Inflammatory Mediators, MMPs, and Nrf2

CFA-induced arthritis in rats showed significant increases in protein expression of iNOS, IκBα, NF-κB p50, NF-κB p65, MMP-1, MMP-3, and MMP-9 compared to the control group ($p < 0.05$). Naringenin administration to arthritic rats considerably lowered the elevated protein levels of the identified inflammatory mediators and MMPs ($p < 0.05$) (Fig. 3). Naringenin treatment corrected ankle iNOS, NF-κB p50, MMP-1, and MMP-9 protein levels in arthritic rats, as they were not statistically different from the normal control group ($p > 0.05$). Treatment with naringenin reduced IκBα, NF-κB p65, and MMP-3 protein expression in arthritic rats, but these levels remained considerably greater ($p < 0.05$) than in normal rats.

On the other hand, CFA-induced arthritic rats showed significantly lower Nrf2 protein expression than normal rats ($p < 0.05$). Naringenin administration to arthritic rats dramatically boosted Nrf2 protein expression ($p < 0.05$) (Fig. 3), but the levels remained significantly lower than the normal control.

Histopathological Effects

Fig. 4 shows the histological changes in the ankle joint across various groups. Hyaline cartilage covers the articular bone, and a capsule with two parts surrounds and joins the ends of the two bones; the inner synovial membrane lines the fibrous capsule and reflects onto the bone, which covers up to the articular cartilage. The outer capsule extends well beyond the articular cartilage of each bone. As a result, either articular cartilage or synovial membrane entirely lines the joint cavity (Fig. 4a).

Synovial hyperplasia, exuberant synovium proliferation leading to pannus formation, and an influx of inflammatory cells into the joint space, with erosion in the bone and cartilage, characterize arthritic joints in CFA rats compared to normal control rats (Fig. 4b–d). Naringenin treatment of arthritic rats reduced inflammation and/or joint destruction significantly. The synovial membrane in the joints has nearly returned to normal, and the joint histology is nearly normal (Fig. 4e,f).

Discussion

Natural product research has greatly benefited modern medicine because it is safe and/or has few side effects [34]. Because some anti-inflammatory drugs cause undesirable and potentially dangerous side effects, new treatments with few or no side effects are still needed. Citrus peel extracts and their flavonoid content are of particular interest to scientists due to their anti-oxidant and anti-inflammatory properties [35]. This study used CFA-induced arthritis as a rat model of RA, which is characterized by the infiltration of inflammatory cells, synovial membrane hyperplasia, and joint destruction. Because of its high similarity to human RA, scientists believe adjuvant-induced arthritis in rats could be a useful model for screening anti-arthritic drugs [36]. Thus, we investigated naringenin's anti-inflammatory and anti-oxidant effects in the RA rat model. In addition, we investigated the impact of naringenin on the NF-κB pathway, MMP activity, and Nrf2 involvement in arthritic rats.

In the current study, naringenin administration significantly reduced right hind paw swelling in arthritic rats after only two weeks, indicating that it has anti-inflammatory properties in treating RA [37]. This, in turn, reflects a reduction in edema, attenuation of the inflammatory process, and synovial tissue hyperplasia, as well as a decrease in serum RF and ACCP levels, as shown in our study and previously reported [26].

To evaluate naringenin's anti-inflammatory effects, serum RF, ACCP, PGE_2, Th1 cytokines (TNF-α, IL-1β), Th2 cytokine (IL-4), and Th17 cytokine (IL-17) were measured two weeks after CFA injection. RF is a circulating antibody directed against IgG's Fc fragment used to diagnose RA [38]. B-lymphocytes are the source of RF and ACCP, which contribute to forming immune complexes in the joints. B-cells respond by releasing cytokines and chemokines, which promote angiogenesis, leukocyte infil-

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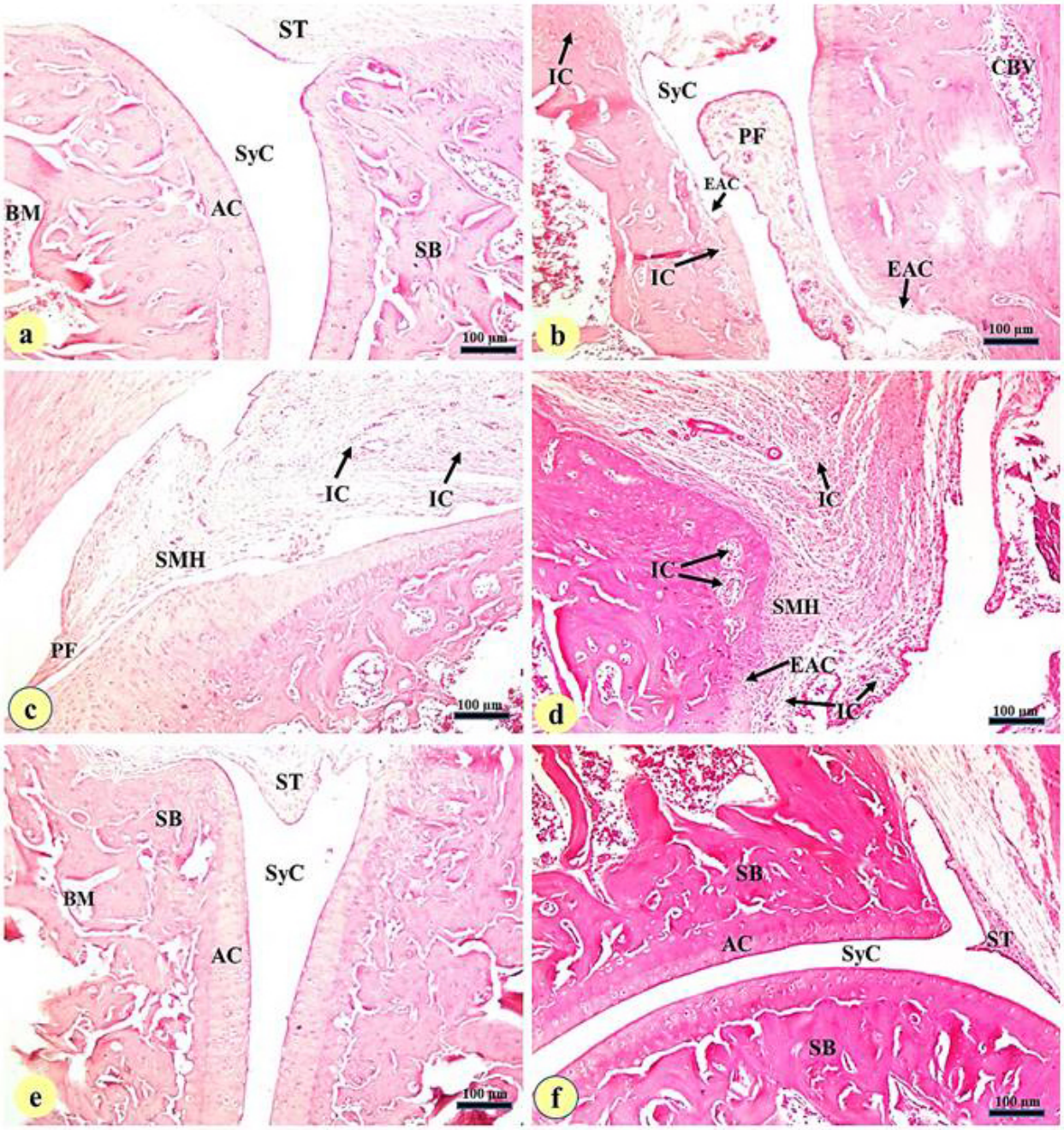

Fig. 4. Photomicrographs of sections of rat ankles of normal (a), arthritic control (b–d), and arthritic group treated with naringenin (e,f). Photomicrograph (a) showed normal articulating cartilage (AC), sponge bone (SB), synovial tissue (ST), synovial cavity (SyC), and bone marrow (BM). Photomicrographs (b–d) showed pannus formation (PF), erosion of articulating cartilage (EAC), synovial cavity (SyC), inflammatory cells' infiltration (IC), and synovial membrane hyperplasia (SMH). Photomicrographs (e,f) showed normal articulating cartilage (AC) and sponge bone (SB), bone marrow (BM), synovial tissue (ST), and synovial cavity (SyC).

tration, and synovial hyperplasia [39]. Naringenin treatment for two weeks significantly reduced arthritic rats' elevated serum RF and ACCP levels. This finding supports citrus flavonoids' antimicrobial, anti-oxidant, and anti-inflammatory properties [40]. In contrast, the rheumatoid joint has an activated PGE_2 pathway, with overexpression of its synthesizing enzymes, cyclooxygenase (COX)-1 and -2, as well as microsomal PGE_2-synthase 1 (mPGES-1) [41]. Furthermore, PGE_2 maintains inflammatory pathways by increasing the number of auto-aggressive T helper17 (Th17) cells [42]. In the current study, serum PGE_2 levels were significantly increased in arthritic rats, and naringenin treatment significantly reduced this elevated level.

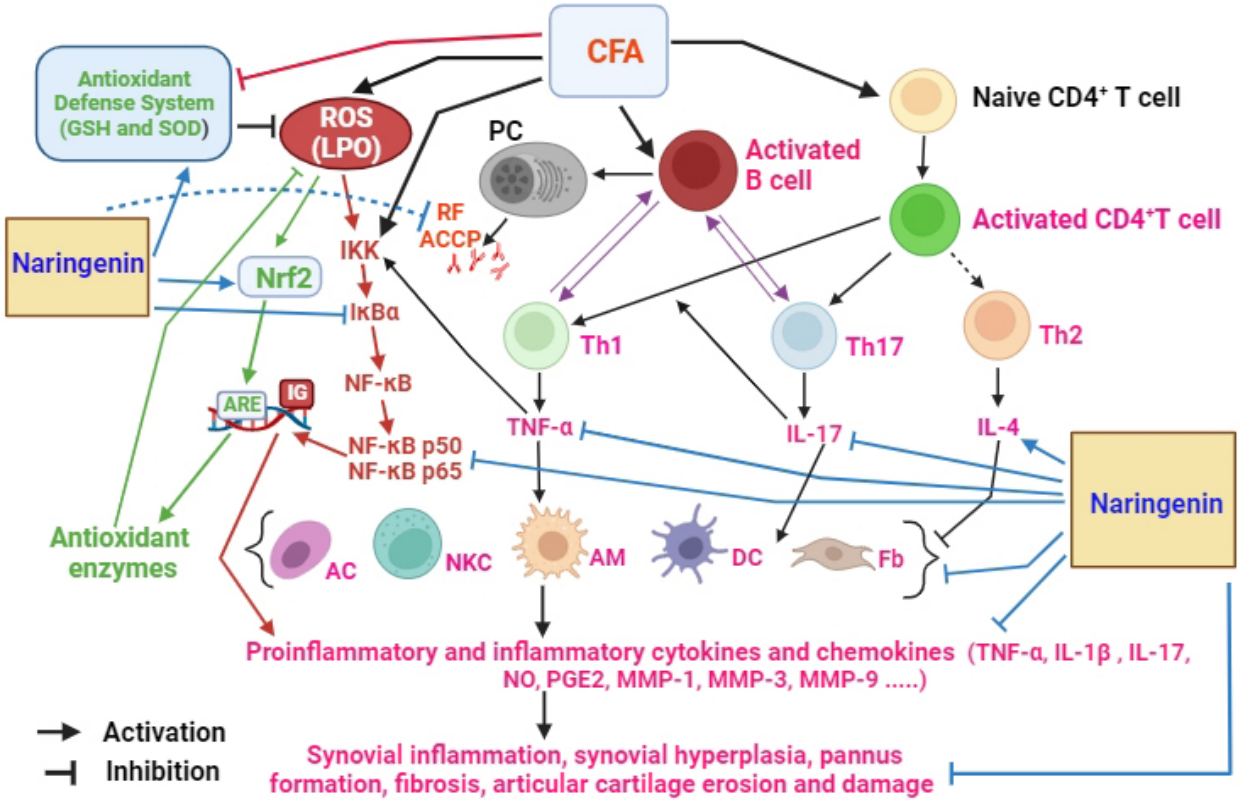

Fig. 5. Schematic figure showing the mechanisms of anti-arthritic actions of naringenin via its modulatory effects on Th1/Th2/Th17 cytokines, oxidative stress, anti-oxidant defense system, MMPs, NF-κB, and Nrf2 pathways. The figure was created by Biorender software (https://www.biorender.com/) on 9 Nov. 2023.

Because free radicals activate pro-inflammatory mediators, using anti-oxidants is expected to reduce cytokine release. Naringenin's ability to reduce inflammation has been proposed to occur via a variety of mechanisms. These mechanisms include increased phosphorylation of extracellular signal-regulated kinases (ERK) 5 and P38, mitogen-activated protein kinase (MAPK), as well as the inhibition of NF-κB [43]. Furthermore, naringenin inhibits the activation of *NF-κB*, resulting in a decrease of the downstream target genes controlled by *NF-κB*, including *iNOS* and *COX-2* [44]. Our study found that naringenin treatment reduced over-expressed NF-κB p50 and p65 proteins, as well as iNOS, in arthritic joints. Subsequent enzymes facilitate the catalysis of NO and PG production induced by oxidative stress, which serves as crucial inflammatory mediators in the development of RA [45]. These results are consistent with our findings, in which naringenin administration significantly reduced the elevated levels of PGE_2 and NO caused by RA.

The study found that untreated arthritic rats had significantly higher levels of serum pro-inflammatory cytokines (TNF-α, IL-1β, and IL-17) but significantly lower levels of IL-4. In RA, Th1 cytokines (TNF-α) promote the production of other inflammatory cytokines, such as IL-1β, which play a role in establishing the inflammatory process in the synovium [46,47]. TNF-α can trigger osteoclast differentiation, leading to bone degradation and joint damage [48]. Furthermore, IL-17 can increase the production of pro-inflammatory mediators [49], differentiate osteoclast progenitors into mature osteoclasts, and promote the production of nuclear factor κB ligand (RANK-L) by osteoblasts and synoviocytes, leading to bone degradation [50].

Naringenin treatment for two weeks significantly reduced serum TNF-α, IL-17, and IL-1β levels while increasing serum IL-4 levels. Naringenin reduces inflammation by normalizing TNF-α levels and inflammatory cell infiltration [40]. Naringenin suppresses the generation of TNF-α and IL-1β in the blood of arthritic rats. It also triggers programmed cell death in AIA synovial cells by regulating the Bcl-2 and Bax expression [35]. Naringenin's anti-oxidant properties reduce ROS generation, TNF-α activity, and the production of superoxide anions and cytokines [51,52].

Arthritic rats in the current studies had significantly higher liver LPO and NO levels than non-arthritic rats. It is thought that oxidative stress causes inflammation [53], which leads to connective tissue damage and synovitis [54]. Increased NO production as a result of oxidative stress is important in the pathophysiology of RA because it induces immune intolerance, which leads to inflammation and cell

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death [55]. LPO oxidizes membrane PUFAs, generating lipid peroxyl radicals that cause oxidation and cell membrane damage [56].

Naringenin treatment of arthritic rats significantly reduced elevated MDA and NO levels. Naringenin has anti-oxidant, metal chelating, and free radical scavenging properties that provide some protection against mutagenesis and LPO [57], and it has been shown to inhibit LPO activity [58]. Treatment with naringenin may increase glutathione peroxidase (GPx) activity, an anti-oxidant enzyme [59], implying that naringenin can inhibit the accumulation of free radicals produced during the LPO process and reduce oxidative stress by lowering lipid peroxide levels [60]. In addition, the B-ring catechol group in naringenin is essential for its anti-oxidant activity [61], as it can stabilize a radical species by donating H^+. Natural flavonoids, such as naringenin, have cytoprotective properties, as evidenced by the significant improvement in total protein levels following therapy [62].

The current study found that GSH levels in the livers of arthritic rats consistently decreased throughout the trial. GSH levels may be reduced because *GPx* and GST use GSH as a substrate [63]. In contrast, SOD activity increased significantly after two weeks of naringenin treatment. Free radicals can damage cartilage in joints directly or indirectly. Free radicals, or secondary messengers, play a role in RA's inflammatory and immune cellular responses [64]. GSH is a free radical scavenging compound that catalyzes the reduction of reactive oxygen species (ROS) to less reactive radicals. Extracellular SOD is the primary catalytic anti-oxidant found in joint fluid [65]. Furthermore, GSH serves as a coenzyme for numerous enzymes involved in cell defense [66]. *GPx* and GST can use GSH as a substrate to detoxify lipid hydroperoxides, H2O2, and electrophilic compounds. While preserving the cell's normal reduced anti-oxidant defense state, the GSH sulfhydryl group functions as a buffer that combats oxidative stress [67].

Arthritic rats showed a substantial elevate in hepatic GSH content after treatment with naringenin. Nonetheless, naringenin treatment of arthritic rats resulted in a significant increase in SOD activity. It has been reported that naringenin has a potent anti-oxidant activity that can reduce oxidative stress in lead-treated liver and kidneys by decreasing lipid peroxide levels [60]. Furthermore, naringenin was found to inhibit LPO, thereby protecting against free radical damage in acetaminophen-induced acute liver injury [68]. Furthermore, in cholesterol-induced hepatic inflammation, naringenin was found to increase hepatic GSH and SOD levels while decreasing MDA content and DNA damage [69]. Arthritic rats exhibited abnormalities in their right rear paw, such as increased size, redness, and ulceration. Furthermore, the affected ankle joint showed synovial hyperplasia, eroded articulating cartilage, and pannus formation. These histological and morphological changes could be attributed to an increase in oxidative stress and a decrease in the anti-oxidant defense mechanism. Furthermore, these changes could be ascribed to an elevation in cytokines associated with pro-inflammatory and inflammatory responses (PGE_2, TNF-α, IL-1, and IL-17), coupled with a reduction in cytokines associated with anti-inflammatory processes (IL-4) (Fig. 5). Treatment with naringenin may help reduce inflammation and joint destruction by decreasing pro-inflammatory cytokines and increasing anti-inflammatory cytokines, resulting in the disappearance of most histological lesions. These advantages could be attributed to a robust anti-oxidant defense system, which reduces oxidative stress and inflammation [70].

Activated chondrocytes and synovial fibrocytes produce matrix metalloproteinases (MMPs), which contribute to articular cartilage damage in RA and osteoarthritis [71]. In the current studies, rats with CFA-induced arthritis had significantly higher MMP-1, 3, and 9 protein expression levels in ankle articular tissues, indicating cartilage damage and erosion. These results are consistent with previous studies [72,73]. Naringenin treatment significantly reduced ankle MMP-1, 3, and 9 protein expression, which was linked to increased ankle histological integrity. Thus, naringenin may reduce articular cardiac erosion and damage by suppressing ankle MMPs.

Nrf2 is involved in anti-inflammatory pathways and anti-oxidant defense, which protects cells from damage caused by high ROS levels [74,75]. It stimulates the expression of anti-oxidant response element (ARE)-dependent genes, increasing anti-oxidant enzyme production and regulating the physiological and pathological effects of oxidant exposure (Fig. 5) [76]. In the current study, naringenin treatment significantly increased ankle Nrf2 protein levels. As a result, naringenin's anti-oxidant and anti-inflammatory properties may be mediated by increasing Nrf2 protein expression.

Conclusions

In conclusion, naringenin has a potent anti-arthritic activity via modulatory effects on Th1 (TNF-α and IL-1β), Th2 (IL-4), and Th17 (IL-17) cytokines, oxidative stress (LPO and NO), anti-oxidant defense system, NF-κB, MMPs, and Nrf2 pathways.

Availability of Data and Materials

The dataset generated in the current study is available from the corresponding author on demand.

Author Contributions

Research conception and design: OMA, RRA, DAA and FESAR; experiments: OMA, MMK, TA, FESAR, AAD, MEM, AME, SA and HIS; statistical analysis of the data: OMA and HIS; draft this manuscript: OMA, MMK, TA, FESAR, AAD, MEM, AME, SA and HIS; interpreta-

tion of the data and writing of the manuscript: all authors; contribute to important editorial changes in the manuscript: all authors; read and approved the final manuscript: all authors.

Ethics Approval and Consent to Participate

All animal procedures adhere to the guidelines of the "Experimental Animals Ethics Committee of the Faculty of Science, Beni-Suef University, Egypt, for the use and care of animals in research (Ethical Approval Number: BSU/FS/2015/20)".

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Conflict of Interest

The authors declare no conflict of interest.

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