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ORIGINAL ARTICLE

Ameliorative Effect of Morin, a Plant Flavonoid against Freund's Complete Adjuvant-Induced Polyarthritis in Rats

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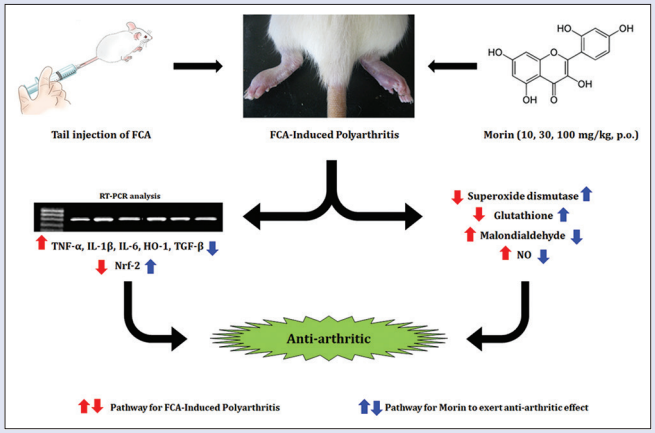
ABSTRACT

Background: Rheumatoid arthritis is a chronic relapsing autoimmune disorder with multifactorial etiology and prognosis. Morin [2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one], a plant flavonoid reported having antioxidant and anti-inflammatory potential. **Objective:** The aim is to study the anti-arthritic activity of the morin against adjuvant-induced polyarthritis in rats. **Materials and Methods:** Polyarthritis was induced in female Wistar rats (150–180 g) using Freund's complete adjuvant (FCA) in the tail. Leflunomide (10 mg/kg, as a standard) and morin (10, 30 and 100 mg/kg) were administered orally daily for 28 days. **Results:** Treatment with morin (30 and 100 mg/kg) showed statistically significant inhibition ($P < 0.05$) in FCA-induced decrease in thermal hyperalgesia and mechanical hyperalgesia. It also showed significant attenuation ($P < 0.05$) in FCA-induced alterations in hematological parameters, aspartate aminotransferase, alanine transaminase, alkaline phosphatase, serum turbidity, albumin, C-reactive protein, blood sugar levels, and erythrocyte sedimentation rate. Altered levels of serum and liver lipid profile were significantly attenuated ($P < 0.05$) by morin (30 and 100 mg/kg). Morin treatment significantly decreased ($P < 0.05$) serum oxido-nitrosative stress, tumor necrosis factor-alpha (TNF-α), and interleukin-1β (IL-1β) levels. Morin (30 and 100 mg/kg) also significantly ($P < 0.05$) inhibited FCA-induced up-regulated liver TNF-α, IL-1β, IL-6, heme oxygenase-1, transforming growth factor-beta and down-regulated nuclear factor erythroid 2-related factor-2 messenger RNA expression. Histopathology alteration-induced by FCA was also reduced by morin treatment. **Conclusion:** Morin showed promising curative properties against FCA-induced polyarthritis in rats via modulation of endogenous biomarkers. Thus, morin can be considered as an alternative treatment regimen for the management of arthritis.

Key words: Arthritis, Freund's complete adjuvant, heme oxygenase-1, interleukin-1β, interleukin-6, morin, nuclear factor erythroid 2-related factor-2, transforming growth factor-beta, tumor necrosis factor-alpha

SUMMARY

- Administration of Freund's complete adjuvant (FCA) in tail induces polyarthritis
- Morin (30 and 100 mg/kg) treatment showed significant inhibition in FCA induced decrease in thermal hyperalgesia and mechanical hyperalgesia
- It also significantly decreased serum oxido-nitrosative stress level
- Morin significantly down-regulated liver TNF-α, IL-1β, IL-6, HO-1, TGF-beta and up-regulated Nrf-2 mRNA expressions
- Morin can be considered as a potential antiarthritic agent for further development.



Abbreviations used: AIA: Adjuvant-induced arthritis, ALT: Alanine transaminase, ALP: Alkaline phosphatase, AST: Aspartate aminotransferase, BSL: Blood glucose level, CRP: C-reactive protein, ESR: Erythrocyte sedimentation rate, FCA: Freund's complete adjuvant, HO: Heme oxygenase, HDL: High-density lipoprotein, IL-1β: Interleukin-1 beta, LDL: Low-density lipoprotein, MDA: Malondialdehyde, NO: Nitric oxide, Nrf2: Nuclear factor-like 2, ROS: Reactive oxygen species, GSH: Reduced glutathione, RT-PCR: Reverse transcription-polymerase chain reaction, SOD: Superoxide dismutase, TC: Total cholesterol, TGF-β: Transforming growth factor-beta, TG: Triglyceride, TNF-α: Tumor necrosis factor-alpha, VLDL: Very low-density lipoprotein.

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease of multi-factorial etiology characterized by synovial hyperplasia, vasculogenesis, cartilage and bone destruction, and joint malformation. It is the most common form of arthritis that leads to pain, inflammation, and tissue damage causing restriction of movements of the limb.[1] The symptoms of RA include pain, swelling, morning stiffness, warmth, redness, and limited functions of the joints. The prevalence of the

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RA estimated in populations from different developed countries was 0.5%–1%.[2] The systemic ramifications of the disease, apart from morbidity and mortality, include cardiopathy, nephropathy, vasculopathy as well as pulmonary and cutaneous disorders.[3] Although the cause of RA is unknown, autoimmunity plays a pivotal role in its chronicity and progression. Thus, it is considered as a systemic autoimmune disease.

Although non steroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs (DMARDs), and corticosteroids appear to be highly efficient drug therapies in the treatment of RA, they may cause side effects that can range in severity from mild to serious. The major adverse drug reactions-associated with NSAIDs are gastrointestinal (perforation, ulceration, or bleeding), and cardiovascular (myocardial infarction) with effects on other systems. Despite their wide clinical uses, the long-term implication of NSAIDs is a major clinical problem due to their side-effects.[4] Some of the NSAIDs were withdrawn from the market because of the risk of heart attacks and stroke.[3] On the other hand, other therapies against arthritis such as DMARDs and biological agents had a risk of immunosuppression and serious infection on long-term usage. Furthermore, ascending morbidity of RA, together with occasional intolerable adverse effects of DMARDs, puts burden over medical researchers to find alternative treatment strategies. Therefore, the search for safer drugs for the management of RA in the long-term uses is still going on. Among the various experimental animal models of arthritis, Freund's complete adjuvant (FCA)-induced arthritis (AIA) in rats is the most reliable animal model.[5] AIA model mimics the human pathophysiological state characterized by chronic swelling in multiple joints due to the accumulation of inflammatory cells, joint cartilage erosion, and bone destruction. Thus, AIA has been used extensively to investigate the potent anti-arthritic agents.[2]

Recently, botanical medicines have become popular as alternative remedies due to their effectiveness, and safe. Morin [2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1- benzopyran-4-one] is one such plant flavonoid bearing potent antioxidant and anti-inflammatory property.[6] Morin widely exhibits in the various plant including *Maclura pomifera* (Osage orange), *Maclura tinctoria* (old fustic), and *Psidium guajava* (common guava).[7] The health and therapeutic benefits of morin have been extensively reviewed in the traditional and modern literature. An array of studies carried out over the past decade revealed anti-cancer, antiulcer, anti-allergic, anti-asthmatic, cardioprotective, renoprotective, and antihypertensive potential of morin.[7-9] Furthermore, studies have reported attenuative potential of morin against oxidative stress via modulation of superoxide dismutase (SOD), catalase, and glutathione peroxidase activities.[10] To the best of our knowledge, no study has been yet reported antiarthritic potential of morin. Hence, the present investigation aimed to evaluate the potential of morin against AIA-induced arthritis by assessing various behavioral, biochemical, and molecular parameters in rats.

MATERIALS AND METHODS

Animals

Female Wistar rats (150–180 g) were obtained from the National Toxicology Centre, Pune, India. The rats were housed in polypropylene cages at a temperature of 24°C ± 1°C with 12 h: 12 h dark–light cycle, with free access to standard pellet feed (Chakan Oil Mill, India) and filtered water. All experiments were carried out between 08:00 h and 17:00 h in a quiet laboratory. The research protocol was approved by the Institutional Animal Ethics Committee and as per Indian norms laid down by the Committee for the Purpose of Control and Supervision of Experimental Animals, New Delhi.

Adjuvant-induced polyarthritis

On day-0 of study, AIA was induced in rats (150–200 g) (five groups, i.e., Group II to VI, n = 12) by single intradermal injection of 0.1 ml FCA (Sigma Aldrich, St. Louis, USA) into the tail of the rats.[11] 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 was allowed to develop arthritis. A separate group of rats (Group I, n = 12) 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 morin (10, 30, and 100 mg/kg, i.e., Group IV to VI)[9] for the next 28 days.

Paw volume was determined by using a plethysmometer (Ugo Basile, Italy).[2] Percentage (%) inhibition of paw volumes was calculated according to the equation reported previously.[2] Pain latency against mechanical hyperalgesia (paw withdrawal latency) was determined by using Randall–Selitto (Ugo Basile Model 7200).[2]

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)[2] and hematological measurements (red blood cell [RBC], hemoglobin [Hb], hematocrit, mean corpuscular volume, mean corpuscular Hb concentration [MCH], mean corpuscular Hb concentration [MCHC], total leukocyte count, lymphocytes, and platelets). The serum was separated by centrifugation using an Eppendorf microcentrifuge and used for serum turbidity measurement.[12] The levels of serum albumin, alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), blood glucose level (BSL), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), very LDL (VLDL), and triglyceride (TG) were measured by a spectrophotometer (ultraviolet-visible spectrophotometer, Jasco V-530, Tokyo, Japan) using commercially available reagent kits according to the procedure provided by the manufacturer (Accurex Biomedical Pvt. Ltd., Mumbai, India). The levels of tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) levels in the serum were determined by enzyme-linked immunosorbent assay using commercial kits (Thermo Fisher Scientific, USA) as per the manufacturers' instructions.

Liver antioxidant and liver lipid analysis

All animals were sacrificed at the end of the study; the liver was immediately isolated. Tissue homogenates (n = 5) were prepared with 0.1 M Tris-HCl buffer (pH 7.4), and supernatant of homogenates was employed to estimate SOD, Reduced Glutathione (GSH), lipid peroxidation (malondialdehyde [MDA]), and nitric oxide (NO) as described previously.[13]

Another portion of liver tissue was homogenized in methanol and filtered through a Whatmann number 1 filter paper. The residue after filtration was scraped and homogenized in chloroform. The residue was once again scraped from the filter paper and ground with 10 ml of a chloroform-methanol mixture (2:1, v/v) and the resulting filtrate was evaporated to dryness. Further, lipids in tissue residue were determined by the previously reported method.[14]

Determination of TNF-α, IL-1β, IL-6, Nrf-2, HO-1 and TGF-β messenger RNA expression in liver

The levels of messenger RNA (mRNA) were analyzed in liver tissue (n = 5) using reverse transcription-polymerase chain reaction (PCR) as described

previously.[13] Single-stranded cDNA was synthesized from 5 μg of total cellular RNA using reverse transcriptase kit (MP Biomedicals India Private Limited, India) as described previously. The primer sequence for TNF-α, IL-1β, IL-6, Nuclear factor erythroid 2-related factor-2 (Nrf-2), heme oxygenase-1 (HO-1), transforming growth factor-beta (TGF-β), and β-actin are presented in the Table 1. Amplification of β-actin served as a control for sample loading and integrity. The intensity of mRNAs was standardized against that of the β-actin mRNA from each sample, and the results were expressed as the PCR-product/β-actin mRNA ratio.

Histopathology of tibiotarsal joint

Ankle joints from the two rats of each group were separated, cleaned, and washed in cold physiological saline, and preserved in 10% of 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 sections were observed, and photomicrographs were taken for histology assessment.

Statistical analysis

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

RESULTS

Effects of morin on FCA-induced alteration in body, spleen, and liver weights in rats

Administration of FCA brought a significant ($P < 0.05$) decreased in body weight and liver weight, as well as an, increase in spleen weight in AIA control group as compared to the normal group. Oral administration of LF (10 mg/kg) showed the significant ($P < 0.05$) increased in body weight, liver weight, and a decreased in spleen weight than that of AIA control group. When compared with AIA control group, morin (30 and 100 mg/kg) treated rats also significantly ($P < 0.05$) inhibited FCA-induced alteration in body, spleen, and liver weights. Morin (10 mg/kg) treated rats also showed significantly ($P < 0.05$) increased body weight than that of the AIA control group. Administration of LF (10 mg/kg) showed that more significant ($P < 0.05$) attenuation in FCA-induced altered body, spleen, and liver weights than that of the morin-treated rats [Table 2].

Effects of morin on FCA-induced alteration in paw volume, joint diameter, percent inhibition, thermal, and mechanical hyperalgesia in rats

Mean paw volumes and joint diameter were increased significantly ($P < 0.05$), whereas thermal and mechanical hyperalgesia were decreased significantly ($P < 0.05$) in AIA control rats as compared to that of the normal group. Treatment of LF (10 mg/kg) and morin (30 and 100 mg/kg) significantly ($P < 0.05$) inhibited FCA-induced alterations in mean paw volumes, joint diameter, thermal, and mechanical hyperalgesia as compared to that of the AIA control rats. The percentage inhibition of paw volume in LF (10 mg/kg) treated rats was 54.87%, whereas in morin (10, 30, and 100 mg/kg) treated groups, it was 3.87%, 23.28%, and 49.59%, respectively [Figures 1 and 2].

Effects of morin on FCA-induced alteration in aspartate transaminase, alanine transaminase, alkaline phosphatase, albumin, serum turbidity, and serum C-reactive protein in rats

AIA control rats showed significantly ($P < 0.05$) increased AST, ALT, ALP, and CRP levels and significant ($P < 0.05$) decreased in albumin and serum turbidity as compared to that of the normal rats. Treatment with LF (10 mg/kg) showed a significant decreased in AST, ALT, ALP, and CRP and significant ($P < 0.05$) increased in albumin and serum turbidity as compared to that of the AIA control group. Administration of morin (30 and 100 mg/kg) also showed significant ($P < 0.05$) inhibition in FCA-induced alterations in levels of AST, ALT, ALP, CRP, albumin and serum turbidity as compared with AIA control group. Administration of morin (100 mg/kg) showed more significant ($P < 0.05$) decreased in AST and ALT levels as well as increased in albumin and serum turbidity as compared to that of the LF (10 mg/kg) treated rats [Table 2].

Effects of morin on FCA-induced alteration in hematological parameters, erythrocyte sedimentation rate, and blood sugar levels in rats

AIA control rats showed a significant decreased ($P < 0.05$) in RBC and Hb as well as a significant increased ($P < 0.05$) in white blood cell (WBC) as compared to that of the normal rats. Treatment of LF (10 mg/kg) showed the significant inhibition ($P < 0.05$) in FCA-induced alterations in hematological parameters, i.e., RBC, WBC, and Hb as compared to that of AIA control rats. Morin (30 and 100 mg/kg) treatment also showed significantly increased ($P < 0.05$) in RBC and Hb as well as significantly decreased ($P < 0.05$) in WBC as compared to that of the AIA control rats. Administration of LF (10 mg/kg) showed a more significant increased ($P < 0.05$) in RBC and Hb as well as a significant decreased ($P < 0.05$) in WBC as compared to morin-treated rats [Table 3].

The ESR and BSL levels were increased significantly ($P < 0.05$) in AIA control rats as compared to that of the normal rats. Treatment with LF (10 mg/kg) showed significant ($P < 0.05$) decreased in ESR and BSL levels as compared to that of the AIA control rats. Morin (30 and 100 mg/kg) treatment showed statistically significant ($P < 0.05$) decreased in ESR and BSL levels as compared with AIA control

Table 1: Primer sequences for TNF-α, IL-1β, IL-6, Nrf-2, HO-1, TGF-β and β-actin

Gense	Sequence		Size (bp)
	Forward primer	Reverse primer	
TNF-α	AAGCCTGTAGCCCATGTTGT	CAGATAGATGGGCTCATACC	295
IL-1β	TGATGTTCCCATTAGACAGC	GAGGTGCTGATGTACCAGTT	290
IL-6	TAGCCGCCCCACACAGACAG	GGCTGGCATTTGTGGTTGGG	479
Nrf2	CCTCACCTCTGCTGCCAGT	GGGAGGAATTCTCCGGTCTC	316
HO-1	TTGTAACAGACTTGCCAGAG	CACTCACTGGTTGTATGCG	202
TGF-β	GTTCTTCAATACGTCAGACATTCG	CATTATCTTTGCTGTCACAAGAGC	309
β-actin	GTCACCCACACTGTGCCCATCT	ACAGAGTACTTGCGCTCAGGAG	764

TNF-α: Tumor necrosis factor-alpha; IL-1β: Interleukin 1-beta; TGF-β: Transforming growth factor-β

Table 2: Effects of morin on FCA induced alterations in body weight, spleen weight, liver weight, serum aspartate transaminase, alanine transaminase, alkaline phosphatase, albumin, serum turbidity, and serum C-reactive protein levels in rats

Treatment	Body weight (g)	Spleen weight (g)	Liver weight (g)	AST (U/ml)	ALT (U/ml)	Alkaline phosphatase (U/L)	Albumin (g/dl)	Serum turbidity (Absorbance at 645 nm)	Serum CRP (mg/L)
Normal	218.4±8.79	1.15±0.02	11.50±0.41	59.16±2.56	42.46±2.25	66.30±3.25	4.28±0.33	1.69±0.10	1.22±0.10
AIA control	168.6±6.85$^{\#}$	2.01±0.44$^{\#}$	8.22±0.31$^{\#}$	99.10±3.17$^{\#}$	85.04±3.12$^{\#}$	183.3±4.62$^{\#}$	1.89±0.30$^{\#}$	0.81±0.18$^{\#}$	3.66±0.18$^{\#}$
LF (10)	199.8±5.83$^{*,\$}$	1.24±0.06$^{*,\$}$	11.01±0.38$^{*,\$}$	67.46±3.41$^{*,\$}$	46.92±2.23$^{*,\$}$	76.74±4.40$^{*,\$}$	3.88±0.37$^{*,\$}$	1.52±0.11$^{*,\$}$	1.76±0.29$^{*,\$}$
M (10)	182.6±4.34*	1.84±0.10	8.97±0.53	91.30±3.55	74.88±1.30	162.2±3.71*	2.51±0.26*	1.08±0.17	3.08±0.32$^{*,\$}$
M (30)	194.8±7.96$^{*,\$}$	1.56±0.08$^{*,\$}$	8.33±0.38*	76.90±2.49$^{*,\$}$	62.22±2.54$^{*,\$}$	110.8±5.39$^{*,\$}$	3.18±0.33$^{*,\$}$	1.24±0.05$^{*,\$}$	2.58±0.28$^{*,\$}$
M (100)	206.6±4.76$^{*,\$}$	1.25±0.54$^{*,\$}$	9.24±0.33$^{*,\$}$	66.12±1.52$^{*,\$}$	46.48±1.83$^{*,\$}$	79.06±5.27$^{*,\$}$	4.06±0.25$^{*,\$}$	1.54±0.10$^{*,\$}$	1.76±0.13$^{*,\$}$

Figures in the parenthesis indicate dose in mg/kg. n=5, data were 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; M: Morin; AST: Aspartate aminotransferase; ALT: Alanine transaminase; CRP: C-reactive protein

Table 3: Effects of morin on FCA-induced alterations in hematological parameters, erythrocyte sedimentation rate, and blood sugar level in rats

Treatment	RBC (×10^6/μL)	WBC (×10^3/μL)	Hb (g/dL)	ESR (mm)	BSL (mg/dL)
Normal	9.33±1.56	12.34±3.21	15.22±2.50	2.87±2.33	74.24±1.36
AIA control	5.55±0.23$^{\#}$	18.22±3.43$^{\#}$	10.98±2.34$^{\#}$	8.99±2.24$^{\#}$	119.00±1.18$^{\#}$
LF (10)	8.53±1.23$^{*,\$}$	13.14±1.45$^{*,\$}$	14.22±2.34$^{*,\$}$	3.55±1.23$^{*,\$}$	85.98±1.45$^{*,\$}$
M (10)	5.76±0.66	17.22±2.12	11.23±2.87	8.09±1.22	115.40±2.09
M (30)	6.87±0.76$^{*,\$}$	15.87±2.22$^{*,\$}$	12.54±2.87$^{*,\$}$	6.89±1.45$^{*,\$}$	106.10±2.06$^{*,\$}$
M (100)	7.88±1.34$^{*,\$}$	14.23±1.56$^{*,\$}$	14.09±2.76$^{*,\$}$	5.44±1.09$^{*,\$}$	89.32±2.97$^{*,\$}$

Figures in the parenthesis indicate dose in mg/kg. n=5, data were 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; M: Morin; RBC: Red blood cells; WBC: White blood cells; ESR: Erythrocyte sedimentation rate; BSL: Blood sugar level

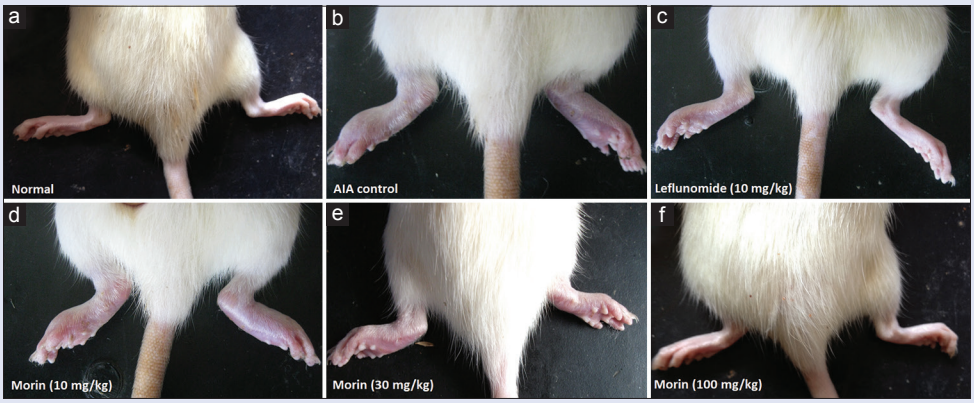

Figure 1: Effects of morin on morphological representations of rat paw after the administration of FCA. Representative images of paw from (a) normal, (b) adjuvant induced arthritis control, (c) Leflunomide (10 mg/kg), (d) Morin (10 mg/kg), (e) Morin (30 mg/kg), and (f) Morin (100 mg/kg)-treated rat

rats. The decreased in ESR and BSL levels was more statistically significant (P < 0.05) in LF (10 mg/kg) treated rats as compared to that of the morin-treated rats [Table 3].

Effects of morin on FCA-induced alteration in serum and liver lipid profile in rats

There was a statistically significant (P < 0.05) increased in TG, TC, LDL-cholesterol (LDL-C), and VLDL-C, whereas significant (P < 0.05) decreased in HDL-cholesterol (HDL-C) level in serum and liver of AIA control rats as compared to that of the normal rats. Treatment with LF (10 mg/kg) showed significant (P < 0.05) attenuation in FCA-induced alterations in serum and liver lipid profile as compared to that of the AIA control rats. Administration of morin (30 and 100 mg/kg) showed significant decreased (P < 0.05) in TG, TC, LDL-C, and VLDL-C, whereas significant increased (P < 0.05) in HDL-cholesterol levels as compared to that of the AIA control rats. The attenuation of FCA-induced alteration in serum and liver lipid profile was more statistically significant (P < 0.05) in LF (10 mg/kg) treated rats when compared with morin-treated rats [Table 4].

Effects of morin on FCA-induced alteration in oxido-nitrosative stress in rats

Administration of FCA resulted in significantly decreased (P < 0.05) SOD and GSH levels, whereas significant increased (P < 0.05) in MDA and NO levels in AIA control rats as compared to that of the normal rats. Administration of LF (10 mg/kg) showed statistically significant increased (P < 0.05) in SOD and GSH levels, whereas significant decreased (P < 0.05) in MDA and NO levels as compared to that of the AIA control rats. Treatment with morin (30 and 100 mg/kg) showed the significant attenuation (P < 0.05) in FCA-induced increased in oxido-nitrosative stress as compared to that of the AIA control rats. The increase in SOD and GSH levels as well as decreased in MDA and NO

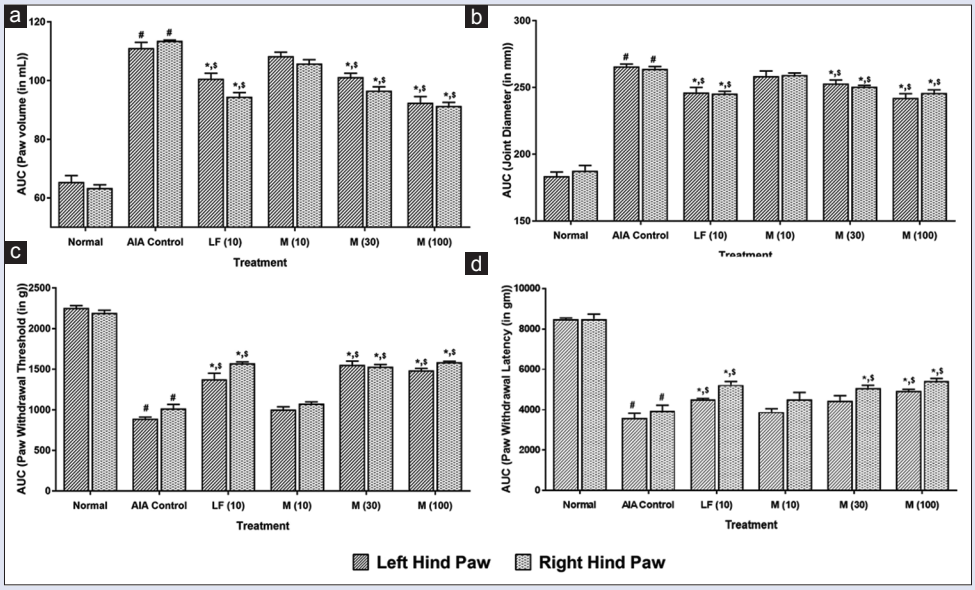

Figure 2: Effects of morin on FCA-induced alterations in (a) change in paw volume, (b) change in joint diameter, (c) paw withdrawal threshold, and (d) paw withdrawal latency in arthritic rats. $n = 5$, Data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. $^{\#}P < 0.05$ 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; AUC: Area under curve; LF: Leflunomide; M: Morin

Table 4: Effects of morin on FCA-induced alterations in plasma and liver lipid profile in rats

Treatment	Tissue	Triglyceride (mg %)	Total cholesterol (mg %)	HDL-C (mg %)	LDL-C (mg %)	VLDL-C (mg %)
Normal	Plasma	74.49±2.51	14.96±0.89	27.59±0.69	4.60±0.24	14.90±0.50
	Liver	52.14±3.27	85.07±3.77	32.48±1.35	27.77±1.57	10.43±0.65
AIA control	Plasma	150.70±1.99$^{\#}$	39.02±1.21$^{\#}$	7.876±0.47$^{\#}$	17.84±0.47$^{\#}$	30.15±0.40$^{\#}$
	Liver	156.30±3.49$^{\#}$	160.70±5.10$^{\#}$	4.73±1.07$^{\#}$	68.78±1.02$^{\#}$	31.26±0.70$^{\#}$
LF (10)	Plasma	81.23±1.64$^{*,\$}$	18.40±1.50$^{*,\$}$	21.85±0.74$^{*,\$}$	6.31±0.29$^{*,\$}$	16.25±0.33$^{*,\$}$
	Liver	63.99±2.47$^{*,\$}$	95.53±1.49$^{*,\$}$	29.11±0.92$^{*,\$}$	32.78±1.08$^{*,\$}$	12.80±0.49$^{*,\$}$
M (10)	Plasma	146.30±1.93	39.02±1.23	9.724±0.83	13.73±0.14	29.27±0.39
	Liver	139.8±2.51	143.70±19.8	8.20±0.99	64.10±1.12	27.95±0.50
M (30)	Plasma	118.60±1.29$^{*,\$}$	29.68±1.36$^{*,\$}$	17.84±1.17$^{*,\$}$	10.27±0.27$^{*,\$}$	23.71±0.26$^{*,\$}$
	Liver	105.80±2.36$^{*,\$}$	120.30±8.13$^{*,\$}$	15.08±0.67$^{*,\$}$	53.61±0.74$^{*,\$}$	21.16±0.47$^{*,\$}$
M (100)	Plasma	95.35±2.19$^{*,\$}$	25.36±1.36$^{*,\$}$	20.76±0.37$^{*,\$}$	7.94±0.41$^{*,\$}$	19.07±0.44$^{*,\$}$
	Liver	75.38±2.72$^{*,\$}$	100.90±5.35$^{*,\$}$	21.73±1.14$^{*,\$}$	41.45±1.00$^{*,\$}$	15.08±0.54$^{*,\$}$

Figures in the parenthesis indicate dose in mg/kg. n=5, data were 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; M: Morin; HDL-C: High-density lipoprotein-cholesterol; LDL-C: Low-density lipoprotein-cholesterol; VLDL-C: Very low-density lipoprotein-cholesterol

levels were more significant ($P < 0.05$) in LF (10 mg/kg) treated rats as compared to that of the morin-treated rats [Table 5].

Effects of morin on FCA-induced alteration in TNF-α and IL-1β as well as hepatic TNF-α, IL-1β and IL-6 mRNA expressions in rats

There was a significant ($P < 0.05$) increased in serum TNF-α and IL-1β levels as well as upregulation in hepatic TNF-α, IL-1β, and IL-6 mRNA expressions of AIA control rats as compared to that of the normal rats. Treatment with LF (10 mg/kg) showed significant ($P < 0.05$) decreased in serum TNF-α and IL-1β levels as well as down-regulation in hepatic TNF-α, IL-1β, and IL-6 mRNA expressions as compared to that of the AIA control rats. Administration of morin (30 and 100 mg/kg) also showed significantly down-regulated ($P < 0.05$) serum TNF-α and IL-1β levels as well as hepatic TNF-α, IL-1β, and IL-6 mRNA expressions as compared to that of the AIA control rats. The attenuation of FCA-induced alteration in serum TNF-α and IL-1β levels, hepatic TNF-α, IL-1β, and IL-6 mRNA expressions was more statistically significant ($P < 0.05$) in LF (10 mg/kg) treated rats when compared with morin-treated rats [Table 5 and Figure 3].

Effects of morin on FCA-induced alteration in hepatic Nrf-2, HO-1 and TGF-β mRNA expressions in rats

The AIA control rats showed significant ($P < 0.05$) down-regulation in hepatic Nrf-2 and significant ($P < 0.05$) upregulation in HO-1 and TGF-β mRNA expressions as compared to that of the normal rats. Oral treatment with LF (10 mg/kg) showed a significant upregulation ($P < 0.05$) in hepatic Nrf-2 and significant ($P < 0.05$) down-regulation in HO-1 and TGF-β mRNA as compared to that of the AIA control rats. Morin (30 and 100 mg/kg) treated rats also show significant ($P < 0.05$) upregulation ($P < 0.05$) in hepatic Nrf-2 and significant ($P < 0.05$) down-regulation in HO-1 and TGF-β mRNA as compared to that of the AIA control group. Administration of LF (10 mg/kg) showed a more significant attenuation ($P < 0.05$) in FCA-induced alterations in

Table 5: Effects of morin on FCA-induced alterations in liver antioxidant parameters and serum cytokines levels in rats

Treatment	SOD (U/mg of protein)	GSH (µg/mg of protein)	MDA (nM/mg of protein)	NO (µg/mL)	Serum TNF-α (pg/mL)	Serum IL-1β (pg/mL)
Normal	3.16±0.11	2.25±0.07	0.42±0.07	111.80±1.94	22.10±2.45	129.07±4.55
AIA control	0.60±0.04$^{\#}$	0.61±0.08$^{\#}$	3.20±0.10$^{\#}$	553.60±3.95$^{\#}$	77.88±2.34$^{\#}$	513.89±12.34$^{\#}$
LF (10)	2.65±0.06$^{*,\$}$	2.08±0.06$^{*,\$}$	0.73±0.07$^{*,\$}$	167.80±3.36$^{*,\$}$	42.30±8.98$^{*,\$}$	234.78±6.77$^{*,\$}$
M (10)	1.00±0.08	0.58±0.12	2.70±0.09	507.20±3.40	72.65±5.78	456.87±10.98
M (30)	1.57±0.10$^{*,\$}$	1.15±0.05$^{*,\$}$	1.48±0.08$^{*,\$}$	336.60±3.57$^{*,\$}$	61.65±3.56$^{*,\$}$	367.77±12.97$^{*,\$}$
M (100)	2.16±0.12$^{*,\$}$	1.66±0.11$^{*,\$}$	0.86±0.12$^{*,\$}$	213.00±3.29$^{*,\$}$	48.42±8.33$^{*,\$}$	216.98±8.87$^{*,\$}$

Figures in the parenthesis indicate dose in mg/kg. n=5, data were 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-1β: Interleukin 1-beta; AIA: Adjuvant-induced arthritis; LF: Leflunomide; M: Morin

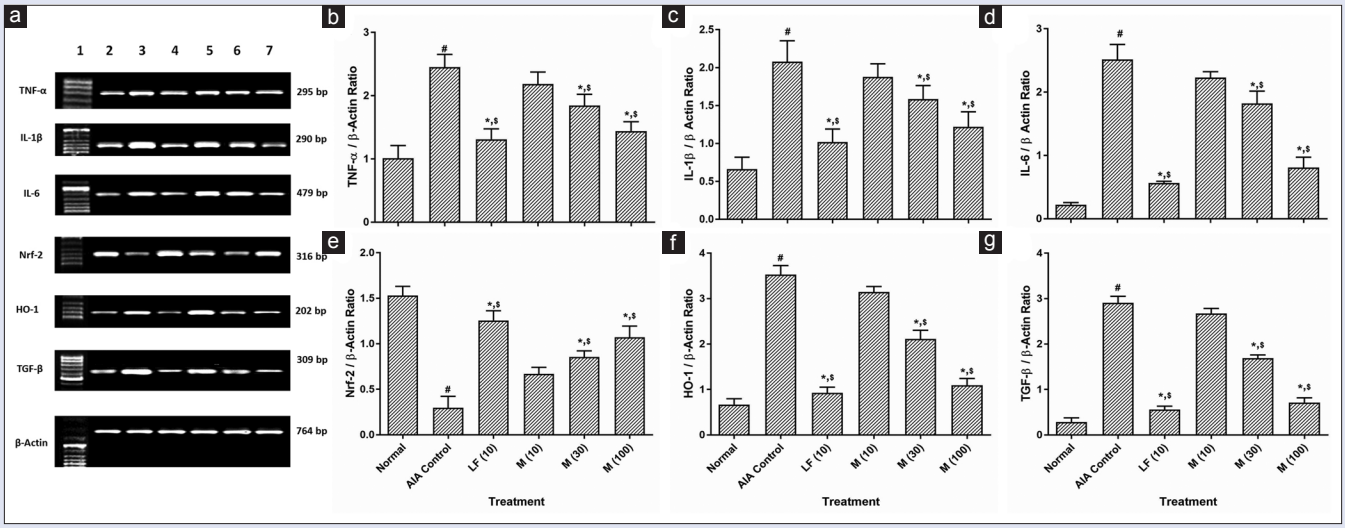

Figure 3: Effects of morin on FCA-induced alterations in hepatic TNF-α, IL-1β, IL-6, Nrf-2, HO-1 and TGF-β messenger RNA expression in rats (a), quantitative representation of the messenger RNA expression of TNF-α (b), IL-1β (c), IL-6 (d), Nrf-2 (e), HO-1 (f), and TGF-β (g). Data are expressed as mean ± standard error of the mean (n = 5) 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: Messenger RNA expression of normal group; Lane 3: messenger RNA expression of AIA control group; Lane 4: messenger RNA expression of Leflunomide (10 mg/kg)-treated group, and Lane 5-7: messenger RNA expression of Morin (10, 30, and 100 mg/kg)-treated group. AIA: Adjuvant-induced arthritis; LF: Leflunomide; M: Morin; TNF-α: Tumor necrosis factor-alpha; IL-1β: Interleukin-1 beta; Nrf2: Nuclear factor erythroid 2-related factor-2; HO-1: Heme Oxygenase-1; TGF-β: Transforming growth factor-beta

hepatic Nrf-2, HO-1, and TGF-β mRNA as compared to that of the morin-treated rats [Figure 3].

Histopathology of the tibiotarsal joint in FCA-induced arthritis in rats

Figure 4a represents the normal architecture of tibiotarsal joint tissues from normal rat. It is devoid of any cellular infiltration, synovitis, edema, or damage to cartilage or bone. Figure 4b represented the architecture of joint tissues from AIA control rat and showed highly abnormal joint histology with severe (grade +++) cellular infiltration (red arrow), synovitis, edema (yellow arrow), and damage to cartilage (black arrow). Figure 4c represents the architecture of joint tissues from LF (10 mg/kg) treated rats, showed inhibition of cellular infiltration (red arrow), and edema (yellow arrow) with mild synovitis and damage of bones with mild (grade +) damage of cartilage tissues (black arrow). Figure 4d represented the architecture of joint tissues from morin (10 mg/kg) treated rats and showed mild edema (yellow arrow) in deeper subcutaneous tissues with moderate (grade ++) cellular infiltration (red arrow), synovitis, cartilage, and bone damage (black arrow). Figure 4e and f represented the architecture of joint tissues from morin (30 and 100 mg/kg) treated rats and showed a reduction in histological alteration-induced by AIA.

DISCUSSION

Adjuvant arthritis is well-established and widely used animal model for the screening of potent antiarthritic agents. In the present investigation, polyarthritis was induced in the Wistar rats that include intradermal injection of FCA with heat-killed mycobacteria in mineral oil in the tail.[11] The tail is preferred as the preliminary site for the injection of FCA, hence that the swelling of both hind paws can be easily measured as well as chances of severe debilitation and interference of acute components of inflammation could be minimized.[11] FCA-induced polyarthritic rats mimics all clinicopathological features of RA which includes joints swelling, inflammatory infiltration, bone destruction, cartilage erosion, and remodeling.[2] The initial reaction includes the edema formation and soft-tissue thickening in both hind paw, whereas the formation of secondary lesions in the tail during late-phase of arthritis is presumed to be a manifestation of cell-mediated immunity.[11] In addition, to mimic real-life clinical situations, arthritis was allowed to develop by keeping rats untreated for 32 days, and morin was administered for the next 28 days.

The AIA is a well-characterized laboratory model of persistent pain in the arthritic study.[15] Mechanical and thermal hyperalgesia are characteristic features of arthritic pain, whereas Randall–Selitto test, Hargreaves test have been reported methods to record these behavioral measures.[16]

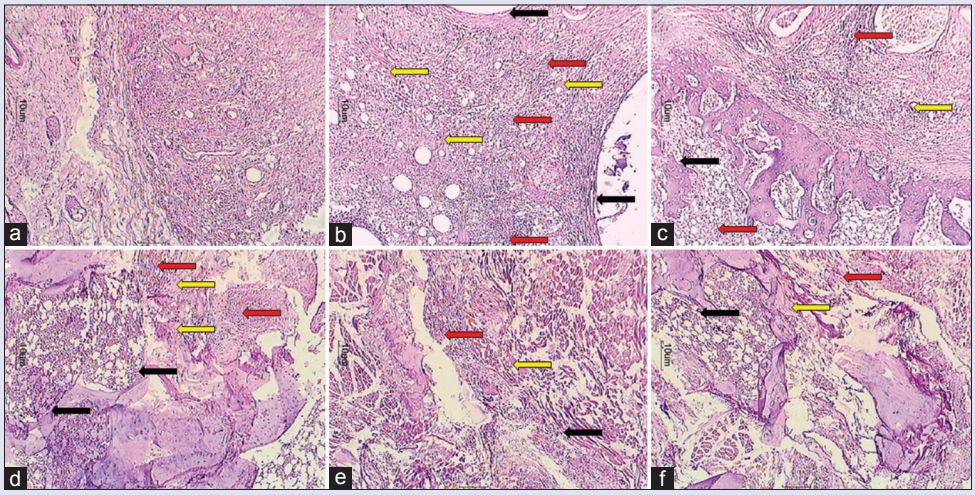

Figure 4: Effects of morin on the histopathology of tibiotarsal joints after administration of FCA. Representative histological images from (a) normal, (b) adjuvant-induced arthritis control, (c) Leflunomide (10 mg/kg), (d) Morin (10 mg/kg), (e) Morin (30 mg/kg) and (f) Morin (100 mg/kg)-treated rat. Images stained with H and E, ×100. Cellular infiltration (red arrow), edema (yellow arrow), damage to cartilage tissues (black arrow)

Intradermal administration of FCA produces a pronounced tibiotarsal joint swelling, and hyperalgesia appeared with no involvement of both hind paw. During the acute phase of arthritis, hyperalgesia is found to occur more frequently, and reduced nociceptive threshold in AIA rats. This can be assessed by spontaneous behavior including protection of the affected paw by curving or elevation of the paw and avoiding the support of the body on the paw.[17] Along with mechanical hyperalgesia, AIA also associated with dysregulated thermal receptor leads to a declined thermal stimuli threshold.[18] This was evident by decreased paw withdrawal latency during the plantar test by AIA control rats. Inhibition of mechanical and thermal hyperalgesia observed after the administration of morin might be a result of an indirect modulation of nociceptor sensitization because it could attenuate release of pro-inflammatory mediators to diminishing sensitization of afferent fibers.

Evaluation of the stability of serum protein against heat denaturation is another important hallmark that reflects the severity of the polyarthritic condition. The release of degradative enzymes from the lysosomes caused significant denaturation in native connective tissue that, in turn, induces an immune response in AIA. Most of the therapeutically used anti-inflammatory drugs for polyarthritis were shown to inhibit the lysosomal enzymes and restore the nature of native connective tissue.[12] In addition, CRP, an acute-phase protein has been identified as an important biomarker for various immune-inflammatory and degenerative diseases including chronic RA.[1] In the present study, AIA-induced rats were associated with decreased serum turbidity and elevated levels of CRP, whereas morin treatment attenuated FCA-induced alterations in serum turbidity, and CRP levels depicting its potential to improve the stability of serum protein.

The typical rise in serum ALP, AST, and ALT in RA are the common features of AIA and has been well-reported previously.[1] Determination of activity of these biomarkers in serum is a simple tool to assess the potential of various anti-arthritic agents against AIA.[19] Increased in the activities of serum AST, ALT, and ALP are good indices of hepatic impairment which is also considered a feature of adjuvant arthritis. In addition, most NSAIDs drugs utilized in the treatment of AIA caused hepatotoxic result in the elevated levels of AST and ALT in AIA. However, administration of morin has significantly prevented the elevation of AST, ALT, and ALP revealed its organo-protective roles in AIA arthritis rats.

Numerous evidence suggests that oxidative stress plays a pivotal role in the pathogenesis of arthritis.[3,11] Arthritis is recognized by an elevation of oxidative stress and massive impairment of antioxidant defense system. An antioxidant system is consists of endogenous enzymes including hydroxyl radical and superoxide anions which composed of SOD, GSH, and GSH-Px that protect against oxidative stress thus alleviates elevated reactive oxygen species (ROS) generation.[13] Furthermore, MDA is responsible for the destruction of liposome which leads to the tissue damage. Degradation of cell membranes polyunsaturated fatty acid by MDA resulted in cell death. Furthermore, Nrf-2 is an antioxidant, plays a central role in the protection of the cells against oxidative stress.[13] In addition, exogenous and endogenous chemical exposure induce oxidative stress that might be associated with Nrf-2/HO-1 pathway in cells. Thus, various researchers have implicated antioxidant with an ability to decrease ROS generation and increase Nrf-2 production for the treatment of arthritis.[13,20] Bioflavonoids have been considered as a potent bioactive moiety against free radicals and oxidative stress. In the present investigation administration of morin, a flavonoid resulted in amelioration of oxido-nitrosative stress via upregulation of Nrf-2 mRNA expression.

A study has reported the role of various mediators including cytokines (TNF-α and IL's), macrophage colony stimulating factor, interferons, and platelet-derived growth factor in the pathogenesis of RA.[21] Activated leucocytes caused the release of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) which have been found in high concentrations in synovium as well as in plasma of RA patients.[22] Elevated TNF-α plays a vital role in pain initiation, joint inflammation, bone deformations, and joint function disability.[1] Release TNF-α cause sensitization of nociceptive neurons via induction of a proinflammatory cytokine cascade involving IL-1β, IL-6, and IL-8 that produces significant mechanical and thermal hyperalgesia.[23] It has been reported that intradermal administration of FCA caused a significant increase in the levels of cytokines in synovium and blood of AIA rats.[4,11] Rats-treated with morin showed the significant inhibition in up-regulated serum, and hepatic expressions of TNF-α, IL-1β, and IL-6 which in turn decreased the redness, swelling, and edema around the ankle joint of the rat. It was also evident in the gross pathological as well as the histological findings of the morin-treated rats. A study carried out by the previous researcher has reported the TNF-α and IL's inhibitory potential of morin

in vivo.[7] The result of the present study corroborates with the findings of the previous researcher.[7]

Extracellular membrane protein activation and matrix degradation subdue is correlated with TGF-β1-induced the development of autoimmunity. TGF-β1 is a most potent multitasking profibrogenic cytokine indulged in the regulation of functional immunomodulatory effects depending upon the environment thus, leading to development polyarthritis.[24] Interestingly, TGF-β signal transduction is greatly connected with the Smads. It has been evident that Smad3 phosphorylation in the nucleus activates the formation of heteromer complex with co-Smad (Smad4) that are receptive to TGF-β.[25] Furthermore, recently it has also been documented that FCA ingestion responsible for the TGF-β/Smad activation.[26] Consistent with this finding, the results of the present investigation showed that there was significant up-regulation in the TGF-β mRNA expression in FCA administered rats. Whereas, administration of morin down-regulated this TGF-β mRNA expression thus, ameliorates FCA-induced arthritis. The results of the present investigation are in accordance with the findings of the previous investigator where morin treatment significantly attenuates TGF-β/Smad signaling.[27]

The current treatment regimen for arthritis includes synthetic drugs such as NSAIDs (including ibuprofen, aceclofenac, naproxen, etc.) or in combination with the steroid hormone (such as cortisone and prednisone) and DMARDs (including methotrexate, cyclosporin A, and leflunomide) which utilized to minimize the degree of pain.[4] However, these therapies provide relief in the only a fraction of patients, and their side effect limits their use. However, in the present study, morin administration did not produce any toxicity.

Nowadays, many chronic diseases involve multiple organ systems and interdependent etiological factors hence, use of single target-and single molecule-based drug development is of worthless.[28] Hence, for have an effective treatment for the disease like arthritis there is a need for a therapeutic agent who takes care of alterations in the multiple mediators and their effects. In the present investigation, morin protected synovial membrane and prevented the destruction of cartilage, which improved health status through the inhibition of release of pro-inflammatory mediators such as TNF-α, IL's, HO-1, and TGF-β. It also demonstrates not only its ability to improve health status in arthritis but also clinical signs including paw edema, thermal hyperalgesia, and histomorphological examinations. These results showed that morin would be an effective long-term anti-arthritic agent from natural origin to overcome serious side effects of synthetic agents.

CONCLUSION

The results from the present investigation suggest that morin showed promising curative properties against FCA-induced polyarthritis in rats through the inhibition of endogenous biomarkers (CRP, TNF-α, IL-1β, IL-6, HO-1, and TGF-β) as well as oxido-nitrosative stress (SOD, GSH, MDA, and NO) along with an increase in Nrf-2 levels. Thus, morin can be considered as an alternative treatment regimen for the management of arthritis

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Conflicts of interest

There are no conflicts of interest.

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