



# *Moringa rivae* leaf extracts attenuate Complete Freund's adjuvant-induced arthritis in Wistar rats via modulation of inflammatory and oxidative stress biomarkers

Ammara Saleem<sup>1</sup> · Mohammad Saleem<sup>1,2</sup> · Muhammad Furqan Akhtar<sup>3</sup> · Muhammad Shahzad<sup>4</sup> · Shah Jahan<sup>5</sup>

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## Abstract

*Moringa rivae* is widely used as a traditional remedy against arthritis. The present research was designed to evaluate the anti-arthritic potential of *Moringa rivae* extracts. Treatment of rats with adjuvant-induced arthritis with methanolic and aqueous extracts of *M. rivae* (150, 300 or 600 mg/kg), and piroxicam (10 mg/kg) was started orally at day 8 post-administration of complete Freund's adjuvant and continued till 28th day. The therapeutic effect of the plant extracts was assessed in arthritic rats by arthritic index, body weight, and haematological and biochemical parameters. Furthermore, the modulatory effect on gene expression (I- $\kappa$ B, IL-4 and IL-10, COX-2, IL-1 $\beta$  and IL-6, NF- $\kappa$ B, and TNF- $\alpha$ ) in the blood was determined using qRT-PCR, while ELISA assay was used to find PGE2 and TNF- $\alpha$  concentrations in the serum. Oxidative stress parameters in the liver and ankle joint histopathology were also evaluated. Moreover, the most effective methanolic extract was further characterized by GC–MS for the presence of phytochemicals. Treatment with the plant extracts significantly restored arthritic index, change in the body weight and immune organ weight, and the histopathological indices. Both extracts significantly ( $p < 0.05$ ) reduced the serum concentration of rheumatoid factor, C-reactive protein, PGE2, and TNF- $\alpha$  in arthritic rats. The extracts persuasively down-regulated the COX-2, PGE2, IL-1 $\beta$ , IL-6, NF- $\kappa$ B, and TNF- $\alpha$ , and up-regulated the mRNA expression of I- $\kappa$ B, IL-4, and IL-10. Both extracts increased the activities of CAT and SOD while reducing the formation of MDA in a dose- dependent manner in the liver. Histopathological evaluation showed that treatment with the plant extracts significantly ( $p < 0.05$ ) reduced joint inflammation, pannus formation, and bone erosion in treatment groups in comparison to arthritic control. Phytochemicals detected by GC–MS in the methanolic extract included esters, alcohols, ketones, fatty acids, and vitamin E. These findings provide evidence of the anti-arthritic potential of *M. rivae* extracts in chronic polyarthritis model.

**Keywords** *Moringa rivae* · Arthritis · Oxidative stress · Inflammatory cytokines · Complete Freund's adjuvant

✉ Ammara Saleem  
amarafurqan786@hotmail.com

✉ Mohammad Saleem  
saleem2978@hotmail.com

<sup>1</sup> Department of Pharmacology, Faculty of Pharmaceutical Sciences, Government College University Faisalabad, Faisalabad, Pakistan

<sup>2</sup> Department of Pharmacology, University College of Pharmacy, University of the Punjab, Lahore, Pakistan

<sup>3</sup> Riphah Institute of Pharmaceutical Sciences, Riphah International University, Lahore Campus, Lahore, Pakistan

<sup>4</sup> Department of Pharmacology, University of Health Sciences, Lahore, Pakistan

<sup>5</sup> Department of Immunology, University of Health Sciences, Lahore, Pakistan

## Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disorder demonstrated by chronic progressive synovitis. It affects numerous joints primarily small diarthrodial joints followed by large joints and extra-articular tissues and ultimately culminates in pain-stricken death (Scheel et al. 2006). The clinical depictions of RA include pain, puffiness, and rigidity in the knee and ankle joints prompting immobility and deformity in advanced phase (Yu et al. 2018). An estimate of RA prevalence is 2–4% internationally; however, probabilities vary among different age groups and ethnic, gender, and geographic clusters. Women are more susceptible to RA than men (Di Ming et al. 2011). However, the association of genetic, hormonal, and environmental factors also

considerably contribute to the etiology and progression of RA (Fattahi and Mirshafiey 2012).

The exact etiology of the disease is still unknown. However, an elevation of pro-inflammatory cytokines mainly tumor necrosis factor (TNF- $\alpha$ ), interleukin (IL)-6, IL-1 $\beta$ , and metabolic enzymes such as cyclo-oxygenases (COX) and lipo-oxygenase, and a decline in anti-inflammatory cytokines mainly IL-4 and IL-10 is evidenced. Furthermore, nuclear-factor-kappa B (NF- $\kappa$ B) known as pro-inflammatory transcription factor influences many cellular processes including immune response to inflammation and cell proliferation. NF- $\kappa$ B is markedly increased in the case of RA (Miller et al. 2010). The presence of reactive oxygen species (ROS) and other free radicals also contribute to the pathogenesis of RA (Afonso et al. 2007).

Routinely, non-steroidal anti-inflammatory drugs (NSAIDs), slow acting anti-rheumatic drugs, steroids, biologics, and disease modifying anti-rheumatic drugs are prescribed to combat RA; however, all these drugs provide symptomatic relief. These therapies adversely affect vital organs and instigate ulceration, hypertension, and stroke. The modern world is turning to herbal therapy because of drug resistance, organ toxicity, and high cost presented by these therapies (Oyeleke et al. 2018).

The plant, *Moringa rivae* Chiov., belongs to Moringaceae family that is a small, slender shrub or tree with gummy bark and indigenously called as Swanjhero. It is native to Africa and Asia. Locally, its gum, leaves, and bark are used to cure arthritis and weakness of thigh and calf muscles (Padayachee and Bajinath 2012). The Moringaceae family comprises of only *Moringa* genus with 33 species. Among the plants of the Moringaceae family, only a few have been scientifically explored. Miracle tree is also known as *M. oleifera*, has profound medicinal, food, agricultural, and industrial values. Most parts of this plant have therapeutic entities. *M. oleifera* has anticancer, anti-arthritic, anti-inflammatory, antimicrobial, hepatoprotective, hypolipidaemic, antihypertensive, antiulcer, and antioxidant activities (Karthivashan et al. 2013).

Recently, we have demonstrated the in vitro anti-inflammatory, anti-arthritic, and antioxidant activities. Furthermore, alkaloid, glycosides, saponins, flavonoids, tannins, and phenols as preliminary phytoconstituents were detected in aqueous and methanol extracts of the plant. Different phenolic and flavonoids such as quercetin, gallic acid, *p*-coumaric acid, caffeic acid, chlorogenic acid, sinapic acid, and kaempferol were present in the plant extracts under study (Saleem et al. 2018). The literature survey indicated that still no scientific work has been carried out to find the anti-arthritic potential of *M. rivae* using chronic polyarthritis model. The present research is aimed to explore the molecular and cellular anti-arthritic mechanism of the plant using Complete Freund adjuvant (CFA)-induced arthritis model

and to explore its efficacy in alleviating joint inflammation and oxidative stress.

## Materials and methods

### Preparation of extracts

Fresh leaves of *Moringa rivae* were collected in April 2016 and identified by a taxonomist from University of Agriculture Faisalabad, who gave voucher no. 101-1-16 for future reference. The leaves of the plant (2 kg) were pulverised after washing and drying and soaked in methanol (1:5) for triple maceration. A semisolid mass of the extract was obtained by evaporation with a rotary evaporator at 40 °C under reduced pressure. The dried extract was dissolved in water, defatted with n-hexane, and then sequentially extracted with ethyl acetate, n-butanol, and water on the basis of increasing polarity through liquid–liquid extraction (Ishtiaq et al. 2014). After filtration, each extract was dried with a rotary evaporator. The aqueous and methanolic fractions were selected for CFA-induced arthritis study in rats based on their maximum in vitro antioxidant and anti-arthritic activities detected in the previous study.

### Experimental animals

Previously acclimatised Wistar rats of both sexes (170–220 g) were randomly allocated into different groups in the animal house of Faculty of Pharmaceutical Sciences, GC University, Faisalabad. These rats were provided with standard laboratory conditions i.e.,  $28 \pm 2$  °C,  $55 \pm 5\%$  humidity, and 12 h light/dark cycles. The animals were given a standard pellet diet and water ad libitum. Approval for the animal study was obtained from the Institutional Animal Ethical Committee, GC University, Faisalabad with reference number GCUF/ERC/19574 (Study no. 19574) prior to the experiment. The experiment was performed strictly under international rules and regulation about laboratory animal ethics (NIH publication no. 85-23) revised in 2002.

### Experimental design

Animals were divided into nine groups ( $n = 6$ ). The polyarthritis was induced by an injection of intradermal 150  $\mu$ l CFA emulsion in the subplantar region of right hind paw at day 0 to all groups except normal (healthy) control. CFA emulsion contained heat-killed *Mycobacterium tuberculosis* suspended in mineral oil (Sigma-Aldrich, St. Louis, Mo, USA). Oral treatment with the extracts, piroxicam, and distilled water was started at 8th day via oral gavage till 28th day (Shabbir et al. 2016). Piroxicam and plant extracts were individually solubilised in distilled water. Normal control

and disease control groups received distilled water (2 ml) by oral gavage. Piroxicam (10 mg/kg/day) was given as standard therapy to arthritic rats only. Other six groups comprising of arthritic animals received 150, 300, and 600 mg/kg/day methanolic or aqueous plant extract.

### Evaluation of polyarthritis

Paw edema was measured at day 0 prior to CFA injection as paw volume and thickness using plethysmometer (Panlab<sup>®</sup>, Spain) and digital Vernier caliper, respectively. Paw volume, paw thickness, and body weight were further determined at days 7, 12, 16, 20, 24, and 28. Percentage inhibition of paw edema was measured.

Clinical severity of polyarthritis (arthritic index) was evaluated using the arthritic scoring system. The presence of edema and erythema was noted in the ipsilateral and contralateral paw and scored from 0 to 4 (0 = no swelling, 1 = swelling of toe joints, 2 = swelling of toes and toe joints, 3 = swelling of ankle joints, and 4 = swelling of entire paw which led to immobility) (Yu et al. 2018).

Animals were anaesthetised with diethyl ether (Sigma-Aldrich<sup>®</sup>, US) and killed on the 29th day by cervical dislocation. The thymus and the spleen were harvested and weighed to find their role in CFA-induced arthritis.

### Biochemical and haematological analysis

Blood was collected from anaesthetised rats by cardiac puncture. Commercially available kit (Antec Diagnostic Products<sup>®</sup>, UK) was used to find out C-reactive protein in serum. Likewise, rheumatoid factor (RF), alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and urea and creatinine levels were also determined by utilizing automated chemistry analyser (Microlab 300<sup>®</sup>, Germany) and kit manufacture protocols (Analyticon Biotechnologies AG, Germany). Complete blood count (CBC) was determined using automated haemocytometer (Sysmex<sup>®</sup>, Roche, Germany).

### Histopathological study

Ankle joints were removed from killed rats and fixed in 10% formalin for 48 h. The joints were decalcified by placing in decalcifying solution (10% EDTA) for 30 days. The tissues were embedded in paraffin. The joint section (5 µm) was cut and stained with haematoxylin and eosin (BDH Chemicals, England). Histopathological changes in the joints were noticed under a photomicroscope (Meiji Techno<sup>®</sup>, Japan) for the presence of pannus formation, inflammation, and bone erosion. Severity of polyarthritis was scored on a 0–4 scale (0 = normal, 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked) (Shabbir et al. 2018).

### Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative estimation of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , IL-4, IL-10, COX-2, NF- $\kappa$ B, and I- $\kappa$ B in the blood was carried out by qRT-PCR. Blood drawn from rats was placed in EDTA-containing tubes. The extraction of RNA from blood samples was carried out using the TRIzol method and yield was accessed by Nanodrop spectrophotometer. Reverse transcription was performed using the kit manufacturer protocol (WizScript<sup>®</sup> cDNA synthesis kit; W2202). For amplification and quantification, the kit protocol of 2 $\times$  Sybr qPCR master mix (GeneAll<sup>®</sup>, Korea) was followed on qRT-PCR (Bio-Rad<sup>®</sup> system). Briefly, forward and reverse primers (1 µl each), nuclease-free water (7 µl), cDNA (1 µl), and Sybr mix (10 µl) were added into the wells of the micro-plate. The micro-plates were placed in a thermal cycler with 40 cycles of denaturation at 95 °C and annealing at 60 °C followed by extension at 72 °C. Primers of various inflammatory markers and housekeeping genes designed using primer blast and Genbank<sup>®</sup> are as shown in Table 1 (Coura et al. 2015).

### Estimation of TNF- $\alpha$ and prostaglandin (PG) E<sub>2</sub>

For quantification of TNF- $\alpha$  and PGE<sub>2</sub> in the serum of rats, enzyme-linked immunosorbent (ELISA) assay was performed using commercial kit protocols (Elabscience Biotechnology<sup>®</sup>, China) (Ruan et al. 2015; Zhu et al. 2015).

### Estimation of oxidative stress biomarkers in liver

The liver was removed from killed rats and 10% w/v homogenate was made according to the previously described method. Protein content was estimated by Lowry's method using bovine serum albumin as standard (Akhtar et al. 2016).

### Activity of superoxide dismutase (SOD)

Total SOD activity in 10% w/v rat liver homogenate was determined using xanthine oxidase method as superoxide generator according to SOD kit maker protocol (Institute of Biological Engineering of Nanjing Jiancheng, Nanjing, China). The activity was expressed in units/mg protein (Sharif et al. 2016).

### Activity of catalase enzyme (CAT)

The catalase activity in 10% w/v homogenate liver tissue was determined by directly adding H<sub>2</sub>O<sub>2</sub> in supernatant and a decrease in absorption measured at 240 nm. One unit of CAT was expressed as a change in absorbance of 0.01 U/min. The activity of CAT was expressed as units/mg protein (Adeyemi and Olayaki 2018).

**Table 1** List of primers used in qRT-PCR analysis

Inflammatory markers	Forward/reverse	Sequence	Gene accession no.
IL-4	Forward	5'-GTACCGGGAACGGTATCCAC-3'	NM_201270.1
	Reverse	5'-TGGTGTTCCTTGTGCGTA-3'	
IL-10	Forward	5'-TTGAACCACCCGGCATCTAC-3'	NM_012854.2
	Reverse	5'-CCAAGGAGTTGCTCCCGTAA-3'	
I- $\kappa$ B	Forward	5'-TGAGTACCTGGACTTGCAGAACG-3'	NM_030867.2
	Reverse	5'-TGTAGATGCCTCTCCAAGGATGG-3'	
TNF- $\alpha$	Forward	5'-ATGGGCTCCCTCTCATCAGT-3'	NM_012675.3
	Reverse	5'-GCTTGGTGGTTTGCTACGAC-3'	
IL-1 $\beta$	Forward	5'-GTCCTCTGCCAAGTCAGGTC-3'	NM_031512.2
	Reverse	5'-CAGGGAGGGAAACACACGTT-3'	
IL-6	Forward	5'-CCCACCAGGAACGAAAGTCA-3'	NM_012589.2
	Reverse	5'-ACTGGCTGGAAGTCTCTTGC-3'	
NF- $\kappa$ B	Forward	5'-CTGAGTCCC GCCCTTCTAA-3'	NM-001276711.1
	Reverse	5'-CTCCACCAGCTCTTTGATGGT-3'	
COX-2	Forward	5'-ATGCTACCATCTGGCTTCGG-3'	NM_017232.3
	Reverse	5'-TGGAACAGTCGCTCGTCATC-3'	
GAPDH	Forward	5'-AGTGCCAGCCTCGTCTCATA-3'	NM_017008.4
	Reverse	5'-ACCAGCTTCCCATTCTCAGC-3'	

### Estimation of malondialdehyde (MDA)

The level of lipid peroxides was determined in 10% w/v liver homogenate by thiobarbituric acid reacting substances (TBARS) method. The absorbance of the reaction mixture was measured at 532 nm. Level of MDA was expressed as  $\mu\text{M}/\text{mg}$  protein (Mueller et al. 2016).

### GC-MS analysis

The GC-MS (Agilent technologies<sup>®</sup>, US, GC model: 7890 A, MS model: 5975 C) conditions for the analysis of the plant methanolic extract were set as follows. Capillary column, HP-5 MS (30 m  $\times$  250  $\mu\text{m}$   $\times$  0.25  $\mu\text{m}$  film thickness) was used as the stationary phase. Helium (99.9%), as a carrier gas, was flown at the rate of 1 ml/min. The sample solution was prepared by dissolving 2 mg plant extract in 10 ml methanol. The sample solution (1  $\mu\text{l}$ ) was injected into the GC-MS in split-less mode. The temperature was set at 60  $^{\circ}\text{C}$  followed by a rise of 10  $^{\circ}\text{C}/\text{min}$  until attaining 310  $^{\circ}\text{C}$  for 4 min. The MS temperature was set at 240–250  $^{\circ}\text{C}$ , whereas the temperature of mass quadrupole analyzer was 150–200  $^{\circ}\text{C}$ . Ionizing voltage was 59 eV. Total run time of the sample was 29 min (Uroos et al. 2017). The GC-MS chromatogram of the plant extract was compared with the database of the National Institute of Standard and Technology (NIST) library to identify unknown compounds.

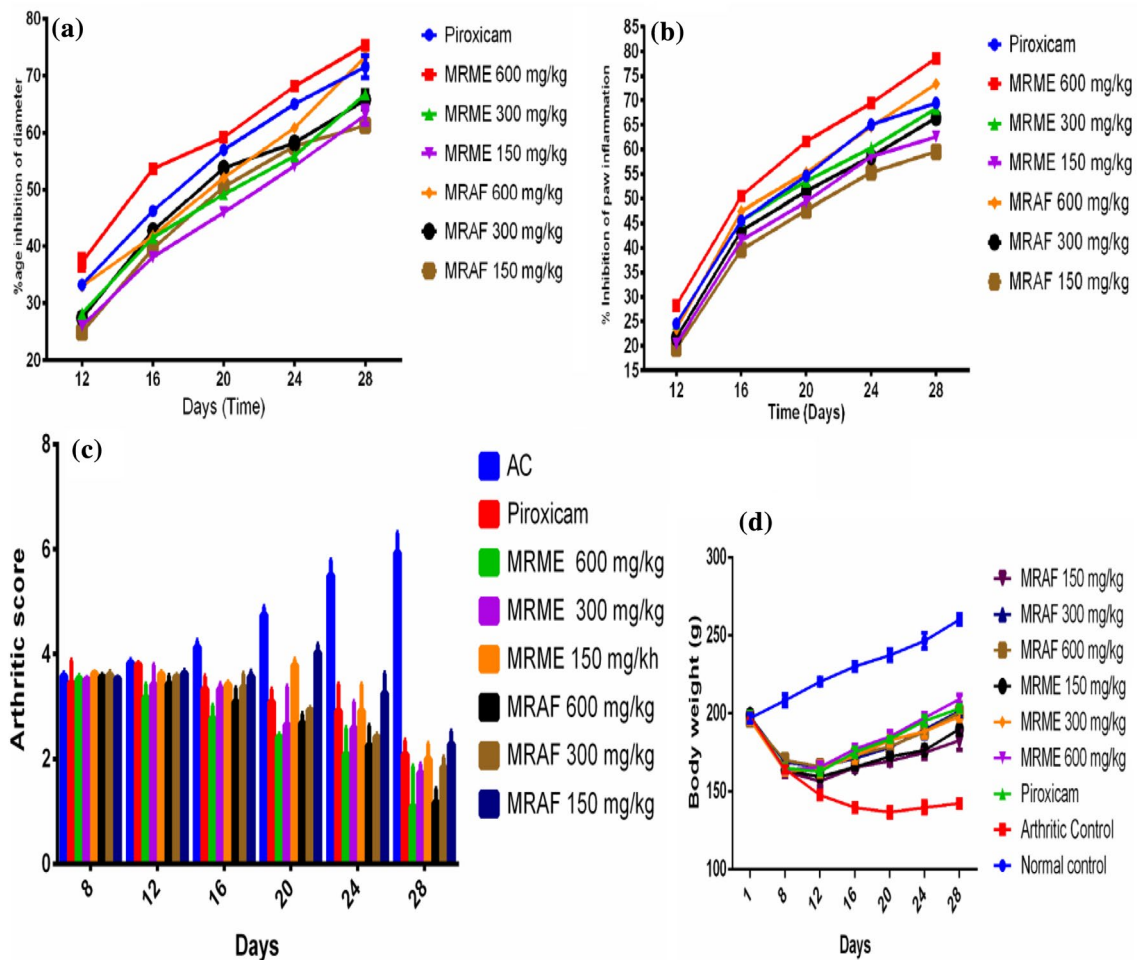
### Statistical analysis

All results were presented as mean  $\pm$  standard deviation. Two-way analyses of variances (ANOVA) were performed on percentage inhibition of paw diameter and volume and effect on weight variation in arthritic rats followed by Tukey's post hoc test. The effect of plant extracts or piroxicam on the arthritic score of the rats, determined on different days, was analysed by Chi square for trend. All other data were analysed by one-way ANOVA followed by Tukey's test using the GraphPad Prism<sup>®</sup> 6.01 software. Familywise error rate was controlled by Bonferroni correction to  $p$  value so as to avoid false positive results.

## Results

### Effect on paw swelling

Subplantar administration of CFA increased inflammation in primary paw (injected) resulting in peak swelling noticed on the 8th day. A momentous elevation in paw volume and paw thickness in the arthritis control group was observed at day 8–28 in contrary to a normal control group. Treatment with methanolic and aqueous extracts alleviated paw volume and diameter in comparison to the arthritic control group with maximum efficacy shown at 600 mg/kg dose (Fig. 1). Percentage inhibition in paw volume exhibited by methanolic extract at 600 mg/kg (78.56  $\pm$  0.26%) and aqueous extracts



**Fig. 1** Effect of *Moringa rivae* extracts on adjuvant-induced arthritis in Wistar rats **a** inhibition of paw diameter by methanolic and aqueous extracts; **b** inhibition of paw volume by methanolic and aqueous extracts; **c** arthritic score of different groups treated with methanolic and aqueous extracts; **d** changes in body weight of arthritic rats treated with methanolic and aqueous extracts. Where MRME, Moringa rivae methanolic extract; MRAF, Moringa rivae aqueous fraction; AC, arthritic control. Results were presented as Mean  $\pm$  SD ( $n=06$ ) and analysed by two-way ANOVA ( $p<0.05$ ) followed by

Tukey's post hoc test, while effect of different treatments on arthritic scores was analysed by Chi square for trend ( $p<0.05$ ). All treatments (**a**, **b**) were significantly different to piroxicam-treated group except MRAF 600 mg/kg (**a**), while MRME 300 mg/kg at 12 and 16th day (**b**). There was statistically insignificant effect on arthritic score (**c**). All values were statistically different ( $p<0.0001$ ) to arthritic control following 12th day to the end of therapy (**d**). All values were significantly different from normal control following 8th day to the end of study (**d**)

at 600 mg/kg ( $73.35 \pm 0.22\%$ ) was significantly higher than piroxicam-treated rats ( $69.45 \pm 0.45\%$ ) at 28th days (Fig. 1b).

**Effect on arthritic index**

The normal control group did not show any swelling in the whole study. The findings illustrated in Fig. 1c showed the continuous rise in arthritic index articulated by an arthritic control group. Treatment with methanolic and aqueous extracts of *M. rivae*, and piroxicam (10 mg/kg) was effectively masked arthritic index in comparison to arthritis control group from day 16 to the end of the study. The maximum arthritic score was noticed at 28th day in arthritic control group ( $5.93 \pm 0.371$ ) that was restored by piroxicam

( $2.08 \pm 0.26$ ), methanolic extract at 600 ( $1.08 \pm 0.77$ ), 300 ( $1.73 \pm 0.16$ ) and 150 mg/kg ( $2 \pm 0.28$ ), and aqueous extract at 600 ( $1.16 \pm 0.25$ ), 300 ( $1.83 \pm 0.19$ ), and 150 mg/kg ( $2.27 \pm 0.25$ ) treatments.

**Effect on body weight**

There was a continuous decline in body weight as inflammation developed. Results showed in Fig. 1d indicated that weight loss was more prominent in the arthritis control group when compared with normal control at  $p<0.0001$  from the 8th day until the end of the study. On the other hand, treatment with the plant extracts and piroxicam

significantly restored body weight of arthritic rats compared to arthritic control group ( $p < 0.0001$ ) from 12 to 28th day.

### Effect on histopathology of joints

The results of ankle joint histopathology at the end of 28 day study revealed significant ( $p < 0.05$ ) infiltration with mononuclear cells ( $3.79 \pm 0.18$ ), bone erosion ( $3.8 \pm 0.2$ ), pannus formation ( $3.75 \pm 0.25$ ), and synovial hyperplasia in arthritic control rats in contrast to normal control. Treatment with the plant extracts reduced pannus formation, inflammation, and bone erosion in arthritic rats at all dose levels in comparison with arthritic rats ( $p < 0.05$ ), as shown in Fig. 7f–h. However, the effect of methanolic and aqueous extracts on pannus formation, inflammation, and bone erosion was comparable to that of piroxicam, as shown in Fig. 3. Though, gross macroscopic examination of the injected paw after CFA-induced arthritis prior to sacrificing is shown in Fig. 2. It was clear from Fig. 3 that there was a marked inflammation in arthritic rats in contrast to normal rats, while treatment groups exhibited minimal (600 mg/kg) to mild and moderate inflammation (300 and 150 mg/kg, respectively) at 28th day.

### Effect on haematological, liver, and kidney function parameters

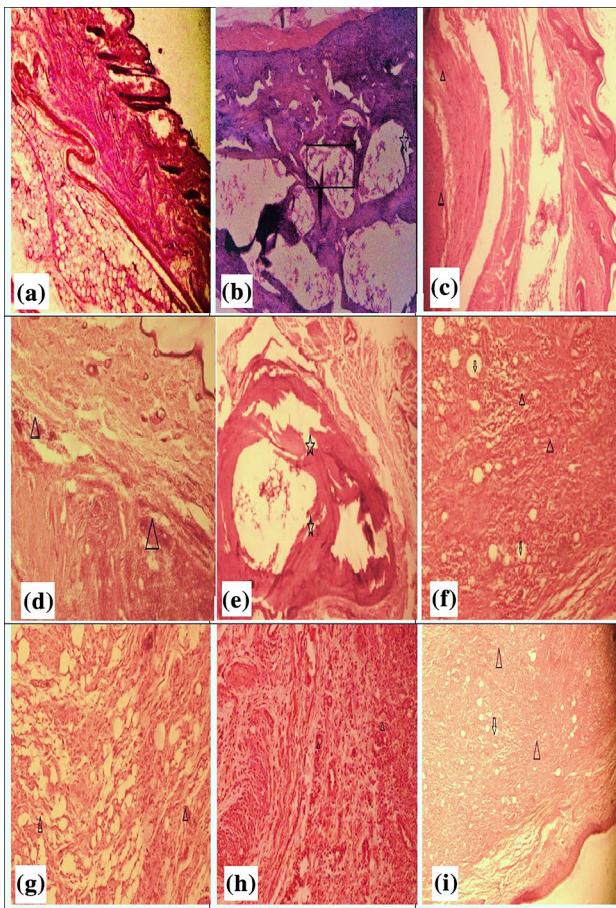
It was found that the induction of Freund's adjuvant-induced arthritis in rats resulted in an increase in ALP, ALT, C-reactive protein (CRP), and RF. Treatment with the plant extracts and piroxicam caused a significant decrease in ALP, CRP, and RF ( $p < 0.0001$ ). However, the ameliorating effect of plant extracts on ALT and AST was statistically insignificant (Fig. 4a, b). The systemic biomarker as CRP and RF were significantly increased ( $43.33 \pm 4.51$  and  $53 \pm 4.58$  respectively) in arthritic control as shown in Fig. 4k, h. However, both extracts significantly attenuated CRP and RF in rats treated with methanolic extract 600 mg/kg ( $10.12 \pm 2.08$  and  $11.66 \pm 1.52$ , respectively) that was comparable to piroxicam treatment ( $17.07 \pm 2.05$  and  $20.11 \pm 2.20$ , respectively).

Induction of polyarthritis and treatment with the plant extract or piroxicam had no significant effect on kidney function tests such as urea and creatinine. There was decreased in Hb and RBCs in rats; however; an increase in WBCs and platelets was seen in arthritic rats in contrary to normal control. Dose-dependent ameliorating effect on these haematological parameters was observed upon administration of aqueous and methanolic extracts as shown in Fig. 4.



**Fig. 2** Gross macroscopic examination of primary paw after complete Freund's adjuvant-induced arthritis before sacrificing. **a** Normal control, **b** arthritic control, **c** piroxicam-treated rats, **d–f** methanolic

extract at 600, 300, and 150 mg/kg treated rats, **g–i** aqueous extract at 600, 300, and 150 mg/kg treated rats



**Fig. 3** Effect of *Moringa rivae* extracts on histopathology of complete Freund's adjuvant-induced arthritic rats at  $\times 40$  magnification: **a** normal control group rat; **b** arthritic group rats; **c** piroxicam treated; **d–f** showed the paw histology rats treated with methanolic extract at 600, 300, and 150 mg/kg; **g–i** showed paw histology of rats treated with aqueous extract 600, 300, and 150 mg/kg treated rats. Where star showed bone erosion, triangle showed focal areas of severe infiltration with mononuclear cells, arrow showed blood vessel, and rectangle showed pannus formation

### Effect on immune organ weight

The result depicted in Fig. 5 illustrated that the weight of the spleen and thymus markedly increased in the arthritic control group in contrast to normal control. Treatment of arthritic rats with the plant extracts restored the weight of the spleen and thymus and maximum action exhibited at 600 mg/kg dose of the extracts.

### Effect on gene expression

The mRNA expression of various inflammatory biomarkers was determined following 28 days of study in Wistar rats. A significant decrease ( $p < 0.0001$ ) in the expression of IL-10 was recorded in the arthritic control group ( $34 \pm 3\%$ ).

However, IL-10 was up-regulated in methanolic extract at 600 ( $60.68 \pm 4.72\%$ ) and 300 mg/kg ( $48.83 \pm 6.05\%$ ), aqueous extract at 600 ( $55 \pm 4\%$ ) and 300 mg/kg ( $47.5 \pm 2.18\%$ ), and piroxicam treatment ( $50.67 \pm 6.65\%$ ) groups in comparison to arthritic control group (Fig. 6a).

It was found that mRNA expression of NF- $\kappa$ B ( $p < 0.0001$ ) was raised in the arthritic control group ( $4.9 \pm 0.3$ -fold). Treatment with methanolic extract at 600 ( $2.63 \pm 0.25$ -fold), 300 ( $3.1 \pm 0.4$ -fold), and 150 mg/kg ( $3.26 \pm 0.20$ -fold), aqueous extract at 600 ( $2.43 \pm 0.30$ -fold), 300 ( $2.8 \pm 0.36$ -fold) and 150 mg/kg ( $3.4 \pm 0.3$ -fold), and piroxicam ( $2.2 \pm 0.2$ -fold) ameliorated this rise in NF- $\kappa$ B in arthritic rats (Fig. 6b).

An exaggerated COX-2 expression ( $p < 0.0001$ ) occurred in the arthritic control group ( $6.36 \pm 0.42$ -fold). Treatment of arthritic rats with methanolic extract at 600 ( $2.4 \pm 0.36$ -fold), 300 ( $2.8 \pm 0.45$ -fold), and 150 mg/kg ( $3.37 \pm 0.45$ -fold), aqueous extract at 600 ( $2.7 \pm 0.4$ -fold), 300 ( $3.46 \pm 0.25$ -fold), and 150 mg/kg ( $4.2 \pm 0.45$ -fold), and piroxicam ( $1.86 \pm 0.15$ -fold) reduced the expression of COX-2 as compared with arthritic control group (Fig. 6c).

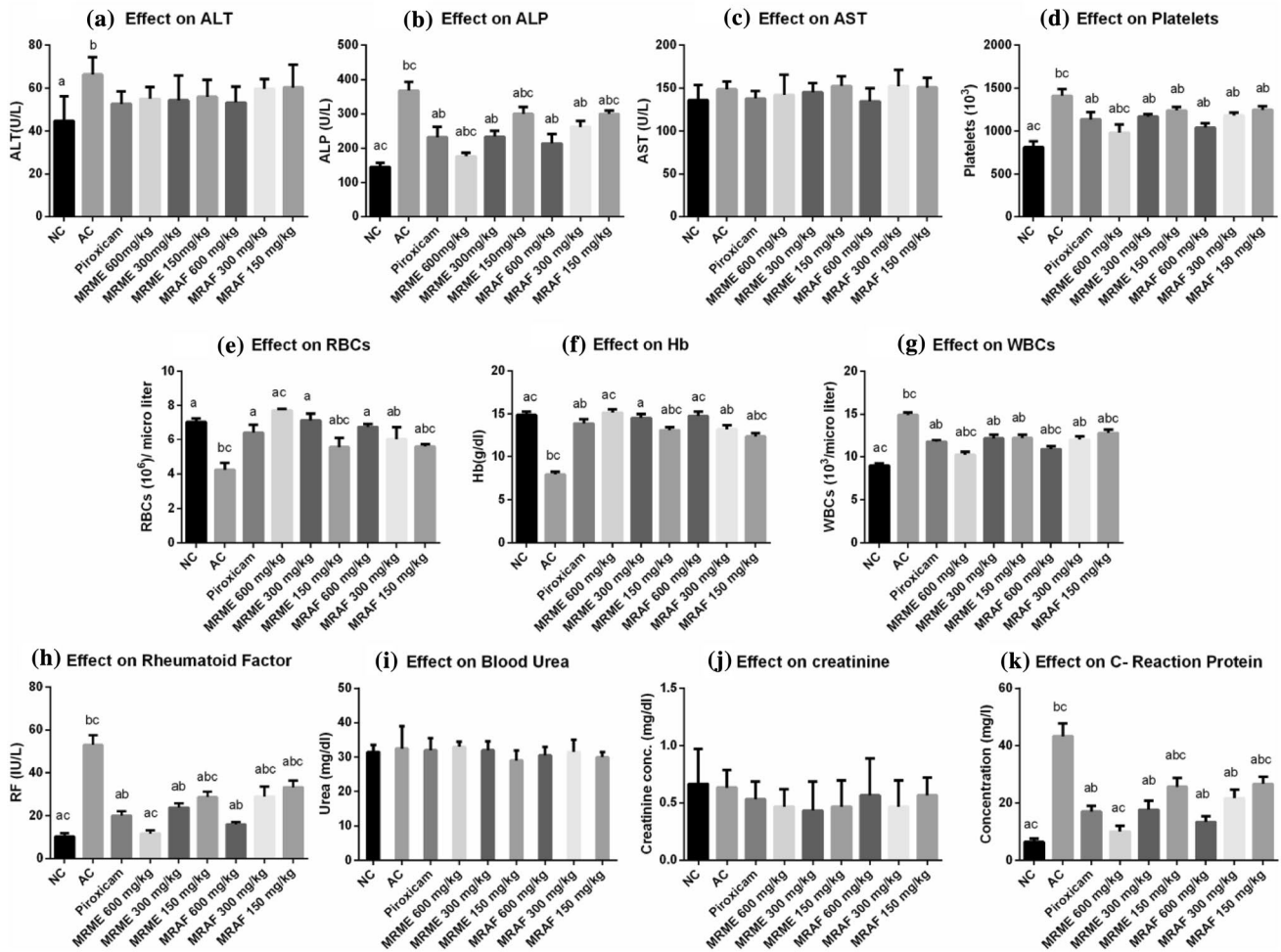
A considerable decrease ( $p < 0.0001$ ) in IL-4 expression was noticed in the arthritic control group ( $40.47 \pm 3.11\%$ ). Treatment with methanolic extract at 600 ( $81 \pm 4.58\%$ ), 300 ( $71.68 \pm 3.51\%$ ), and 150 mg/kg ( $64.67 \pm 2.52\%$ ), aqueous extract at 600 ( $66 \pm 3\%$ ), 300 ( $64.66 \pm 4.93\%$ ) and 150 mg/kg ( $56.70 \pm 5.51\%$ ), and piroxicam therapy ( $83.46 \pm 2.15\%$ ) significantly increased the expression of IL-4 in comparison to arthritic control group as shown in Fig. 6d.

A significant alleviation in I- $\kappa$ B expression was observed in the arthritic control group ( $48 \pm 3.60\%$ ) that up-surged significantly ( $p < 0.0001$ ) in response to methanolic extract at 600 ( $70 \pm 2.64\%$ ) and 300 mg/kg ( $63 \pm 2.64\%$ ), aqueous extract at 600 ( $67.5 \pm 1.32\%$ ) and 300 mg/kg ( $64 \pm 3\%$ ), and piroxicam ( $84 \pm 5.29\%$ ) therapy, as shown in Fig. 6e.

A significant upsurge ( $p < 0.0001$ ) in IL-6 expression was obvious in the arthritic control group ( $5.3 \pm 0.5$ -fold) than the normal control group as exhibited in Fig. 6f. However, methanolic extract at 600 ( $2.18 \pm 0.15$ -fold) and 300 mg/kg ( $3.03 \pm 0.57$ -fold), aqueous extract at 600 ( $2.3 \pm 0.2$ -fold) and 300 mg/kg ( $3.06 \pm 0.56$ -fold), and piroxicam ( $2.16 \pm 0.22$ -fold) therapy subdued this elevation of IL-6 in arthritic rats.

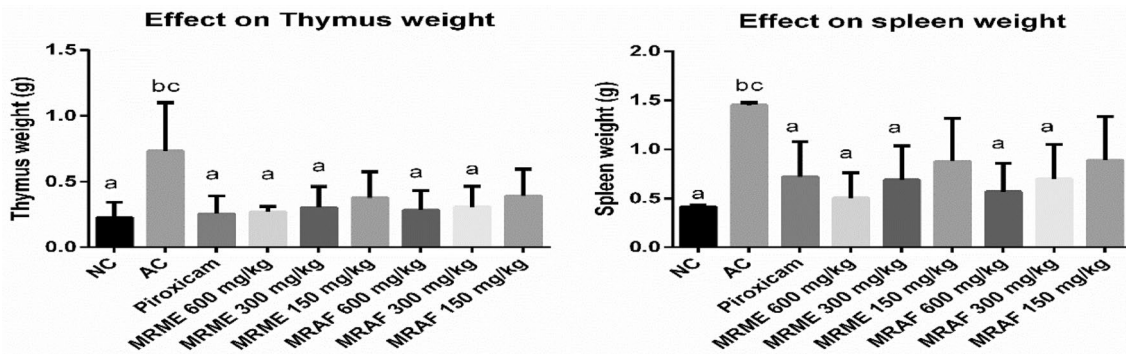
A significantly elevated expression ( $p < 0.0001$ ) of TNF- $\alpha$  was evidenced in arthritic control group ( $4.73 \pm 0.55$ -fold) that declined in rats treated with methanolic extract at 600 ( $2.5 \pm 0.1$ -fold), 300 ( $2.65 \pm 0.21$ -fold) and 150 mg/kg ( $2.93 \pm 0.41$ -fold), aqueous extract at 600 ( $2.5 \pm 0.2$ -fold), 300 ( $3.1 \pm 0.1$ -fold), and 150 mg/kg ( $3.55 \pm 0.12$ -fold), and piroxicam ( $2.03 \pm 0.21$ -fold), as shown in Fig. 6g.

The mRNA expression of IL-1 $\beta$  (fold) notably increased ( $p < 0.0001$ ) in the arthritic control group ( $6.37 \pm 0.28$ -fold) than the normal control, as shown in Fig. 6h. The elevated expression of IL-1 $\beta$  was decreased by methanolic extract at



**Fig. 4** Effect of *M. rivae* extracts on haematological, liver, and kidney function parameters of arthritic rats. Where ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Hb, haemoglobin; RBC, red blood cells; WBC, white blood

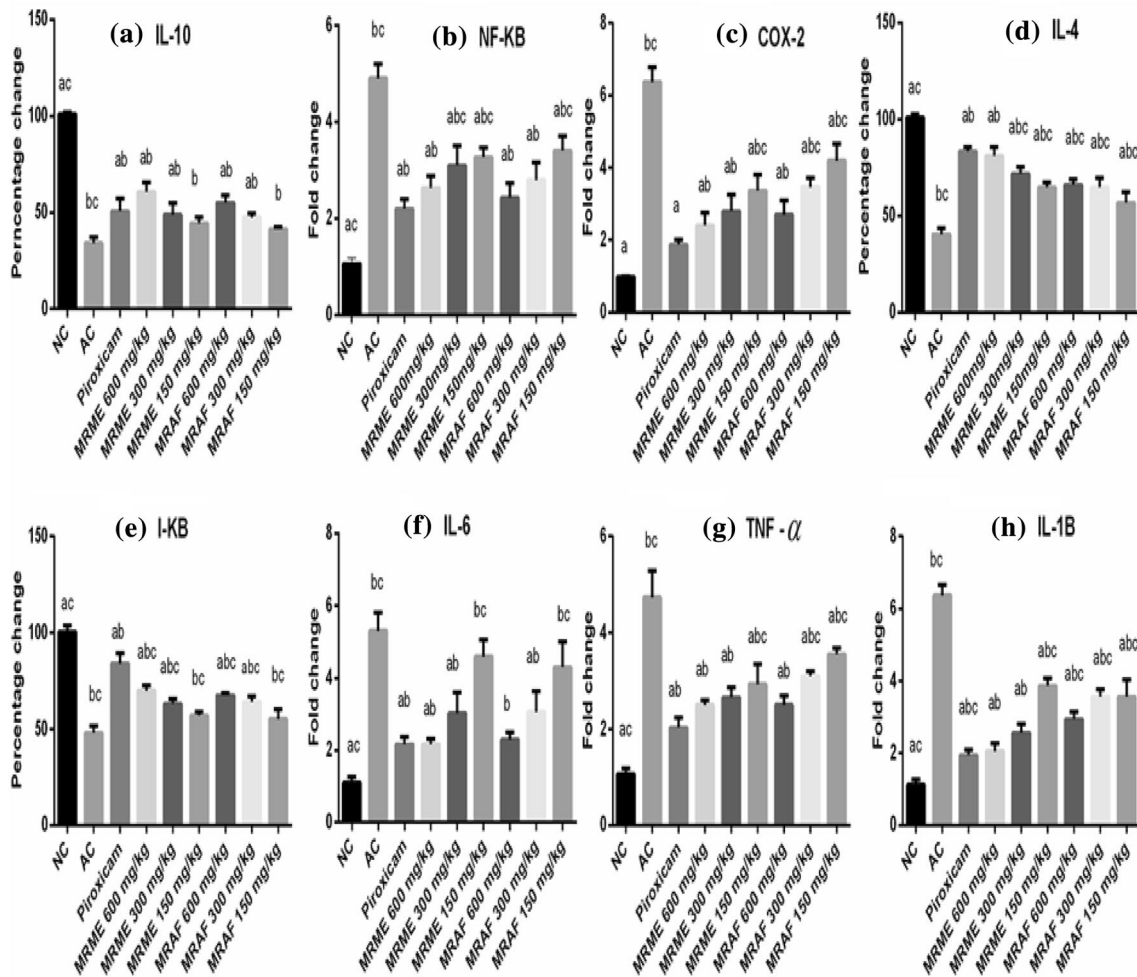
cells. Results are presented as Mean ± SD ( $n=06$ ) and analysed by one-way ANOVA ( $p<0.05$ ) followed by Tukey's post hoc test. **a–c** Statistically different as compared with arthritic control, normal control, and piroxicam therapy, respectively



**Fig. 5** Effect of *Moringa rivae* extracts on immune organs weight of arthritic rats. Where results are presented as Mean ± SD ( $n=06$ ) and analysed by one-way ANOVA ( $p<0.05$ ) followed by Tukey's post hoc

test. **a–c** Statistically different as compared with arthritic control, normal control, and piroxicam therapy, respectively





**Fig. 6** Effect of *Moringa rivae* extracts on mRNA expression of various inflammatory mediators in arthritic rats. Where IL, interleukin; I-κB, inhibitor of kappa B; COX-2, cyclo-oxygenase-2; NF-κB, nuclear-factor-kappa B; TNF-α, tumor necrosis factor. Results are

presented as mean ± SD ( $n=06$ ) and analysed by one-way ANOVA ( $p < 0.05$ ) followed by Tukey's post hoc test. **a-c** Statistically different as compared with arthritic control, normal control, and piroxicam therapy, respectively

600 mg/kg ( $2.06 \pm 0.20$ -fold), aqueous extract at 600 mg/kg ( $2.93 \pm 0.21$ -fold), and piroxicam therapy ( $1.94 \pm 0.15$ -fold) in arthritic rats.

aqueous extract at 600 mg/kg ( $190.74 \pm 58.64$  pg/ml), and piroxicam ( $108.82 \pm 76.37$  pg/ml) (Fig. 7e).

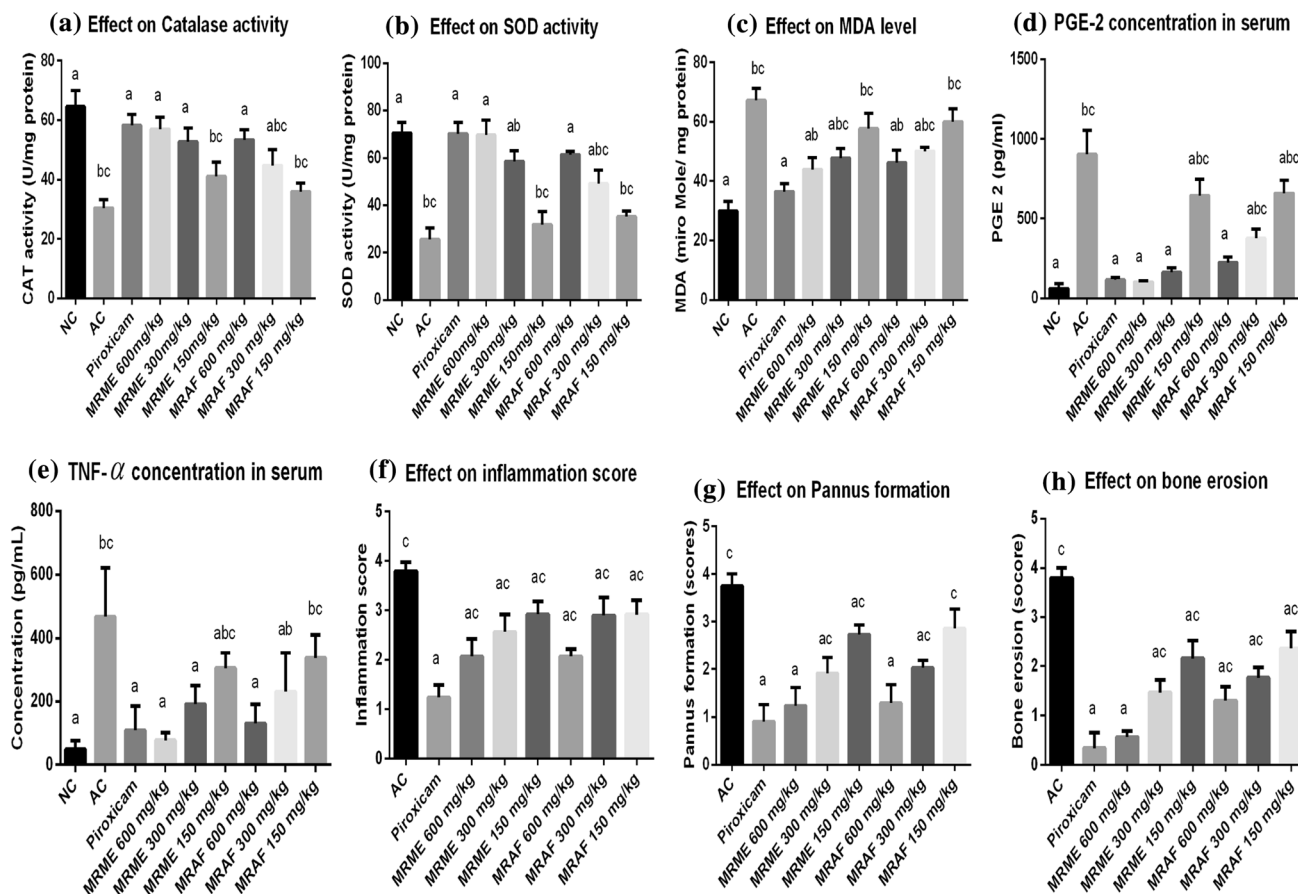
**Effect on serum concentration of PGE2 and TNF-α**

**Effect on oxidative stress**

An elevated level of PGE2 ( $p < 0.0001$ ) in the serum of the arthritic control group ( $906.07 \pm 147.28$  pg/ml) was noticed. However, the elevation in PGE2 was significantly mitigated by the treatment with methanolic extract at 600 ( $103.25 \pm 9.07$  pg/ml), aqueous extract at 600 mg/kg ( $225.18 \pm 35.17$  pg/ml), and piroxicam ( $116.27 \pm 16.08$  pg/ml) in comparison to arthritic control (Fig. 7d).

The findings of CFA-induced oxidative stress and ameliorating effect of methanolic and aqueous extracts are presented in Fig. 7. There was a significant decline ( $p < 0.0001$ ) of SOD ( $25.63 \pm 4.88$ U/mg protein) and CAT activities ( $30.34 \pm 2.94$  U/mg protein) in the arthritic control group in comparison to a normal control group (SOD:  $71.61 \pm 4.28$  U/mg protein and CAT:  $64.52 \pm 5.35$  U/mg protein). However, piroxicam (10 mg/kg), methanolic and aqueous extracts of *M. rivae* reinstated ( $p < 0.0001$ ) the activities of CAT and SOD in arthritic rats, as shown in Fig. 7a, b, respectively. There was also an elevated level of MDA in the liver of arthritic control group ( $67.32 \pm 3.98$  μM/mg protein) at ( $p < 0.0001$ ) as compared

An abnormally high level of TNF-α was observed in the arthritic control group ( $467.65 \pm 153.70$ ). This elevated level was significantly ( $p < 0.0001$ ) attenuated by treatment with a methanolic extract at 600 mg/kg ( $77.71 \pm 23.71$  pg/ml),



**Fig. 7** Effect of *Moringa rivae* extracts on oxidative stress in the liver, serum inflammatory mediators, and histopathological parameters of arthritic rats. Where SOD, superoxide dismutase; MDA, malondialdehyde (MDA); PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; TNF- $\alpha$ , tumor necrosis

factor. Results are presented as mean  $\pm$  SD ( $n=06$ ) and analysed by one-way ANOVA ( $p < 0.05$ ) followed by Tukey's post hoc test. **a–c** Statistically different as compared with arthritic control, normal control, and piroxicam therapy, respectively

with the normal control group ( $29.98 \pm 3.29 \mu\text{M/mg protein}$ ). The level of MDA was significantly reduced by treatment with piroxicam, 300 and 600 mg/kg dose of methanol or aqueous extract in arthritic rats, as shown in Fig. 7c.

### GC–MS analysis

Phytochemicals detected in the plant methanolic extract included esters, alcohols, ketones, fatty acids, and hydrocarbons. The 10-octadecenoic acid and hexadecanoic acid methyl ester exhibited the maximum percentage area in GC–MS spectrum. The characteristics of phytochemicals in the plant extract confirmed by GC–MS spectrum are summarized in Table 2.

### Discussion

CFA-induced arthritic model has been widely employed in rodents for preclinical testing due to similarities with human RA in pathology. The CFA-induced arthritis is biphasic; the

first phase is acute, lasting for 0 to 10th days and arises due to discharge of histamine, serotonin, and PG by immune cells followed by a chronic phase lasting for 11–28th day. The later period corresponds to disruption of pro-/anti-inflammatory homeostasis that is manifested as synovitis, infiltration, hypertrophy, bone erosion, etc. (Foyet et al. 2015).

There is a direct relationship between weight loss and the severity of joint inflammation. Weight loss/slow gain in body weight might be due to muscle proteolysis, malabsorption from the intestine, altered metabolism of protein and lipids, disease associated distress, reduction in food intake, hyper-algesia, and allodynia (Taksande et al. 2017). Weight loss occurred in the arthritic control group that was significantly restored by the plant extracts as co-evidenced along with the improvement of other polyarthritis symptoms such as a decrease in joint swelling. A hyper-functional immune system is recognized by splenomegaly, splenitis, and lymphadenopathy (Issekutz and Sapru 2008). This study reported that both extracts

**Table 2** Phytochemicals detected in GC–MS spectrum of the *Moringa rivae* leaf methanolic extract

Peak no.	Retention time (min)	Chemical compound	Molecular formula	Molecular weight	Peak area (%)
1	15.247	Hexadecanoic acid, methyl ester	C <sub>17</sub> H <sub>34</sub> O <sub>2</sub>	270	25.853
2	16.843	9,12-Octadecadienoic acid, methyl ester	C <sub>19</sub> H <sub>34</sub> O <sub>2</sub>	294	3.269
3	16.897	10-Octadecenoic acid	C <sub>19</sub> H <sub>36</sub> O <sub>2</sub>	296	29.436
4	17.033	Phytol	C <sub>20</sub> H <sub>40</sub> O	296	13.369
5	17.121	Heptadecanoic acid, 16-methyl-, methyl ester	C <sub>19</sub> H <sub>38</sub> O <sub>2</sub>	298	10.707
6	19.762	Phenol, 2,2'-methylenebis [6-(1,1-dimethylethyl)-4-methyl-	C <sub>23</sub> H <sub>32</sub> O <sub>2</sub>	340	2.372
7	20.651	9,10-Secocholesta-5,7,10(19)-triene-3,24,25 triol,(3,β,5Z,7E)	C <sub>27</sub> H <sub>44</sub> O <sub>3</sub>	416	2.478
8	22.878	(22S)-6.α.,11.β.,21-Trihydroxy16.α.,17.α.,-propyl-methylenedioxypregna-1,4-diene-3,20-dione	C <sub>25</sub> H <sub>34</sub> O <sub>7</sub>	446	2.076
9	24.786	Vitamin E	C <sub>29</sub> H <sub>50</sub> O <sub>2</sub>	430	10.441

notably suppressed the hyper-functioning of immune organs which might be due to phytochemicals present in this plant (Wang et al. 2016).

Elevation of ALP usually corresponds to bone erosion and peri-articular osteoporosis (Moon et al. 2013). This study showed that both extracts had restored the level of ALP in arthritic rats. One of the clinical features of RA is anaemia; this study revealed that both extracts had restored the anaemia in comparison to an arthritic control group which was reported previously (Thite et al. 2014). It is attributed to the apparent healing of inflamed joints as supported by histological slides of treatment groups as compared with the arthritic control group. Moreover, serum CRP and RF are biomarkers of systemic inflammation, which represent active inflammation. Increased level of CRP and RF indicated the progression of arthritis. Elevated level of IL-6 and TNF- $\alpha$  aggravates the production of CRP as evidenced from the previous studies (Kumar et al. 2013). Therefore, it can be proposed that the treatment with the plant extracts reduced systemic inflammation as depicted by a low level of CRP and RF via reduction of IL-6 and TNF- $\alpha$ , as shown in the previous studies (Kalaiselvan and Rasool 2016).

After CFA immunisation, TNF- $\alpha$ , IL-6, and IL-1 $\beta$  are released from macrophages and monocytes. Subsequently, TNF- $\alpha$  activates the discharge of other inflammatory mediators such as IL-6 and IL-1 $\beta$ , which result in the transport of more leukocytes, infiltration, and vasodilatation at the site of edema. Furthermore, these pro-inflammatory cytokines stimulate chemokines, which attract neutrophils and monocytes towards inflamed joints (Voon et al. 2017). To avoid bone and cartilage destruction, it is necessary to block pro-inflammatory cytokines (TNF- $\alpha$ ) involved in gene expression of matrix metalloproteinase (MMPs), particularly MMP1 and MMP13 (Srirangan and Choy 2010). This study expressed a marked decrease in the expression of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 at all three dosage levels in contrast to the arthritic control group.

IL-4 and IL-10 are immunoregulatory cytokines, which halt the process of inflammation, bone erosion, and cartilage damage during RA in contrast to inflammatory cytokines. IL-10 not only diminishes pro-inflammatory T helper (Th)1 cell generated cytokines mainly TNF- $\alpha$  and IL-1, but also maintains the integrity of joint tissues during the RA. Likewise, IL-4 blocks Th1 cells, but stimulates immunomodulatory Th2 cells (Shabbir et al. 2014). The results of the present study showed a raised level of IL-4 and IL-10 in treatment groups, in contrast to the arthritic control group.

It was also established that PGE2 exerted pleiotropic actions chiefly pain and swelling. Inflammatory cytokines critically provoke the COX-2 expression and indirectly augmented the PGE2 level in activated synovial cells (Sano 2011). Both extracts alleviated synovitis and prevented angiogenesis through decreased expression of COX-2 and PGE2 levels. As NF- $\kappa$ B regulate innate and adaptive immune system through transcription of inflammatory markers such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and metabolic enzyme nitric oxide synthase and COX. NF- $\kappa$ B is inactivated by I- $\kappa$ B protein family. Activation of NF- $\kappa$ B worsens the RA (Lawrence 2009). The arthritic control group showed decreased expression of I- $\kappa$ B and increased expression of NF- $\kappa$ B, while the opposite was observed in groups treated with aqueous and methanolic extracts.

It is suggested that SOD and CAT activities had reduced, while MDA was raised in RA, as evidenced in the present study. This oxidative stress wrongly influences gene transcription (Thite et al. 2014). Stimulus like Freund's adjuvant is thought to activate NF- $\kappa$ B pathway, but also raises the level of ROS and inflammatory cytokines that further provoke immune cells to release inflammatory cytokines and enzymes to exacerbate the arthritis (Kalaiselvan and Rasool 2016). Therefore, it can be speculated that the reduction in oxidative stress by *M. rivae* is one of the main mechanisms for inhibition of gene expression of inflammatory cytokine and COX-2 in RA.

Piroxicam reduced the expression of COX-2, PGE2, TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and NF- $\kappa$ B while increasing the expression of I- $\kappa$ B, IL-4, and IL-10 in arthritic rats similar to aqueous and methanol extracts of the plant, in addition to reducing paw swelling and restoring body weight. These findings are supported by several studies on herbal drugs and piroxicam, which showed a significant reduction in paw swelling following 21 day therapy (Shabbir et al. 2018; Kumar et al. 2013).

The GC–MS chromatogram revealed the presence of multi-functional fatty acids, including vitamin E ( $\alpha$ -tocopherol) in the methanolic extract of the plant leaves. Vitamin E has been widely recognized as an antioxidant, hypolipidemic, and antiplatelet. Vitamin E is also previously reported in other plants of *Moringa* genus such as *M. oleifera* (Gopalakrishnan et al. 2016). Hexadecanoic acid methyl ester has shown antibacterial and antifungal properties (Chandrasekaran et al. 2011). Furthermore, 9,12-octadecadienoic acid has demonstrated chemo-preventive, anti-cancer, and anti-inflammatory characteristics previously (Yu et al. 2005). Plant polyphenols, flavonoids, fatty acids, and vitamin E act as free radical scavengers, and are beneficial against oxidative stress-related diseases like RA, diabetes, and Parkinson's disease (Oyeleke et al. 2018). A high-performance liquid chromatography analysis of *M. rivae* extracts had shown the occurrence of variable quantities of phenolic and flavonoid compounds such as quercetin, gallic acid, *p*-coumaric acid, caffeic acid, chlorogenic acid, sinapic acid, and kaempferol (Saleem et al., 2018). The present GC–MS study failed to detect phenolic and flavonoids in the plant extract in contrast to the previous study. This apparent discrepancy may be due to acquiring GC–MS spectrum without derivatisation as polar non-volatile compounds such as polyphenol and flavonoids required it before being subjected to GC–MS (Rohloff 2015). The alleviation of joint edema, oxidative stress, and inflammation may be attributed to various phytochemicals such as fatty acids and vitamin E detected in the plant extract (Shabbir et al. 2018).

## Conclusions

It can be inferred from the present study that the methanolic and aqueous extracts of *M. rivae* mitigated the hallmarks of RA such as weight loss, edema, pannus formation, bone erosion, and anaemia, and decelerated the progression of polyarthritis such as recovering synovitis, immune organ weight, articular damage through down-regulation of pivotal cytokines, i.e., COX-2, PGE2, IL-1 $\beta$  and IL-6, NF- $\kappa$ B and TNF- $\alpha$ , and up-regulation of I- $\kappa$ B, IL-4, and IL-10 in dose-dependent manner. This modulation of gene expression, and articular and systemic alterations is probably due to scavenging of ROS by the plant extracts. The anti-arthritic

activity of *M. rivae* extracts suggests the need for activity-based fractionation and isolation of particular compounds responsible for the anti-arthritic potential of the plant. These compounds must be evaluated for their molecular targets and signaling pathways in attenuating arthritis.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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