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RESEARCH PAPER

# ANTIARTHRITIC ACTIVITY OF METHANOL EXTRACT OF *ZIZIPHUS MAURITIANA* LAM LEAVES IN CFA INDUCED ARTHRITIS IN RATS

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***Ziziphus mauritiana*** **leaves was used by traditional Indian practitioners to treat inflammation and gastrointestinal aliments. The present study was aimed at evaluating the antiarthritic potential of the methanol extract of *Z. mauritiana* Lam leaves (MEZ) and identifying its mechanism. The antiarthritic activity was examined by CFA induced arthritis in rats and were treated orally with MEZ 500 mg/kg, twice daily throughout the treatment period. The arthritic score, cytokines, protein (COX-2), histopathology of joints, ulcer protective actions and gene expression (PGE2 and iNOS) were analyzed. MEZ 500 mg/kg significantly reduced the arthritic score and cytokine levels and reduced the COX-2 expression in arthritic rats. Similarly, the inflammatory gene expressions were significantly reduced in the treatment groups. Similar results were recapitulated in histological observations. Thus, this study provides evidence to support the traditional claim of *Z. mauritiana* as an effective anti-arthritic drug.**

**Key words:** *Ziziphus mauritiana*, Inflammation, Arthritis, Cytokines, Prostaglandins, Cyclooxygenase.

## INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease that primarily affects synovial joints and, if left untreated, can cause irreversible damage [1]. WHO estimated that 18 million people are living with RA being women are affected mostly than men and the elderly patients are mostly affected by it. It affects multiple parts of the body including wrists, feet, ankle, elbows, shoulders and produce, pain, stiffness, tenderness and swelling in the joints are common symptoms [2]. The causes of RA are unclear, but the speculation being around the autoimmunity which affects the synovial joints provokes disease. This leads to chronic inflammation, bone erosion and cartilage destruction caused by the production of auto antibodies like anti-CCP, IL-1, IL-6 and TNFα worsen the conditions. Other factors such as genetic predisposition, infection, smoking, obesity and pollution also cause RA [3]. Current arthritis treatments, such as disease-modifying antirheumatic medications (DMARDs) and non-steroidal anti-inflammatory drugs (NSAIDs), provide symptomatic relief but are frequently linked to serious side effects and have limited

ability to slow the progression of the condition [4]. The chronic usage of NSAIDs induces erosions and ulcers in the gastrointestinal (GI) tract in approximately 50 % of individuals, in which 2 to 4% of individuals develop bleeding and leads to death [5]. As a result, using herbs, herbal products, or phytoconstituents is enticing as the first option for their gentle action and lack side effects [6]. Medicinal plants are intended to treat arthritis by conventional medicinal practitioners worldwide. The search for an ideal anti-arthritic drug also had been extended to herbal medications in search of new molecules which afford better protection and decrease the incidence of relapse. The therapeutic potential of herbal medicine in the treatment of chronic illnesses, such as arthritis, has drawn a lot of attention in recent years [7]. Indian jujube, or *Z. mauritiana* Lam that is used extensively in traditional medicine. The plant contains numerous bioactive substances including flavonoids, alkaloids, and saponins are abundant in this plant's leaves, fruits, and bark. These substances have a variety of pharmacological characteristics, such as immunomodulatory, antioxidant, and anti-inflammatory effects. Indian traditional practitioners use *Z. mauritiana* (Rhamnaceae) to treat gastrointestinal, hepatic, cardiovascular, and urinary conditions. The leaves are used to treat eczema, diarrhea, obesity, diabetes, and insomnia. We studied the analgesic and anti-inflammatory activities [8] of *Z. mauritiana* leaves and studied the anti-asthmatic activities in mice [9]. We also found that the leaves contain apigenin, betulin, betulinic acid (BA), kaempferol, luteolin, myricetin, catechin, and kaempferol [10]. Thus, the present study aims to investigate antiarthritic potential of *Z. mauritiana* leaves in CFA induced arthritis in rats by identifying its mechanism to justify its traditional claim.

## MATERIALS AND METHODS

### *Chemicals*

Complete Freund's adjuvant (C.F.A.) were procured from Sigma-Aldrich, USA. All the ELISA kits were procured from Ray Biotech, USA. HPLC solvents were procured from Merck, India. Madras Pharmaceuticals, Chennai, gifted the prednisolone. All the AR grade solvents used were purchased from Fisher Scientific, India.

### *Collection and extraction of plant materials*

The leaves of *Z. mauritiana* Lam were collected from wild sources of Western Ghats of Tamil Nadu. The extraction and UPLC quantification of the extract was mentioned in our previous studies [9, 10].

### *Experimental animals*

Male Wistar rats (180-220 g) was procured from Sri Venkateshwara Enterprises in Bengaluru, Karnataka, India. The animals were housed in standard polypropylene cages and provided regular pellet diets (23% protein, 5% fat) and water ad libitum. The animals were maintained under standard lab conditions with 12-hour light/dark cycles. The study was approved by the Institutional Animal Ethical Committee of Anna University, Tiruchirappalli (AUROT/IAEC/ NOV/2021/0017 Dt. 26.11.2021).

### *Acute toxicity study*

An acute toxicity study was performed as per the guidelines of OECD 423 to observe whether the methanol extract would produce any toxic effects. Our earlier research [11] reported the acute and chronic toxicity of MEZ.

### *Complete Freund's adjuvant-induced arthritis in rats*

Rats were split into four groups of six in each group. Group 1 is standard control. Except for the control group, all the other group rats were injected intradermally with 100 µL of CFA on the left hind paws to develop arthritis [12]. Group 2 is arthritic control (only CFA). Groups 3 and 4 were treated with Prednisolone 10 mg/kg, *p.o.* and MEZ 500 mg/kg, *p.o.* All the treatment continued for 28 days. The arthritic score was calculated on each rat throughout the experimental period. They scored on a scale 0–4:

Score 0 = no swelling/ inflammation
Score 1 = Mild swelling
Score 2 = moderate swelling
Score 3 = swelling below the ankle
Score 4 = All joints and ankles were swollen

At the end of the study, rats were euthanized by an overdose of ketamine (100 mg/kg) and xylazine (20 mg/kg) *i.p.*, blood was collected from the heart in a blood tube, centrifuged at 10,000 rpm for 10 min to collect the serum [13]. Serum was used to analyze the cytokine levels. The thymus, spleen and left limb joints were collected, weighed and stored in 10 % formalin for histopathological sections. The synovial tissues were excised and stored at -80°C for the western blot analysis.

***Detection of COX-2 expression by western blotting in synovial tissues***

The COX-2 protein expression was analyzed by western blotting. The synovial tissues were homogenized in PBS and centrifuged at 6000 × g for 10 min. The supernatants were used for protein quantification using the BCA method (Invitrogen, USA). The protein was resolved in 10% SDS polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 10 % NFD milk for 30 min and incubated with primary mouse monoclonal COX-2 antibody (sc-19999, Santacruz biotechnology, USA) with 1:400 in 10 % NFD milk at 4°C overnight [14] and β-actin was used as standard. After washing, the membrane was incubated with rabbit IgG secondary antibody (Santa Cruz Biotechnology, Inc.) conjugated with HRP in 10 % NFD milk at room temperature for one hour. After washing, the blots were incubated with Pierce ECL western blotting substrate (Bio-Rad, USA) for 5 min at room temperature. The membranes were imaged using a Bio-Rad ChemiDoc Imager.

***Total mRNA isolation and RT-PCR analysis***

The triazole reagent (DSS Takara, India) was used to extract the total mRNA from paw samples. Using β-actin as an internal reference, RT-PCR was performed from cDNA using RT-PCR (C1000 Touch Thermal Cycler, Bio-Rad, USA). A 2% agarose gel containing ethidium bromide was used to resolve the products and observed under UV light. ImageJ software (Bio-Rad, USA) measured each band's density (**Table 1**) [15].

**Table 1**. Primers for RT-PCR analysis

| Targets | Sequences |
|---|---|
| PGE2 | F-5'-ATGGCAGGACATCATGGAC-3'<br>R-5'-TCAGGAGGAGGAGGAGAG-3' |
| iNOS | F-5'-CACCACCCTCCTTGTTCAAC-3'<br>R-5'-CAATCCACAACTCGCTCCAA-3' |
| *β*-actin | F-5'-TCATGAAGTGTGACGTTGACATCCGT-3'<br>R-5'-CCTAGAAGCATTTGCGGTGCACGATG-3' |

***Histopathology of ankles***

The paraffin-embedded ankles were made into 4-5 µm thick sections on glass slides. These slides were stained with hematoxylin-eosin and examined for pathological changes under light microscope [16].

***Statistical analysis***

All the results were expressed as mean±standard error mean (SEM). We used Graph pad prism Software Version 11.0. for testing. We used analysis of variance (ANOVA) wherever applicable, followed by and $p < 0.05$ considered as statistical significance.

**RESULTS AND DISCUSSION**

Secondary metabolites play a crucial role in the process of drug discovery by aiding in the identification and exploration of new and innovative compounds. The findings of our study indicate the existence of phenols, flavonoids, glycosides, terpenes, alkaloids, and carbohydrates. ROS have been associated with various pathological conditions, such as atherosclerosis, diabetes, cancer, and arthritis, among others. We studied the free radical scavenging capacity of MEZ using the DPPH, and ABTS, in which MEZ continued to exhibit superior activities [17]. Rat adjuvant-induced arthritis progresses in three stages, like human rheumatoid arthritis. They consist of induction phase, early synovitis and late synovitis during which the joints gradually deteriorate [18]. In the current study, CFA injections in the hind paw produced pain, swelling and inflammation in rats. The rats have experienced severe pain and restricted movement and cannot support their rear paws while standing. The paw began to inflame from day 4 onwards, but a significant difference was observed (Peak swelling and inflammation) on day 7 compared to the control rats (**Figure 1**). Prednisolone treatment significantly reduced the swelling and inflammation in the rats from day 14 onwards; the effect was observed throughout the treatment period. Similarly, treatment with MEZ significantly reduced the swelling from day 17, which continued throughout the experiment.

The induction of inflammation through the subcutaneous injection of CFA elicits the secretion of inflammatory mediators due to the host's inflammatory response. Following

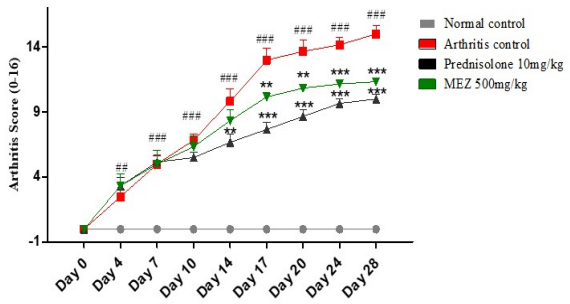


**Fig. 1**. Effect of MEZ on arthritic score in rats

Values are expressed as mean±SEM, **p*<0.05, ***p*<0.01 and ****p*<0.001 compared to arthritic control, ##*p*<0.01 and ###*p*<0.001 significant different in normal control *vs* arthritic control

the activation, the synovial inflammation leads to producing cytokines like TNF-α, IL-6 and PGE2 [19]. During the inflammatory process, the presence of TNF*α* exacerbates the synthesis of prostaglandin (PGE2), hence augmenting the experience of pain and inflammation. The concurrent impact of elevated levels of NO and TNFα, under the influence of IFNγ, leads to the occurrence of tissue damage in the presence of inflammation. The cytokines play a crucial role in the control of inflammation, hematopoiesis, and immunological responses [20]. Arthritic inflammation substantially increases the inflammatory cytokine levels in the blood. It significantly elevated the proinflammatory cytokines like IL-6, IL-17 and IL-1β in the arthritic group compared to the control group (**Figure 2**).

MEZ treatment significantly reduced the IL-17 and IL-1*β* (p<0.05) levels, but a reduction trend is observed in the IL-6 levels, even though it is non-significant. Prednisolone treatment significantly (p<0.01) reduced the pro-inflammatory cytokine levels.

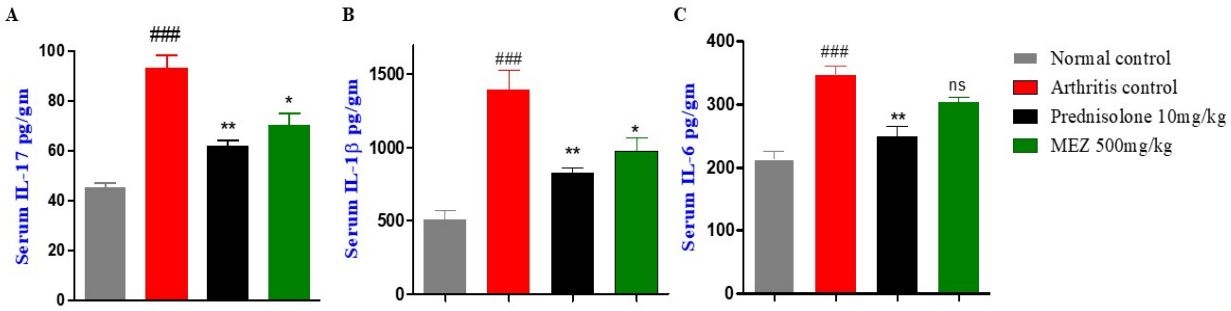


**Fig. 2**. Effect of MEZ on serum cytokine levels on CFA induced arthritic rats

Values are expressed as mean±S.E.M. *p<0.05, **p<0.01 and ***p<0.001 compared to arthritic control, ##p<0.01 and ###p<0.001 significant difference in normal control *vs* arthritic control

MEZ contains bioactive compounds including, *β*-sitosterol, apigenin-7-*O*-glucoside, kaempferol 3-*O*-glucoside, and luteolin-7-*O*-glucoside, showed strong anti-inflammatory effects by working on several different molecular pathways. *β*-sitosterol stops the production of NO by blocking iNOS and lowers inflammation by changing NF*κ*B [21]. Apigenin-7-*O*-glucoside stops the production of iNOS and ROS and lowers IL-6 and TNF*α* by changing NF*κ*B. Kaempferol 3-*O*-glucoside lowers NO and ROS and turns on Nrf2 to make antioxidant enzymes, which reduces inflammation even more [22].

By stopping iNOS, getting rid of ROS, and stopping the NF*κ*B, luteolin-7-*O*-glucoside successfully blocks NO, ROS, and pro-inflammatory cytokines [23]. The present investigation observed that the administration of MEZ at 500 mg/kg reduced cytokine levels to reduce the arthritic inflammation.

One important factor in RA is the transformation of the body's immune system. Spleen is the vital organ for immune cell activation, and it is vulnerable to alterations in inflammatory diseases like arthritis. By acting as central and peripheral immune organs, the thymus and spleen could regulate the immune response. The increase in spleen weight implies a stimulatory effect on the immune system, especially T and B lymphocytes, which expand drastically against the "foreign" antigens [24]. Unlike the control group, CFA injections exhibit immunological modulation, as seen by increased spleen and thymus weight (**Figure 3**). We observed that MEZ and prednisolone treatments significantly reduced the increased weight of spleen and thymus, indicating protective actions against immunomodulation.

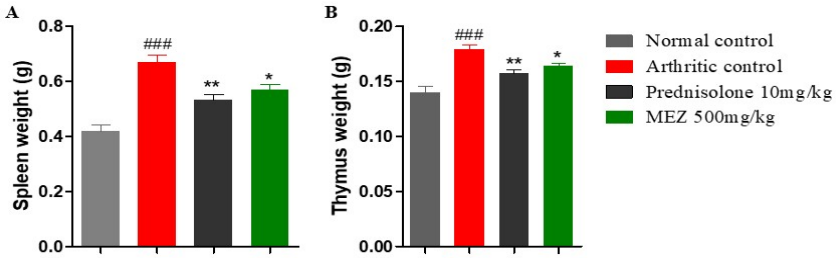


**Fig. 3**. Effect of MEZ on A) spleen and B) thymus weights in arthritic rats.

*Values are expressed as mean±S.E.M. *p<0.05, **p<0.01 and ***p<0.001 compared to arthritic control, ##p<0.01 and ###p<0.001 significant different in normal control *vs* arthritic control

The histopathological analysis revealed the injection of CFA in rats produced severe damage to the joints (**Figure 4**).

Rats in the control group had normal articular joints with normal chondrocytes. CFA induced accumulation of inflammatory cells in the bone marrow, cartilage destruction, synovial hyperplasia, and bone damage. Treatment with prednisolone and MEZ minimizes these effects. Similarly, the spleen of the arthritic rats showed a notable rise in the width of lymphoid follicles together with hyperplastic proliferation and the growth of the germinal center of lymphoid follicles. It showed more tangible macrophages with cytoplasmic engulfed apoptotic debris. Trabeculae thickened, and the red pulp became dense.

Similarly, the white pulp is enlarged by the influx of inflammatory cells. MEZ and prednisolone treatments minimized these effects.

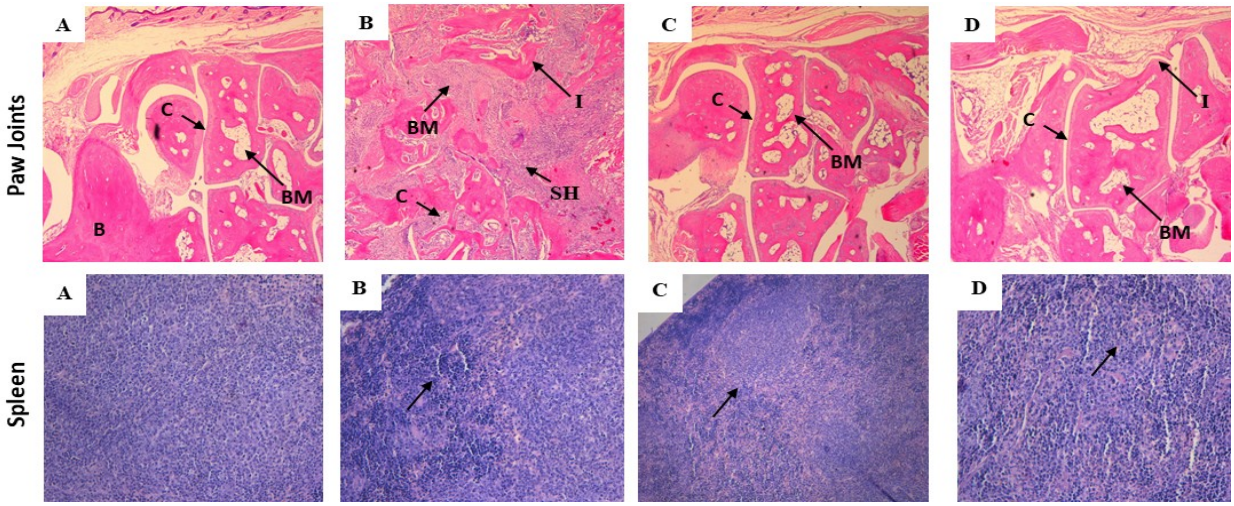


**Fig. 4**. Histopathological observation of joints and spleen in control and treatment groups.
A) Normal control; B) Arthritic control; C) Prednisolone treated; D) MEZ treated. B – Bone; BM – Bone marrow; BD – Bone damage; C – Cartilage; I – Inflammation; SH – Synovial hyperplasia

The cyclooxygenase (COX) enzymes produce prostaglandins, which are crucial inflammatory mediators in arthritis. Both human and rodent synovial tissues have been shown to regulate the expression of the COX-2 gene. Endotoxins, cytokines such as IL-1$\beta$ and TNF$\alpha$, endothelial cells, and chondrocytes all cause an increase of COX-2 [25]. Furthermore, it was demonstrated that IL-1b increased COX-2 *de novo* production but not COX-1. In rats with arthritis, the inhibition of COX-2 activity also changed the production of cytokines both locally and systemically. Increased TNF$\alpha$ and IL-6 levels were linked to the onset of arthritis [26]. Adjuvants likely cause affected joints to produce more IL-1$\beta$ and TNF-$\alpha$, and IL-1$\beta$ is crucial for controlling the long-term synthesis of COX-2 polypeptides in arthritic response. The effect of MEZ on the COX-2 levels was observed in the synovial tissues isolated from the ankle joint using western blot analysis (**Figure 5**). CFA injection significantly ($p<0.001$) increased the expression of the COX-2 levels, which is evident from the densitometry analysis. Treatment with

prednisolone (p<0.001) and MEZ (p<0.01) significantly reduced the COX-2 levels in the arthritic rats. These findings imply that the antiarthritic impact of MEZ may be facilitated through inhibiting the cyclooxygenase (COX) enzyme pathway.

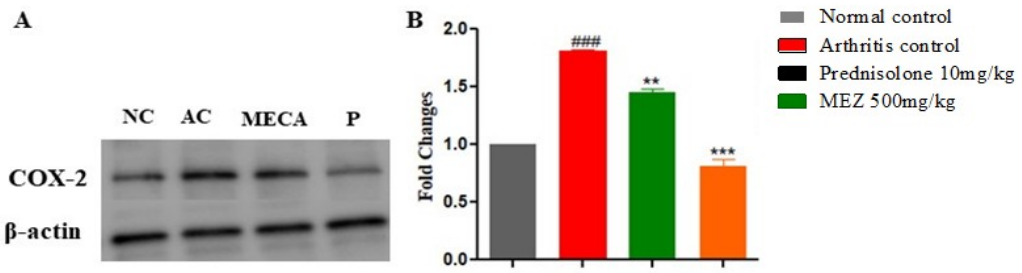


**Fig. 5**. A) Western blot analysis; B) Densitometric analysis of the COX-2 expression levels in control and experimental groups.

Values are expressed as mean±S.E.M. **p<0.01 and ***p<0.001 compared to arthritic control, ###p<0.001 significant difference in standard control *vs* arthritic control

Prostaglandins, important lipid mediators, are produced in large quantities by rheumatoid synovium and other inflammatory tissues. The PGE2 pathway has a well-established role in the pathogenic process of RA, and most research has concentrated on PGE2 and the enzymes that synthesize it in response to inflammation [27]. PGE2 most likely contribute to synovial inflammation by enhancing local blood flow and accelerating the effects of pro-inflammatory mediators that promote vasopermeability, such as bradykinin and IL-1$\beta$ [28]. In RA joints, PGE2 are strong inducers of inflammation, joint degradation, and bone resorption [29]. Through the stimulation of COX-2, cytokines including IL-1$\beta$ and TNF$\alpha$ have been shown to activate synoviocytes and other cells to significantly enhance the synthesis of PGE2. Costa et al. believe that the cause of RA is closely linked to nitric oxide metabolites and total free radical capture antioxidant properties. The results show that iNOS inhibition is regarded as a possible therapeutic approach for the management of RA [30]. The results of the inflammatory gene expressions through RT-PCR analysis are shown in **Figure 6**. The expression of inflammatory genes like PGE2 and iNOS was significantly increased in the arthritic groups compared to the control group. These inflammatory expressions were reduced in the MEZ treatment group. Similar results were seen with the prednisolone group.

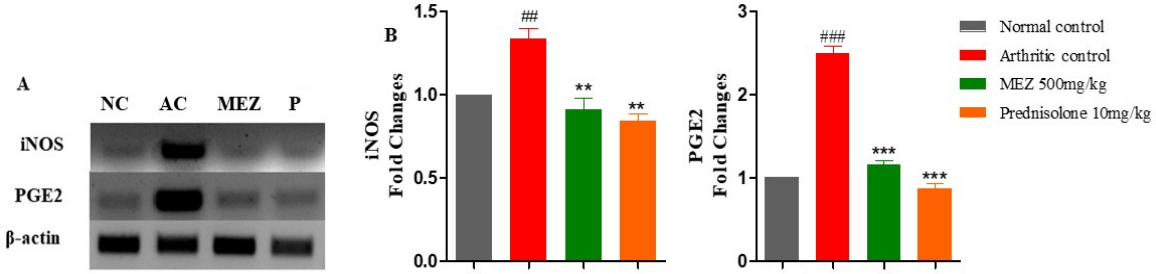


**Fig. 6**. MEZ inhibits inflammatory genes iNOS and PGE2 A) Gene expression; B) Densitometric analysis in control and experimental groups

Values are expressed as mean ± S.E.M. **p<0.01 and ***p<0.001 compared to arthritic control, ###p<0.001 significant difference in standard control *vs* arthritic control

## CONCLUSIONS

The results of this study indicated that the antiarthritic effects of MEZ are facilitated by multiple mechanisms, such as the inhibition of arachidonic acid metabolism and degradation, as well as the inactivation of prostaglandins. These data support the traditional claim that *Z. mauritiana* is utilized within the folk medical

system to manage inflammatory ailments. The advantage of the current study is that it provides the initial proof of concept for MEZ antiarthritic effects. The only drawbacks of the study are its limited cytokine profile and short-term assessment. Long-term evaluations are required to resolve constraints, and future assessments should focus on proper molecular mechanisms to prove the antiarthritic effects.

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