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RESEARCH ARTICLE

Ameliorating Effect of *Guaiacum officinale* Mother Tincture on Adjuvant-induced Arthritis Model by Modulating Oxidative Stress and Inflammation

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Abstract

Background: Rheumatoid Arthritis (RA) is a chronic, systemic inflammatory disease that primarily affects the joints, leading to pain, stiffness, and progressive functional impairment. Despite the availability of several synthetic drugs for RA management, many of these treatments are associated with adverse effects and limited long-term efficacy. This has drawn growing interest towards complementary and alternative therapies. *Guaiacum officinale* is a well-established homoeopathic remedy, and it has been used for managing pain and inflammation. Further scientific studies are needed to better understand its effects. **Aim:** The present study aimed to investigate the efficacy of *G. officinale* Mother Tincture (GUA) as an anti-arthritic agent using the complete Freund's adjuvant-induced arthritis model. **Methods:** Male Wistar rats were divided into four groups: Normal control, arthritic control, standard drug-treated and sample-treated group. Arthritis was induced by subcutaneous injection of complete Freund's adjuvant. After the experimental period, the efficacy of the drug was evaluated by assessing parameters such as antioxidant status, lipid peroxidation end product, pro-inflammatory markers (IL-6 and TNF-α), nitric oxide level and histopathological changes. **Results:** The results clearly demonstrated that in the GUA-treated group, there was a significant reduction in joint swelling, inhibition of inflammatory mediator activation and infiltration, and an enhancement in antioxidant status. Histopathological analysis also revealed attenuated inflammation and reduced joint destruction in the GUA-treated group. **Conclusion:** These findings suggest that *G. officinale* mother tincture possesses potent anti-inflammatory properties and has the potential to ameliorate the symptoms of arthritis.

Major Findings: *Guaiacum officinale* mother tincture markedly lowered arthritis severity by modulating the levels of inflammatory mediators, enhancing antioxidant defence mechanism, and decreasing joint swelling, highlighting its therapeutic potential in managing rheumatoid arthritis.

Keywords: Antioxidants, Arthritis, Cytokines, *Guaiacum officinale,* Inflammation

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1. Introduction

The immune system plays a vital role in maintaining the body's defence mechanisms and overall homeostasis. However, any dysregulation or imbalance can lead to a hyperactive immune response, which is the underlying cause of several autoimmune disorders[1]. RA is a chronic, progressive, inflammatory autoimmune disorder primarily affecting the synovial membrane and joints. It leads to the gradual destruction of cartilage and bone, accompanied by symptoms such as severe pain, swelling, stiffness, and reduced mobility[2]. Globally, RA affects approximately 0.5–1% of the population, with a higher prevalence in women and increasing incidence with age[3].

The pathophysiology of RA is complex and multifactorial, involving persistent inflammation, oxidative stress, and immune dysregulation. During the disease progression, excessive production of free radicals occurs as a by-product of various cellular processes, leading to the overexpression of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α)[4]. These cytokines play a central role in promoting synovial inflammation, leukocyte infiltration, and the proliferation of synovial fibroblasts, resulting in pannus formation and subsequent cartilage and bone erosion. The uncontrolled generation of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) further exacerbates tissue injury by damaging lipids, proteins, and nucleic acids, thereby inducing a state of oxidative stress[5]. Chronic inflammation and oxidative stress together disrupt the delicate balance between oxidants and antioxidants, as persistent free radical generation suppresses endogenous antioxidant defence mechanisms[6].

Currently, there is no definitive cure for RA, and available therapeutic strategies mainly focus on symptom management and slowing disease progression. Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) and Disease-Modifying Anti-Rheumatic Drugs (DMARDs) are the primary pharmacological options. Although effective, long-term use of these drugs is often associated with adverse effects such as gastrointestinal irritation, ulcers, nausea, and diarrhoea, limiting their prolonged administration[7].

In recent years, Complementary and Alternative Medicines (CAM) have gained increasing attention as potential adjuncts or alternatives to conventional therapy due to their natural origin, lower toxicity, and holistic benefits. Homoeopathy, one such system of medicine, has been widely used for managing various chronic and inflammatory conditions. *Guaiacum officinale,* commonly known as *Lignum vitae*, is a well-known homoeopathic remedy which is traditionally used for the treatment of arthritic and rheumatic conditions[8]. The bark, wood, flowers, and leaves of this plant contain bioactive saponins, guaianin and guaiacin, which possess notable anti-inflammatory and analgesic properties[9]. These phytoconstituents are believed to alleviate joint inflammation, swelling, and pain associated with arthritis.

Therefore, the present study aimed to investigate the anti-arthritic potential of *G. officinale* mother tincture in an experimental arthritic model and evaluate its role in modulating oxidative stress and inflammation.

2. Materials and Methods

2.1 Chemicals

All the reagents, chemicals, and biochemicals utilised in this study were high-quality analytical grade. Complete Freund's adjuvant was purchased from Sigma Aldrich Co., USA. Solvents were purchased from Merck, India. IL-6 and TNF-α ELISA kits were procured from Sigma-Aldrich, USA and PGE-2 ELISA kits were purchased from Cayman Chemicals, Ann Arbour, MI, USA.

2.2 Preparation of Samples

GUA was purchased from Kerala State Homoeopathic Co-Operative Pharmacy Ltd. (HOMCO), Mararikulam South, Alappuzha district, Kerala.

For the preparation of the sample, GUA was taken, and the solvent in the mother tincture was removed using a rotary vacuum evaporator at a temperature of 60°C. The resulting residues were then collected and stored at 4°C for further analysis.

2.3 Animals

Adult male Wistar rats (150 ± 10 g) were obtained from the departmental animal facility. Animals were maintained under standard laboratory conditions (temperature: 24–26°C; relative humidity: 55–60%; photoperiod: 12 h light/12 h dark). They were provided with a standard pellet diet (Amrut

Laboratory Animal Feeds, India) and water ad libitum. All animal experiments were conducted according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines and were approved by the Institutional Animal Ethics Committee (Approval No: CBLRC/IAEC/06/01-2023). Before initiating the experiments, the rats were allowed to acclimate to the laboratory conditions for one week.

2.4 Experimental Design

The rats were randomly assigned to four groups by a complete randomised method, and each group consisted of six rats.

Animals were grouped as follows:

Group 1: Normal Control (NC)

Group 2: Treated with Complete Freund's Adjuvant (CFA)

Group 3: Induced with CFA and treated with standard drug (CFA+INDO;10mg/ kg body weight)

Group 4: Induced with CFA and treated with *G. officinale* mother tincture (CFA+GUA; 12mg dissolved in 1ml distilled water/100g body weight).

For immunisation, 0.1ml of CFA containing heat-killed mycobacteria in paraffin oil was injected into the right hind paw of the animals except the normal control. GUA and INDO were administered orally on a daily basis after 7 days of post-immunisation for a duration of 21 days. The dosage of GUA (12 mg/mL/100g body weight) was determined from a preliminary dose–response study.

Paw oedema was measured using a plethysmometer on the 7th, 14th, and 21st days of treatment. The percentage inhibition of oedema was calculated relative to the arthritic control group. At the end of the experimental period, animals were fasted overnight and euthanised under anaesthesia. Blood samples were collected via retro-orbital puncture for serum isolation, and paw tissues were excised for biochemical and histopathological analyses. All experimental procedures, including the sample treatments and data collection, were performed by investigators blinded to the group allocations.

2.5 Analytical Procedures

2.5.1 Antioxidant Assays

Superoxide Dismutase (SOD) activity was estimated using the Kakkar *et al.*[10] method. Tissues were homogenised and the supernatant added to a mix of sodium pyrophosphate buffer, phenazine methosulphate, nitroblue tetrazolium, and NADH. After incubation, reactions were terminated with glacial acetic acid, and the chromogen was extracted with n-butanol. Absorbance was measured at 560nm. One unit of SOD activity was the amount inhibiting Nitroblue Tetrazolium (NBT) reduction by 50%, reported as units per mg protein.

Catalase (CAT) activity was measured following the method of Maehly and Chance[11]. Tissue homogenates were prepared in Tris buffer (pH 7.4), and the supernatant was used for assays. The reaction mix contained phosphate buffer (pH 7.0), hydrogen peroxide, and enzyme extract. Results were expressed as units per mg protein, with one unit defined as the decomposition of 1 µmol H_2O_2 per minute.

Glutathione Peroxidase (GPx) activity was measured following Agergaard and Jensen[12]. Tissue homogenate was prepared in sucrose and centrifuged at 10,000×g for 30 minutes. The resulting supernatant was used for the assay. Enzymatic activity was assessed by adding 0.2 mL of enzyme solution to a reaction mixture containing phosphate buffer, Ethylenediaminetetraacetic Acid (EDTA), sodium azide, reduced glutathione, Nicotinamide Adenine Dinucleotide Phosphate (NADPH), and water, followed by incubation at room temperature for 5 minutes. The reaction was initiated by introducing H_2O_2, and the decrease in absorbance at 340nm was recorded every 20 seconds. Enzyme activity was defined as the amount of NADPH oxidised per minute per milligram of protein.

2.5.2 Estimation of Glutathione Content (GSH)

Glutathione content was measured using the method described by Benke[13]. Tissue samples were homogenised using 5mL precipitating solution and incubated at room temperature for 5 minutes, followed by filtration through coarse filter paper. 3mL of 0.3M phosphate solution and 1mL of 0.04% DTNB were added to 0.2mL of the filtrate. The tubes were then capped, inverted to mix, and the absorbance was measured at 412nm within 4 minutes.

2.5.3 Estimation of Malondialdehyde (MDA)

Malondialdehyde (MDA) level was quantified using the method established by Ohkawa[14]. One gram of tissue

was homogenised in nine volumes of 1.15% KCl, then centrifuged to collect the supernatant. To 0.2mL of the tissue homogenate, 0.2 mL of 8.1% SDS, 1.5mL of 20% acetic acid (pH 3.5), and 1.5mL of an aqueous solution of TBA were added. The total volume was adjusted to 4 mL with distilled water and then heated in a boiling water bath at 95°C for 60 minutes. After cooling, 1mL of distilled water and 5mL of n-butanol: pyridine reagent were added and mixed thoroughly. The mixture was then centrifuged at 4000 rpm for 10 minutes, and the organic layer was separated. The absorbance of the organic layer was measured at 532nm against a butanol blank.

2.5.4 Estimation of Nitric oxide Level

The assay was performed using Griess reagent, which consists of 1% sulfanilamide with 0.1% Naphthylethylenediamine. For the estimation, equal volumes of Griess reagent and the sample were mixed and incubated for 10 minutes. The absorbance was then measured at 540nm.

2.5.5 Estimation of Pro-inflammatory Cytokines and Prostaglandin Using ELISA

The serum concentration of TNF-α, IL-6, and PGE 2 was quantified using ELISA kits, following the manufacturer's instructions.

2.5.6 CRP Level and White Blood Cell Count

Serum CRP level was determined using the Agappe Diagnostic kit, and White Blood Cell (WBC) count was performed using a hemocytometer.

2.5.7 Histopathological Analysis of Paw Tissue

Paw tissue was dissected, and tissue sections (5µm) were fixed in 10% formalin solution at room temperature. For histological analysis, paraffin-embedded sections of paw tissue were stained with Hematoxylin–Eosin (H and E). The stained tissue samples were examined and photographed under a light microscope to assess structural abnormalities. The degree of inflammation in the tissue was evaluated by two independent and blinded observers, who were unaware of the experimental condition.

2.5.8 Estimation of Protein

Protein analysis was done by the method of Lowry *et al*[15].

2.6 Statistical Analysis

The data were analysed using the SPSS/PC+ statistical software, version 11.0 (SPSS Inc., Chicago, IL, USA). A minimum of six animals per group was chosen based on previous similar studies, which demonstrated sufficient power to detect statistically significant differences. All results were expressed as mean ± standard deviation (SD). One-way ANOVA was used to assess significant differences between groups, and pair-fed comparisons were performed using Duncan's multiple range tests. A P value of < 0.05 was considered statistically significant.

3. Results and Discussion

3.1 Percentage Inhibition of Paw Volume by GUA

The induction of arthritis with CFA resulted in significant paw swelling, accompanied by cartilage degradation, synovial inflammation, and immune cell infiltration within the joints and thereby developing a polyarthritis condition which is similar to human arthritis[16,17]. Paw swelling serves as a key indicator for evaluating the anti-arthritic potential of a drug. Consistent with the previous findings, the CFA-induced group showed the signs of inflammation and swelling as compared to the normal control[18]. However, supplementation with GUA produced a noticeable and statistically significant ($p < 0.05$) reduction in paw oedema over time. As shown in Table 1, GUA exhibited progressive inhibition of paw volume from day 7 to day 28, and it shows maximum oedema inhibition of 86% on day 28 as compared to the CFA control and INDO-treated groups ($p<0.05$).

Table 1. Effect of *Guaiacum officinale* (GUA) and indomethacin (INDO) on percentage inhibition of paw edema in CFA-induced arthritic rats. Values are expressed as mean of six rats per group (n = 6). [a]Significantly different from the CFA group at $p < 0.05$ (ANOVA followed by Duncan's multiple range test). Abbreviations: CFA – Complete Freund's Adjuvant; GUA – *Guaiacum officinale* mother tincture; INDO – Indomethacin

Group	% Inhibition of paw volume		
	Day 7	Day 14	Day 21
CFA	0	0	0
CFA+GUA	36[a]	58[a]	86[a]
CFA+INDO	32[a]	47[a]	78[a]

Thus, the result suggests that GUA can be effectively mitigated inflammatory progression likely through the downregulation of pro-inflammatory mediators.

3.2 Effect of GUA on WBC and CRP levels

Haematological parameters are also crucial for assessing inflammatory diseases. CRP and WBC count are commonly used haematological markers. Alterations in haematological parameters have been observed during the progression of RA. CRP is an acute-phase protein produced primarily by the liver in response to pro-inflammatory cytokines, most notably IL-6 and plays multifaceted roles in the immune response[19]. It is involved in the development and progression of RA by actively participating in both molecular and cellular mechanisms, and it will directly influence endothelial cells, neutrophils, and macrophages by triggering the activation of downstream inflammatory signalling pathways[20].

WBCs are essential components of the immune system and play a key role in inflammatory responses and associated disorders. During the progression of RA, WBC count will increase rapidly, which leads to the production of macrophages, granulocytes, and colony-stimulating factors[21]. The result obtained from the study showed that in the CFA group, inflammation was induced after the injection of adjuvant and thus, an increased level of CRP and WBC was observed. The elevated level of these biomarkers indicates the active state of the disease condition in the control group. In contrast, the GUA group suggest its potential role in maintaining a balance of haematological parameters in comparison with CFA-treated rats (Figure 1).

3.3 Effect of GUA on Enzymatic Antioxidant Activity

Oxidative stress and inflammation are two closely linked and significant aspects of rheumatoid arthritis. Several studies have shown that individuals with RA exhibit increased oxidative stress along with elevated inflammatory responses[22]. Upon CFA induction, an inflammatory cascade is activated, characterised by the release of pro-inflammatory mediators and the infiltration of immune cells into the affected joint tissues. This inflammatory microenvironment instigates the activation of diverse signalling pathways, including those involved in the generation of ROS and subsequent oxidative stresss[23]. This surge in ROS overwhelms the antioxidant defence mechanisms, leading to a decline in their levels within the body. SOD, CAT and GPx are the key enzymatic antioxidants, and they work together to defend against the free radicals.

Assessment of enzymatic antioxidants like SOD, CAT and GPx provides a valuable insight into oxidative stress and disease severity. SOD plays a significant role in the dismutation of superoxide radicals released at the inflammatory sites. However, its levels are often insufficient to detoxify the substantial radical burden observed in RA patients[24]. In the case of CAT and GPx, it will prevent the oxidised lipids accumulated in the cell membrane of mitochondria produced as a result of oxidative stress. It helps in the detoxification of toxic metabolites of oxygen and other reactive electrophiles produced during the metabolism of endogenous or exogenous substance[25]. Both CAT and GPx use hydrogen peroxide as their substrate and play a pivotal role in the removal of hydrogen peroxide free

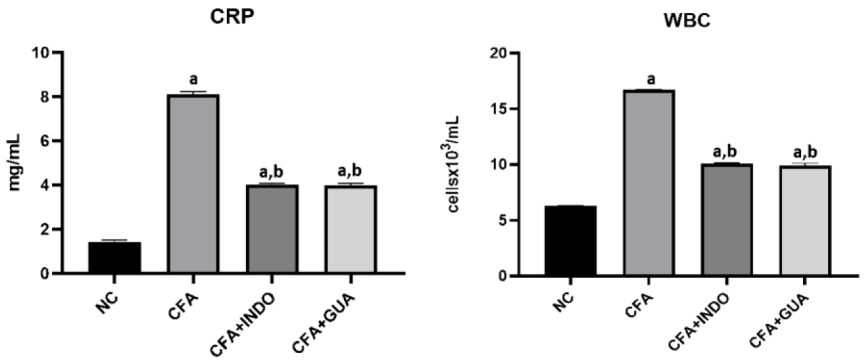


Figure 1. Effect of GUA on serum CRP levels and total WBC count in CFA-induced arthritic rats. Values are expressed as mean ± SD (n = 6). a – significant difference compared with the normal control (NC) group ($P < 0.05$); b – significant difference compared with the CFA group ($P < 0.05$). Units: CRP (mg/mL), WBC (cells×10^3/mL).

radical[26]. Previous reports of RA patients have also shown reduced CAT and GPx activity due to increased oxidative stress[27,28]. Consistent with these findings, our study demonstrated a significant reduction in SOD, CAT, and GPx ($p<0.05$) levels in CFA rats as compared to normal controls. In contrast, administration of GUA boosts the activity of SOD, CAT and GPx by mitigating ROS production and significantly enhances the antioxidant status as compared to the CFA control group (Figure 2).

3.4 Effect of GUA on GSH level

GSH is another important antioxidant which helps to maintain and protect the cells by detoxifying the xenobiotics, scavenges free radicals and regulates the immune functions[29]. Previous investigations in RA have highlighted a significant depletion in GSH levels due to the inflammatory milieu and concomitant ROS generation[30]. In line with these results, our research noted a significant ($p<0.05$) reduction in GSH level in CFA-treated rats as compared to normal controls. The upregulation of GSH by GUA likely stems from reduced ROS load and enhanced enzymatic synergy between GPx and CAT. This action alleviated the stress condition, highlighting the antioxidant attributes of GUA. The effect of GUA on GSH level is shown in Figure 3.

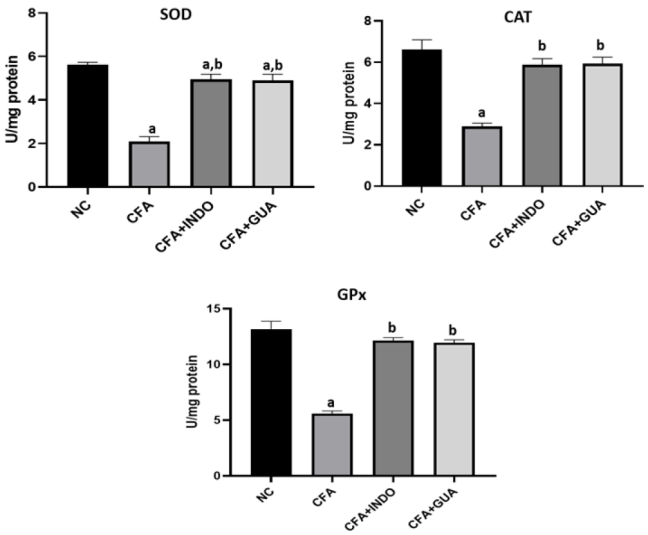


Figure 2. Effect of GUA on antioxidant enzyme activities (SOD, CAT, and GPx) in tissue homogenates of CFA-induced arthritic rats. Values are expressed as mean ± SD (n = 6). a – significant difference compared with the NC group ($P < 0.05$); b – significant difference compared with the CFA group ($P < 0.05$).

3.5 Effect of GUA on MDA level

The consequence of reduced antioxidants was outstripped lipid peroxidation, which was the reason for high MDA levels in RA patients[31]. ROS attack the lipids and proteins on the biomembrane and alter its normal function by changing membrane fluidity. As a result, the permeability of the membrane could be affected, potentially leading to membrane disruption and leakage of intracellular enzymes into the exterior of cells[32]. The result revealed that there was a significant increase in MDA level in the arthritic control group,

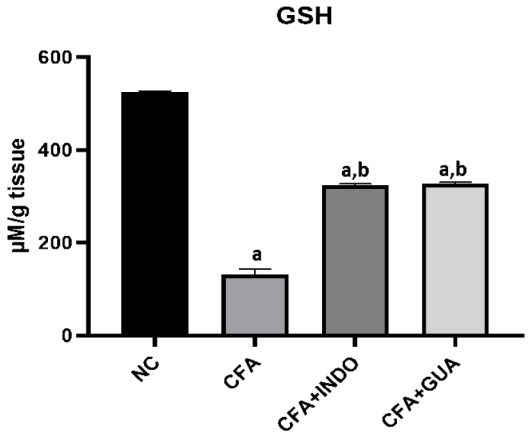


Figure 3. Effect of GUA on reduced GSH levels in tissue homogenates of CFA-induced arthritic rats. Values are expressed as mean ± SD (n = 6). a – significant difference compared with the NC group ($P < 0.05$); b – significant difference compared with the CFA group ($P < 0.05$).

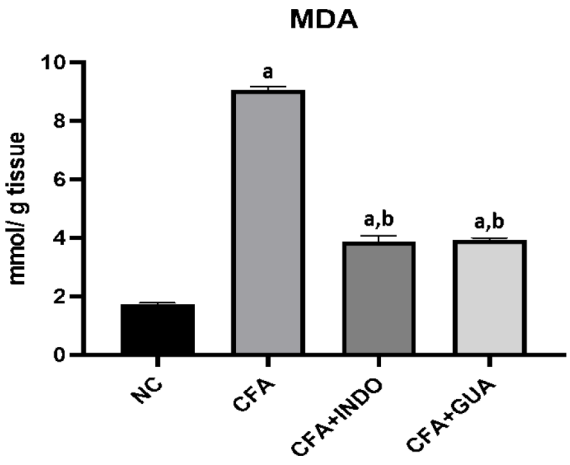


Figure 4. Effect of GUA on MDA level. Values are expressed as mean ± SD (n = 6). a - significant difference compared with the NC group ($P < 0.05$); b - significant difference compared with the CFA group ($P < 0.05$).

which was due to the high lipid peroxidation rate. On the other hand, the rats treated with GUA showed a significant reduction in the level of MDA as compared to the CFA group (Figure 4).

3.6 Effect of GUA on Pro-inflammatory Cytokines and Prostaglandin

Pro-inflammatory cytokines such as IL-6 and TNF-α play critical roles in activating the immune system and triggering inflammatory responses in patients with RA[33]. These cytokines promote the release of various enzymes, particularly Matrix Metalloproteinases (MMP), which contribute to articular cartilage damage[34]. Additionally, cytokine activation leads to the overproduction of other inflammatory mediators and free radicals at the site of inflammation, exacerbating the severity of RA. Following CFA administration, pro-inflammatory cytokines such as IL-6 and TNF-α are released from macrophages and monocytes. Subsequently, TNF-α stimulates the release of other inflammatory mediators, including IL-6 and MMPs, resulting in cellular infiltration, leukocyte leakage and vasodilation at the site of oedema. Moreover, these cytokines promote neutrophil and monocyte activation, contributing to joint inflammation[35]. Another important biomarker involved in arthritis is prostaglandin, which plays a significant role in mediating pain and inflammation. The synthesis of COX-2-dependent Prostaglandins (PG) is responsible for pain perception, making COX-2 a primary therapeutic target for many NSAIDs that inhibit PG synthesis[36]. PGE_2, which can be produced by synovial fibroblasts in response to cytokine stimulation, has been linked to severe cartilage destruction[37]. The results of the study showed a marked increase in the levels of pro-inflammatory cytokines (IL-6 and TNF-α) and PGE_2 in the CFA group compared to the normal control group. The increased expression of these cytokines and prostaglandins correlates with the progression of arthritis. Conversely, treatment with GUA significantly reduced the levels of pro-inflammatory cytokines and excessive production of PGE_2, thereby protecting against cartilage destruction due to its potent anti-inflammatory activity compared to the CFA-treated group (Figure 5).

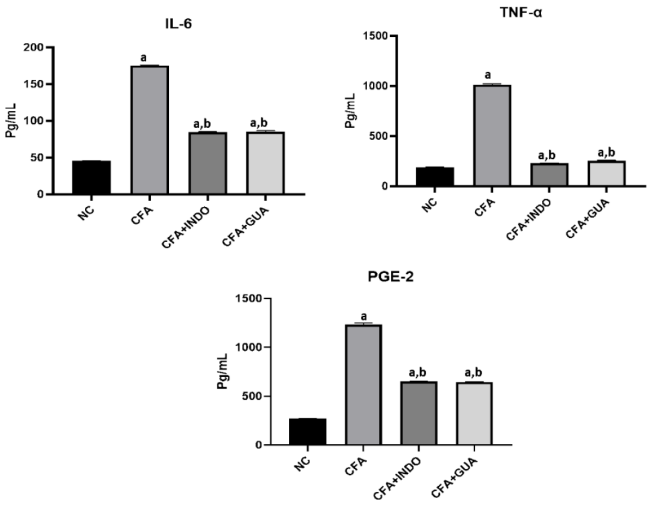


Figure 5. Effect of GUA on pro-inflammatory cytokines (IL-6 and TNF-α) and PGE-2 levels in serum of CFA-induced arthritic rats. Values are expressed as mean ± SD (n = 6). a – significant difference compared with the NC group (P < 0.05); b – significant difference compared with the CFA group (P < 0.05).

3.7 Effect of GUA on NO level

NO has been identified as a toxic radical gas and serves as a key mediator in the pathophysiology of arthritis, playing a dual role in both the initiation and resolution phases of the disease[38]. In arthritic conditions, NO is primarily produced by inducible nitric oxide synthase (iNOS) in response to inflammatory stimuli. Excessive NO production contributes to the chronic inflammation, cartilage degradation, and bone erosion characteristic of rheumatoid arthritis[39]. In this study,

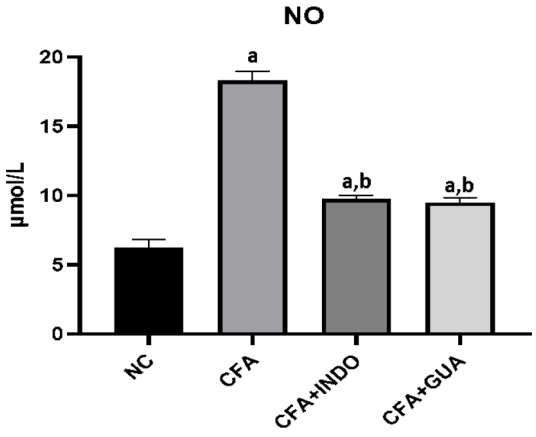


Figure 6. Effect of GUA on NO levels in serum of CFA-induced arthritic rats. Values are expressed as mean ± SD (n = 6). a – significant difference compared with the NC group (P < 0.05); b – significant difference compared with the CFA group (P < 0.05).

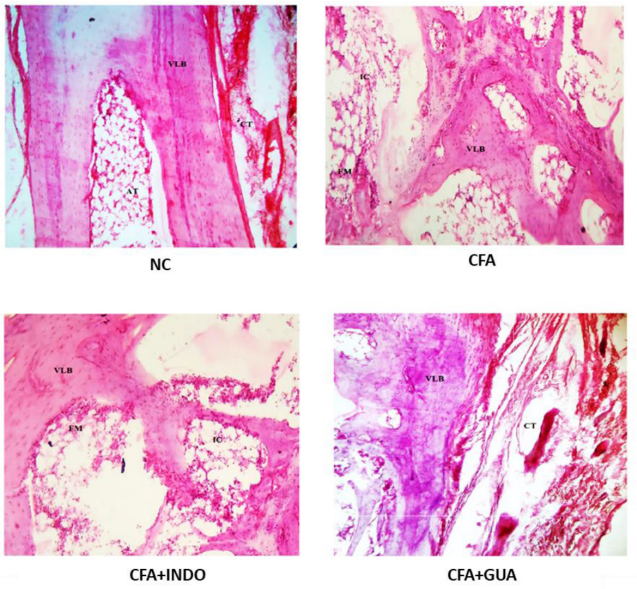


Figure 7. Histopathological analysis of paw tissue. Representative micrographs showing paw tissue architecture in (A) normal control, (B) CFA control, (C) CFA + INDO, and (D) CFA + GUA groups. Sections were stained with hematoxylin and eosin (H and E, ×40 magnification).

a similar pattern was observed, as the arthritic control group showed a significant ($p \leq 0.05$) increase in NO production compared to the normal control group. Treatment with GUA in arthritic rats significantly reduced the elevated level of NO, indicating that GUA possesses anti-inflammatory potential, possibly by inhibiting the activation of iNOS (Figure 6).

3.8 Histopathological Analysis of Paw Tissue

Histopathological analysis serves as a cornerstone in understanding the underlying mechanisms and pathological alterations associated with the experimental conditions, providing invaluable insights into the efficacy and potential mechanisms of action of the tested interventions[40]. In the present study, histopathological examination of paw tissue provided further evidence supporting the anti-arthritic effect of GUA. The result showed that in the CFA group, rats exhibited marked infiltration of inflammatory cells in and around the Vital Lamellate Bone (VLB) and Fatty Marrow (FM) regions. In contrast, the GUA-treated group showed noinfiltration of Inflammatory Cells (IC) in VLB and in Connective Tissue (CT) regions. INDO-treated group exhibited mild inflammatory cell infiltration as compared to the GUA group and the normal control group (Figure 7).

4. Conclusion

The present study demonstrates that *G. officinale* mother tincture exhibits significant anti-arthritic and anti-inflammatory potential. The treatment effectively modulated hyperactive immune responses, attenuated inflammatory cytokine production and cellular infiltration, and enhanced systemic antioxidant defence mechanisms. Moreover, it helps to maintain the normal structural integrity of paw tissues without inducing any apparent damage. These findings collectively suggest that *G. officinale* mother tincture may serve as a promising therapeutic candidate for the management of rheumatoid arthritis.

5. Acknowledgement

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6. Author Contribution

Nisha M: Investigation, Writing - original draft, Data interpretation; Sreelatha S: Data acquisition, Writing - review and editing; Ratheesh M: Conceptualization, Methodology, design and Supervision; Sheethal S: Statistical analysis; Svenia P Jose: Data curation; Vijayan TK: Formal analysis, Resources; Sajeev Martin George: Data curation. All authors have read and agreed to the published version of the manuscript.

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