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Mechanistic Evaluation of Antiarthritic Effects of Citronellol in CFA-Induced Arthritic Rats

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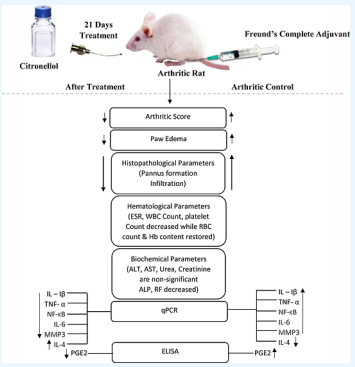
ABSTRACT: Rheumatoid arthritis (RA) is an autoimmune disease characterized by systemic inflammation, joint tissue damage, pain, and synovitis. It leads to deformity of joints, disability, and even premature death. Markers of inflammation are highly expressed in synovium fluid and serum of arthritic patients and play an important role in the pathophysiology of RA. These transcription factors promote the fabrication of type I interferons and inflammatory cytokines. In RA, degradation of synovial cartilage and bone results from stimulation of proinflammatory cytokines. Citronellol (Ct), a monoterpene alcohol, is found in citrus fruits and essential oils of many aromatic plants. It possesses numerous pharmacological properties such as antioxidant activity and potential antinociceptive and anti-inflammatory effects. Keeping in view the significant anti-inflammatory role of Ct, a trial of 28 days was conducted. Ct was administered orally at three different doses (25, 50, and 100) mg/kg in Freund's adjuvant-induced arthritic rats, and the results were compared with piroxicam, chosen as the standard drug. The antiarthritic activity of the compound was evaluated through measurements of arthritic scoring and plethysmometry before and after treatment. The blood biochemical and hematological parameters and histopathological analyses were performed. Additionally, qPCR was conducted to analyze the mRNA expression levels of TNF-α, IL-1β, NF-κB, MMP3, IL-6, and IL-4 in the blood. ELISA was performed to evaluate the levels of PGE2. The results demonstrated that Ct showed significant results at all doses, but the highest dose proved to be most significant in terms of decreasing arthritic scoring and paw edema, indicating the antiarthritic potential of Ct. Furthermore, the compound was found to downregulate all the proinflammatory cytokines (TNF-α, IL-1β, NF-κB, MMP3, and IL-6) and upregulate the anti-inflammatory cytokine (IL-4). The levels of PGE2 were also reduced which further supported the antiarthritic effects of Ct and validated it as a potential antiarthritic candidate.



1. INTRODUCTION

Rheumatoid arthritis (RA) is characterized by chronic inflammation and is autoimmune. The common target tissue is the bone cartilage, which due to inflammation and autoimmunity is eroded and damaged. Prominent indicators of this condition encompass pain, stiffness, and swelling of the joints along with systemic effects comprising fever that leads to body weight loss and fatigue.[1] Due to the autoimmune nature of RA, multiple joints of the body are bilaterally affected.[2] The worldwide prevalence of RA ranges between 0.5 and 1% across different populations.[3] The reported yearly occurrence of RA is about 40 in 100,000 worldwide.[4−8] Apart from physical symptoms, new technology introduces laboratory procedures that are measurable signs of RA and help in identifying biomarkers and therefore diagnosis. Laboratory tests indicate elevated levels of the C-reactive proteins along with an increased erythrocyte sedimentation rate (ESR) and the presence of autoantibodies, for example, rheumatoid factor (RF) and anticitrullinated protein antibodies.[9] Treatment methods for RA that are quite historic include colloidal gold and aspirin, but these treatments are only for relieving the symptoms. However, with the use of steroids, there are some impressive benefits in terms of disease-modifying and rapid improvement in relieving the symptoms, but long-term use of these steroids is typically linked with having serious adverse effects.[10] Disease-modifying antirheumatic drugs have many pharmacological effects, such as the treatment of RA, psoriatic arthritis, and ankylosing spondylitis. They are also used in the treatment of other disorders, for instance, connective tissue diseases such as systemic sclerosis, systemic lupus erythematosus, Sjogren syndrome, inflammatory myositis, vasculitis, uveitis, inflammatory bowel disease, and some types of cancers.[11−13] They also have an increased risk of commonly occurring and serious infections, for instance, bacterial, fungal, and viral infections. Moreover, reactivation of tuberculosis,

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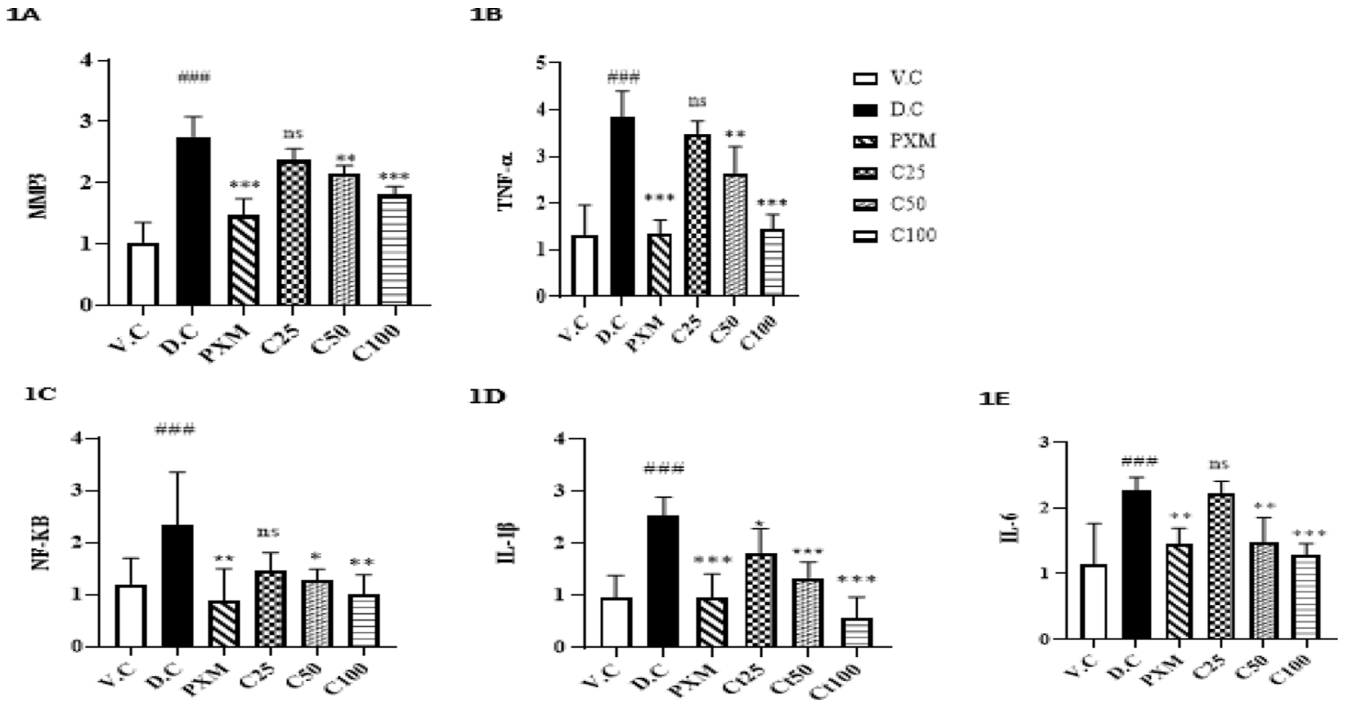

Figure 1. Values of proinflammatory cytokines are depicted as the mean ± SEM ($n = 6$). MMP3 (1A), TNF-α (1B), NF-κB (1C), IL-1β (1D), and IL-6 (1E). * $p < 0.0001$ in comparison to the arthritic control group, * = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$ shows significance where *** reflects highest significance level. #### shows the difference between two control groups, i.e., normal control vs disease control.

herpes zoster, and hepatitis B/C are also reported side effects of DMARDs.[14] Nonsteroidal anti-inflammatory drugs (NSAIDs), such as naproxen and ibuprofen, were also used as treatment methods for RA, but they have the least effects on preventing or reducing the damage caused to the joints. Therefore, these drugs no longer remain the preferred treatment according to the guidelines recently presented.[15] Citronellol (Ct), a monoterpene alcohol, is commonly present in the essential oils of different kinds of aromatic plants, for example, *Cymbopogon citratus*, *Lippia alba*, and *C. winterianus*.[16] Many studies reported pharmacological functions of Ct such as antibacterial, antifungal, antihypertensive, vasodilation, antioxidant, and anti-inflammatory.[17−19] Ct lowers the nociception which in turn minimizes inflammation.[18] Ct significantly downregulates the nuclear factor kappa-B, tumor necrosis factor-α, matrix metallopeptidases-1, interleukin-6, and cyclooxygenase-2, whereas anti-inflammatory cytokine interleukin-10 is significantly upregulated.[16] The aim of the present study was to evaluate the potential antiarthritic effect of Ct, by keeping in view its anti-inflammatory properties, in FCA-induced arthritic rats.

2. MATERIALS AND METHODS

2.1. Chemicals Used. All of the chemicals used were of analytical grade and purchased from Merck (Germany) except Ct (Alfa Aesar, China) and FCA (Sigma-Aldrich).

2.2. Animals Used. A total of 36, Sprague−Dawley female rats were used to carry out an experiment evaluating the antiarthritic activity of Ct. The weights of the rats were between 250 and 350 g. The purchase of these animals was made from the University of Lahore and they were fed standard rat chow under standard conditions of temperature (24 ± 2 °C) and relative humidity (60−70%). Before the experiment began, they were kept in this environment for 7 days. All the protocols and experiments were conducted after obtaining the approval of the Institutional Research Ethics Committee (IREC-2022-47) at the University of Lahore.

2.3. Induction of Arthritis. Arthritis was induced by injecting FCA (0.15 mL) into the subplantar region at day 0 and animals were sacrificed on day 28.[20,21]

2.4. Grouping of Animals. Animals were divided into 6 groups with 6 rats in each group ($n = 6$). Group-1 was the negative control group, where the animals were given distilled water orally from day 8 to day 28. Group-2 was the arthritic control group, where the animals were induced with arthritis by giving FCA (0.15 mL) into the subplantar region at day 0. Groups-3, -4, -5, and -6 were the treatment groups and induced with arthritis at day 0 and treated with piroxicam (10 mg/kg b.w., i.p.; Group-3) and Ct (25, 50, and 100 mg/kg b.w., orally) starting from day 8 to end of the trial on day 28.[22]

2.5. Assessment of mRNA Levels of Pro- and Anti-inflammatory Cytokines. The animals were sacrificed on day 28 and blood samples were drawn for RNA extraction using the TRIzol reagent according to the manufacturer's protocol. The isolated RNA was quantified by using a Nanodrop 1000 spectrophotometer. cDNA synthesis was done using a reverse transcriptase kit (Thermo Scientific, Waltham, USA). The qPCR was done using an SYBR green maxima qPCR kit (Thermo Scientific, Waltham, USA). GAPDH was used as a reference gene. The relative expression was determined using the $2^{-\Delta\Delta CT}$ method. The primers were designed using previously published data.[23]

2.6. Assessment of Arthritic Progression. Arthritic scoring was performed macroscopically for paw inflammation, swelling, and redness. A score of 0 for normal, a score of 1 for minimal, a score of 2 for mild, a score of 3 for moderate, and a score of 4 for severe edema were given on days 7, 14, 21, and 28, respectively.[24]

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2.7. Evaluation of Paw Volume. Arthritis was induced at day 0 and then paw volume (mL) was measured by using a digital water displacement plethysmometer at days 7, 14, 21, and 28.[25]

2.8. Determination of Serum PGE2 Levels. PGE2 levels were measured by using an ELISA kit (Elab Science, E-EL-0034). The optical density was measured by a plate reader (BioTek, ELx-800) at 450 nm wavelength.[26]

2.9. Determination of Blood Biochemical Parameters. For blood biochemical parameters like aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea, creatinine, and RF,[27] commercially available kits were used and measurement was taken using an automated chemistry analyzer (Humalyzer 3500).

2.10. Determination of Hematological Parameters. The blood samples were collected after a cardiac puncture in a vacutainer containing EDTA. The levels of ESR,[27] hemoglobin, red blood cells, white blood cells, and platelet counts[20] were evaluated using an automated hematology analyzer (Sysmex XT-1800i).

2.11. Histopathological Evaluation. On day 28, rats were sacrificed and ankle joints were removed, bisected longitudinally, and immersed in 10% formalin for fixation. The samples were decalcified and implanted in liquid paraffin, segmented, and stained with H&E stain (hematoxylin and eosin). The slides were examined by a single histopathologist for the evaluation of bone erosion, infiltration of cells, and inflammation.[28]

2.12. Radiographic Assessment of Paw Joint. The ankle joints of rats were detached and exposed for radiographic examination to a computerized radiographic system.[29]

2.13. Statistical Analysis. Data were expressed as mean ± standard error of the mean (SEM) and analyzed by using one-way analysis of variance technique. Tukey's post hoc test was run to arbitrate the significant differences among groups using GraphPad Prism (9.5.1). The *P*-value of <0.05 was considered significant.

3. RESULTS

3.1. Effects of Ct on Expressions of Proinflammatory Cytokines. The mRNA expression levels of the proinflammatory cytokines (IL-6, TNF-α, IL-1β, NF-κB, and MMP3) were higher in the disease control in contrast to treatment groups ($p < 0.05$). The doses of Ct (25, 50, and 100 mg/kg) exhibited a significant reduction in the expression levels of all of the measured proinflammatory cytokines, especially at the higher doses (Figure 1).

3.2. Effects of Ct on Expressions of IL-4. The levels of the mRNA for anti-inflammatory cytokine were low in the diseased control group in contrast to the treatment groups ($p < 0.05$). All of the doses of Ct exhibited a significant improvement in the expression levels of IL-4, especially at the higher doses (Figure 2).

3.3. Effects of Ct on Hematological Parameters. The levels of the hematological parameters including the counts of red blood cells and white blood cells, hemoglobin, ESR, and platelets were significantly higher in the arthritic control group in contrast to the treatment groups ($p < 0.05$). All the doses of Ct imparted a significant improvement and restoration of hematological levels to normal values of the control group, especially at the higher doses (Table 1).

3.4. Effects of Ct on Blood Biochemical Parameters. The levels of the blood biochemical parameters including ALT, AST, ALP, RF, urea, and creatinine were significantly increased in the arthritic control group in contrast to the treatment groups ($p < 0.05$). All the doses of Ct significantly improved these parameters. However, the levels of ALT, AST, urea, and creatinine remained unchanged in all of the experimental groups (Table 2).

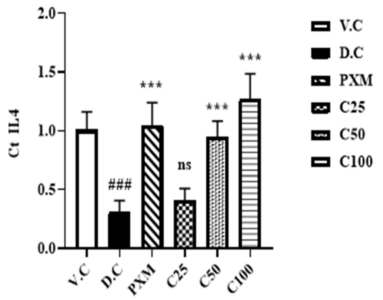

Figure 2. Values of anti-inflammatory cytokine are depicted as mean ± SEM ($n = 6$). * $p < 0.0001$ in comparison to the arthritic control group. + $p < 0.0001$ in comparison to the control group * = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$ shows significance where *** reflects highest significance level. #### shows the difference between two control groups, i.e., normal control vs disease control.

3.5. Effects of Ct on PGE2α Levels. The levels of PGE2α were higher in the arthritic control group in contrast to the treatment groups ($p < 0.05$), except in the 25 mg/kg group (Figure 3).

3.6. Effect of Ct on Arthritic Scoring. Table 3 shows the significantly higher arthritic scores in the CFA induction. The significant reduction in arthritic score is noticed gradually in all the treatment groups as compared to the arthritic control group ($p < 0.0001$).

3.7. Effect of Ct on Paw Edema. The results in Table 4 depict paw edema as compared to the arthritic control group. The treatment with piroxicam and Ct shows a significant reduction in paw edema as compared to the arthritic control group.

3.8. Effect of Ct on Histopathology. The histopathological examination of the rat paws conducted on day 28 illustrated the progression of the disease in the arthritic control group and then suppression of the disease with the treatment over the course of the trial. The findings clearly indicated a marked reduction of RA symptoms such as pannus formation, infiltration, and bone erosion in the arthritic rats induced by FCA. Ct and piroxicam treatment groups displayed a significant decrease in the severity of disease as presented in Figure 4.

3.9. Effect of Ct on Radiograph of Ankle Joint. The X-ray images of the ankle joints at the conclusion of the trial are essential for evaluating the extent and advancement of the disease within the arthritic control group. The images clearly revealed reduced damage in the Ct and piroxicam treatment groups, as depicted in the provided figures (Figure 5)

4. DISCUSSION

Ct is present in the essential oils of several aromatic plants such as *C. citratus*,[30] *Cymbopogon winterianus*,[31] and *Lippa alba*.[32] Several researchers have described certain pharmacological properties of Ct that include vasorelaxant, hypotensive, and anticonvulsant as well as antimicrobial and antispasmodic activities.[17,33,34] Ct also demonstrates antinociceptive and anti-inflammatory activity.[35] In the current study, the CFA-induced

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Table 1. Effects of Different Concentrations of Ct on Hematological Parameters in the Arthritis-Induced Rats[a]

parameters	vehicle control	arthritic control	piroxicam	Ct 25	Ct 50	Ct 100
Hb (g/dL)	13.40 ± 0.67	8.64 ± 0.40###	11.29 ± 0.31**	11.77 ± 0.46***	13.77 ± 0.50***	15.02 ± 0.29***
RBC (106/Ul)	5.19 ± 0.23	1.96 ± 0.22###	4.23 ± 0.27***	3.79 ± 0.22***	5.6 ± 0.19***	5.64 ± 0.25***
WBC (103/Ul)	7.48 ± 0.40	16.70 ± 0.47###	13.81 ± 0.27***	13.96 ± 0.25***	12.22 ± 0.18***	13.63 ± 0.41***
platelets (103/Ul)	772 ± 21	1467 ± 19###	842 ± 36***	809 ± 35***	966 ± 55***	1072 ± 42***
ESR (mm/h)	2.83 ± 0.27	8.40 ± 0.33###	5.79 ± 0.33***	6.46 ± 0.43**	4.04 ± 0.30***	1.76 ± 0.30***

[a]Ct and piroxicam. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ in contrast to the arthritic control group, * = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$ shows significance where *** reflects highest significance level. #### shows the difference between two control groups, i.e., normal control vs disease control.

Table 2. Effects of Different Concentrations of Ct on Blood Biochemical Parameters in Arthritis-Induced Rats[a]

parameters	vehicle control	arthritic control	piroxicam	Ct 25	Ct 50	Ct 100
AST (U/L)	43.50 ± 2.61	47.67 ± 1.35	48.33 ± 2.87	46.83 ± 3.07	51.17 ± 1.77	48.50 ± 2.65
ALT (U/L)	16.75 ± 1.85	17.33 ± 1.60	17.00 ± 1.03	16.83 ± 2.18	17.83 ± 1.77	19.50 ± 1.70
ALP (U/L)	208 ± 6.70	403 ± 9.97	279 ± 5.85***	396 ± 5.09	350 ± 12.52**	321 ± 11.06***
creatinine (mg/dL)	0.48 ± 0.06	0.53 ± 0.04	0.43 ± 0.03	0.40 ± 0.03	0.51 ± 0.04	0.53 ± 0.06
urea (mg/dL)	20.17 ± 1.19	23.00 ± 0.96	22.25 ± 1.06	20.83 ± 1.92	20.50 ± 1.99	23.33 ± 1.33
RF (IU/mL)	6.61 ± 0.28	36.17 ± 1.42	9.167 ± 0.60***	34.83 ± 1.44	22.33 ± 1.54***	13.17 ± 1.16***

[a]*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ in contrast to the arthritic control group.

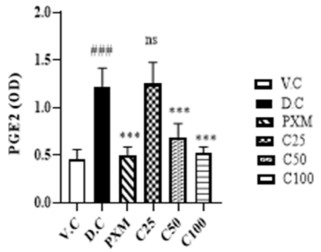

Figure 3. Values are depicted as mean ± SEM ($n = 6$). * $p < 0.0001$ in comparison to the arthritic control group. + $p < 0.0001$ in comparison to the control group, * = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$ shows significance where *** reflects highest significance level. #### shows the difference between two control groups, i.e., normal control vs disease control.

arthritis murine model is chosen due to its resemblances with the arthritic disorders in human beings that are described by bone erosion, cartilage destruction, vascular formation, and synovial hyperplasia.[36] The CFA is composed of heat-killed *Mycobacterium tuberculosis* mixed up with liquid paraffin that can stimulate a cellular immune response that in turn potentiates the production of specific antibodies.[37] CFA triggered an inflammatory response within 3−5 days. The rat paw swelling is an easy, sensitive, and robust procedure to assess the inflammatory response and the drugs' curative efficacy.[38] The inhibition of paw swelling by Ct might be due to the inhibition of neutrophil infiltration, a crucial event of inflammation, like NSAIDs[39] and this is hinting toward its antiarthritic potential. In a previously conducted study on the anti-inflammatory properties of Ct, it was reported that it has the potential to inhibit leukocyte migration and reduce paw edema volume by modulating cyclo-oxygenase (COX)-1 and -2, the enzymes responsible for the biosynthesis of prostaglandins from arachidonic acid.[40] Reducing the cell migration and paw edema is suggestive of Ct's ability to inhibit the synthesis of inflammatory cytokines. These findings further support the results of the current study for reducing paw edema in Ct treatment groups compared to the arthritic group.

A variety of stimuli can activate the NF-κB and include components of bacteria and viruses, growth factors, and cytokines, among many others. Activation of NF-κB in the immune cells is vital to induce the expression of multiple inflammatory and immune cytokines.[41] NF-κB acts as a transcription factor for the activation of interleukin-6, tumor necrosis factor-α, and interleukin-1β. These cytokines are involved in the recruitment and activation of the neutrophils along with the activation of the Th1 response.[42] In our study, the reduced mRNA expression levels in the treatment groups suggested the role of Ct in Th1 inhibition.

IL-6 was initially identified as a secret factor responsible for stimulating immunoglobulin production.[43] Various cells such as T-lymphocytes, monocytes, endothelial cells, and fibroblasts can produce IL-6. RA patients' synovium contains IL-6 which is secreted by the B-cells and synovial fibroblasts.[44] Additionally, IL-6 plays a significant role in upregulating VEGF production, a crucial cytokine involved in the genesis of RA.[45] The inflammation of joints in RA is linked with the migration of immune cells and induction of angiogenesis which further enhances the progression of the disease.[46] Moreover, IL-6

Table 3. Effects of Different Concentrations of Ct on Arthritic Scoring in the Arthritis-Induced Rats[a]

days	arthritic control	piroxicam	Ct 25	Ct 50	Ct 100
7	3.08 ± 0.08	3.08 ± 0.08	3.08 ± 0.08	3.16 ± 0.10	3.16 ± 0.10
14	3.41 ± 0.08	2.25 ± 0.11***	2.83 ± 0.16*	2.67 ± 0.16**	2.75 ± 0.11**
21	3.50 ± 0.12	2.50 ± 0.18***	2.91 ± 0.15*	2.50 ± 0.12***	2.58 ± 0.08***
28	3.58 ± 0.15	2.08 ± 0.08***	2.50 ± 0.12***	2.48 ± 0.01***	2.40 ± 0.06***

[a]* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$ shows significance where *** reflects highest significance level. #### shows the difference between two control groups, i.e., normal control vs disease control.

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Table 4. Effects of Different Concentrations of Ct on Paw Edema (mL) in the Arthritis-Induced Rats[a]

days	arthritic control	piroxicam	Ct 25	Ct 50	Ct 100
7	0.63 ± 0.009	0.54 ± 0.016	0.40 ± 0.009	0.38 ± 0.008	0.37 ± 0.007
14	0.74 ± 0.010	0.63 ± 0.007***	0.65 ± 0.017**	0.62 ± 0.018***	0.61 ± 0.022***
21	0.88 ± 0.006	0.77 ± 0.041**	0.70 ± 0.009***	0.66 ± 0.011***	0.63 ± 0.007***
28	0.95 ± 0.013	0.80 ± 0.016***	0.76 ± 0.018***	0.72 ± 0.019***	0.68 ± 0.020***

[a]* = $p < 0.05$, ** = $p < 0.001$, *** = $p < 0.0001$ shows significance where *** reflects highest significance level. #### shows the difference between two control groups, i.e., normal control vs disease control.

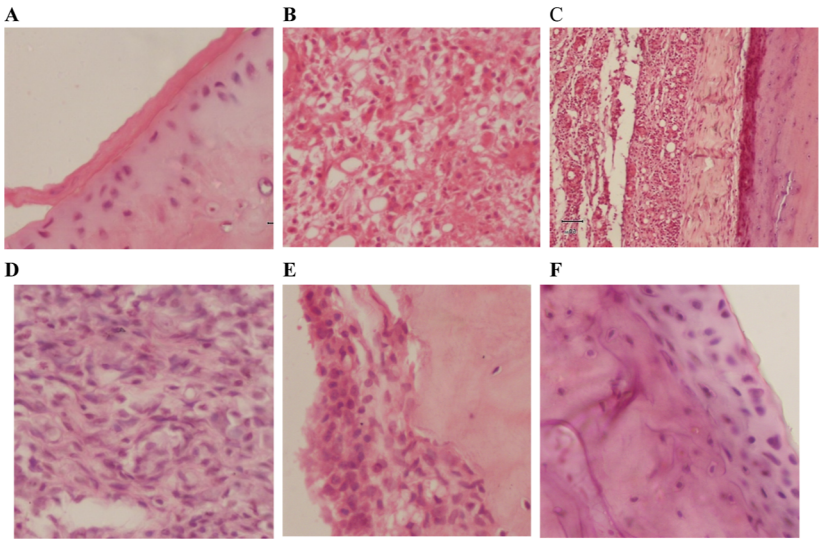

Figure 4. Hematoxylin and eosin staining of the rat's paw. (A) Vehicle control showing normal bone and cartilage, (B) disease control exhibiting infiltration of mononuclear inflammatory cells in the area of dermis, (C) PXM (10 mg/kg) showing moderate pannus formation, (D) Ct (25 mg/kg) presenting fibrosis and infiltration, (E) Ct (50 mg/kg) with cartilage erosion, and (F) Ct (100 mg/kg) with almost normal bone displaying no erosion and infiltration of inflammatory cells. All the pictures' magnification is ×400. (40 × 10).

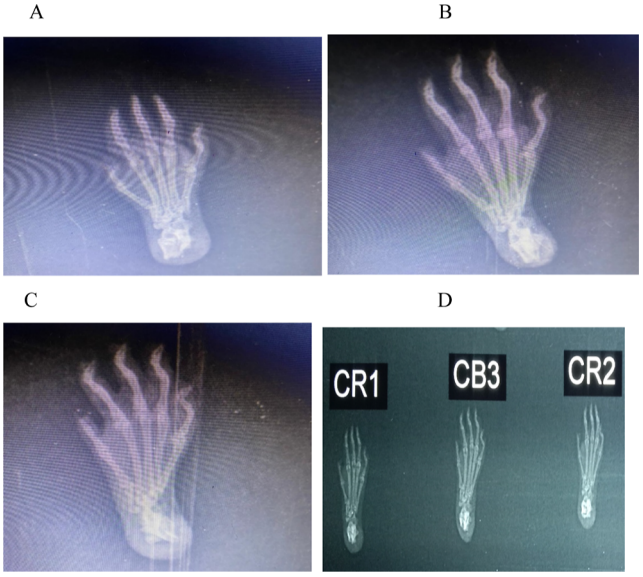

Figure 5. All radiographs of the ankle joints of the treated and untreated rats taken at the end of the trial, where A (vehicle control), B (arthritic control), C (piroxicam), and D (Ct-treated 25, 50, 100) mg/kg, indicated the loss of bones and development of space between the bones at the ankle joints as shown in (B). These damages were either absent or very little in the Ct-treated groups (D) given doses of 25, 50, and 100 mg/kg, respectively.

promotes osteoporosis and joint damage in individuals suffering from RA. TNF-α and IL-1β also stimulate IL-6. Inhibition of IL-6 has shown promising results in preventing osteoclast activity in RA patients.[47] In the present study, Ct had an effective attenuation of IL-6 particularly at higher doses. This attenuation potentially helps in suppressing osteoporosis and suggests that Ct could be a suitable choice for future RA treatment.

TNF-α has a critical role in the pathogenesis of RA, serving as a key regulator. In RA patients, there is an upsurge in the expression of TNF-α. Additionally, transgenic animal studies have demonstrated that overexpressing TNF-α leads to the development of autoimmune arthritis.[48,49] In the pathogenesis of RA, TNF-α signaling exhibits a multidirectional impact. It triggers the activation of proinflammatory cells, including macrophages, synovial fibroblasts, and endothelial cells. These recruited cells, in turn, produce various proinflammatory cytokines (IL-6, IL-1β, and TNF) that take part in the inflammatory process.[49−52] Additionally, TNF-α also regulates the development of Th1 and Th17 cells, influences immunoglobulin production, and plays a role in osteoclast differentiation.[53−55] TNF-α induces the activation of fibroblasts and promotes cartilage destruction by triggering the production of matrix-degrading enzymes.[36] Ct was found to significantly decrease the mRNA expression levels of TNF-α. This suggests that Ct may play a role in suppressing Th1 cells and monocyte-derived macrophages, which are the sources of TNF-α production.

IL-1β, produced by the monocytes and macrophages, plays a pivotal part in the advancement of RA. This is also synthesized by many other cells such as neutrophils, synovial cells,

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endothelial cells, T and B lymphocytes, and natural killer cells. The IL-1β boosted monocyte/macrophage responses that led to the enhancement of inflammation. Additionally, IL-1β prompts the proliferation of fibroblasts and synovium. Moreover, it also triggers the activation of chondrocytes and osteoclasts leading to cartilage destruction and bone resorption, respectively.[56] In RA patients, the presence of IL-1β is highly prominent in the damaged tissues, as well as in the lining of inflamed synovial tissue. Elevated concentrations of IL-1β are also observed in lymphatic drainage of affected joints. Furthermore, IL-1β remains confined within the affected joints, contributing to synovial inflammation in individuals with arthritis.[20] In the current study, Ct effectively minimizes the inflammatory expression of IL-β.

Another proinflammatory cytokine MMP3 is a crucial element in the resorption of bone during RA and leads to the sloughing of the outer osteoid layer, thus allowing the osteoclasts to closely attach to the exposed bone and induce bone damage. MMP3 is a part of the proteinase or component of the extracellular matrix of bone and cartilage families such as RANK/OPG.[57] It is involved in pannus invasion and cartilage degradation.[58] These effects trigger the damage to the connective tissue via activation of pro-collagenase that directly affects the extracellular matrix components.[59] The role of MMP3 in RA development is indicated by the presence of this protein at higher levels in the inflamed synovial fluid.[60] Many research models reported the systemic presence of this protein and designated it as a laboratory biomarker of RA.[60−65] Ct significantly downregulated all of the above-stated proinflammatory cytokines in our study compared with the arthritic control group. This is suggestive of a Ct role as an antiarthritic agent.

IL-4, recognized as an anti-inflammatory cytokine, is responsible for inhibiting the activation of macrophages.[66] Furthermore, IL-4 is involved in the negative regulation of NF-κB, which is a key transcription factor in inflammation. IL-4's inhibitory effects are also extended to the osteoclasts, which play a critical role in cartilage destruction and bone erosion. Recombinant IL-4 therapy has shown promising results in RA patients by suppressing cytokine production and inflammatory processes.[67] In our study, Ct significantly augmented the expression of IL-4 in all of the treatment groups.

Hematological parameters including hemoglobin and red blood cell count declined in the arthritic control group, resulting in anemia. It might result from reduced plasma iron, as influenced by IL-1 or abnormal iron loading in the reticuloendothelial system and synovial tissue. Other factors may include premature destruction of RBCs, reduced erythropoietin levels, and bone marrow failure.[68−70]

The surge in the platelets and white blood cell counts was observed in the arthritic rats. During the process of inflammation, thrombocytosis occurs which leads to thrombosis and later develops many cardiovascular problems.[71,72] Inflammation causes disturbance in the physiological anticoagulant mechanisms due to which the coagulation is induced with IL-6 and TNF-α.[73] The confirmation of the role of the platelets in inflammatory rheumatoid was observed during flow cytometric analyses that detected an extensive number of platelet microparticles in RA synovial fluid.[74] Platelets/leucocytes association in RA has been reported in several studies. Platelets themselves have no migratory properties. Platelets stick to leukocytes in blood circulation and augment leukocyte accumulation on the endothelial wall to facilitate the transport of platelets to the joint space.[75,76] Synovitis is an extreme cellular infiltration in the synovium and synovial membrane in RA.[77] Therefore, RA patients have higher counts of macrophages, monocytes, plasmocytes, and dendritic cells.[78] The levels of all of the hematological parameters were improved in the treatment groups given piroxicam and Ct. The elevated ESR levels in the arthritic control group indicated an inflammatory response[29] and its levels were remarkably improved in the treatment groups given piroxicam and Ct.

The blood biochemical parameters including urea, creatinine, ALT, and AST remained unaffected in all the experimental groups[79] and suggested that Ct is safe at the studied doses.[80] The elevated ALP levels in the arthritic control group might be due to increased bone erosion and activation of lysosomal enzymes.[81] The increased levels of ALP are responsible for bone destruction and mineralization.[82] The significant reduction in the levels of ALP in the treatment groups might suggest the role of Ct in reducing bone erosion and improving the stability of the lysosomal membranes.

RF, an autoantibody, is involved in the activation and recruitment of inflammatory responses and inflammatory cells, respectively.[83] RF is formed against the Fc portion of autoantibody and starts the immune reactions leading to the progression of RA. The decline in the serum RF levels in the treatment groups given piroxicam and Ct reflects their efficacy in reducing the generation of RF.

The histopathology of the hind paws depicts the reduction in bone erosion and immune cell infiltration in the treatment groups receiving higher doses of Ct. Moreover, an X-ray examination of the Ct-treated groups showed a reduction in the rate of cartilage destruction.

In the lipoxygenase pathway, 5-lipoxygenase produces leukotrienes (LTs) like LTC4 and LTD4 that take part in inflammation mediation and induce edema by increasing the microvascular permeability. This indicates the involvement of these compounds in the pathophysiology of various inflammatory processes. PGE2 contributes to the progression of the disease by interacting with the EP4 receptor.[84] The PGE2 production is obvious in rheumatoid synovium by the metabolism of arachidonic acid by the COX-2 pathway.[85,86] Increased levels of PGE2 induce vasodilation, fluid extravasation, and bone and cartilage erosions.[87] In the current study, PGE2 levels declined in the treatment groups, in contrast to the arthritic control group. It is assumed that Ct might have suppressed prostaglandin synthesis, followed by arachidonic acid metabolism inhibition.

5. CONCLUSIONS

Ct is a natural acyclic compound and is found in many citrus fruits and their essential oils. In our study, Ct reduced the signs and symptoms of inflammation, such as paw edema and arthritic scoring. Furthermore, it upregulated mRNA expression levels of IL-4 (anti-inflammatory cytokine) and downregulated the mRNA levels of IL-6, MMP3, IL-1β, TNF-α, and NF-κB (proinflammatory cytokines) along with a reduction in the levels of PGE2. These effects, altogether, led to a reduction in arthritis symptoms in Ct-treated rats. This study provides valuable insights into the potential therapeutic benefits of Ct in managing arthritis, which suggests that Ct may be a safe option for treating arthritis. The main limitation of the study is that it is conducted on CFA-induced arthritis, which partially mimics RA; therefore, the effects of Ct should also be evaluated on a genetically induced arthritis model.

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Optimizing the effective and safe doses of Ct for human use and the development of targeted therapies based on the mechanism of Ct for RA are some of the practical implications of this study.

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Notes

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