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Clinical, Histological and Biochemical Aspects Effect of Gotu Kola (*Centella Asiatica* L.) on Experimental Arthritis

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Rheumatoid Arthritis (RA), as a significant chronic disease, requires the ongoing search for new drugs or supportive agents capable of minimizing current therapy dosages. Therefore, this study aimed to evaluate the therapeutic effects of Gotu Kola (*Centella asiatica* L.; CA) on RA using a Freund's Complete Adjuvant (FCA)-induced rat model. Sixty female *Wistar* rats were divided into six groups: FCA, FCA+CA30, FCA+CA100, FCA+DXM, CA+100, and Control. Arthritis was induced in the first four groups using FCA. For 28 days, oral CA was administered (30 mg/kg or 100 mg/kg) or dexamethasone (DXM) was given intramuscularly (0.1 mg/kg). The study evaluated clinical parameters, total antioxidant/oxidant status (TAS/TOS), and levels of RANKL and OPG. The FCA+DXM and FCA+CA100 groups had the lowest arthritis index scores (P<0.05). Histological scores for the left paw samples were significantly different between the FCA+CA30 group and the FCA+DXM and FCA+CA100 groups (P<0.001). No significant differences were observed in the plasma levels of TAS/TOS, RANKL, and OPG among the groups (P>0.05). In conclusion, CA (100 mg/kg) and DXM demonstrated similar efficacy in FCA-induced model, reducing arthritis severity and reversing histopathological changes. CA could be a useful adjuvant for reducing standard doses of drugs which have some side effects.

Key words: Animal model, Rheumatoid arthritis, Antioxidant status, RANKL, OPG

Introduction

Rheumatoid Arthritis (RA) is a chronic, systemic autoimmune disease marked by symmetrical joint inflammation and synovial hyperplasia[1-3]. This inflammatory connective tissue disease affects approximately 0.5-1% of the adult population worldwide, being two to three times more prevalent in females than in males[4]. RA causes functional impairments that significantly reduce patients' activities of daily living and overall quality of life[5].

The purpose of the RA experimental model is to investigate arthritis-related inflammation and to assist the development of anti-arthritis drugs. Researchers frequently utilize the adjuvant-induced arthritis experimental model when conducting preclinical evaluations for RA. A widely accepted model of chronic inflammation in experimental RA studies is the rat model of Freund's Complete Adjuvant (FCA)-induced arthritis[6,7]. The development of polyarthritis, marked by inflammation in the ankle, tarsal, and interphalangeal joints, typically occurs 10-12 days after the administration of FCA[8,9].

Gotu Kola (*Centella asiatica* L.; CA) is a widely recognized medicinal plant with a long-established history of use in traditional medicine for a variety of conditions, including neurological, dermatological, immunological, and metabolic disorders. The bioactivity of CA extracts is mainly attributed to asiaticoside, madecassoside, and triterpenoids. CA has demonstrated positive effects on antioxidant activity, anti-inflammatory responses, and wound healing, potentially through increased microcirculation[10,11]. Madecassoside topical formulations are widely recognized for their healing power on connective tissue[12,13].

This study was conducted to experimentally evaluate the anti-inflammatory action of CA in the context of RA. In pursuit of this objective, clinical parameters including weight change, arthritis index scores, histopathologic scores, total antioxidant status/total oxidant status (TAS/TOS), and Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) and Osteoprotegerin (OPG) levels were

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assessed in the study. The present study will contribute to understanding the dual role of CA, as a dietary supplement and as a therapy.

Material and Method

Study design

Following approval from the Aydin Adnan Menderes University Animal Experiments Local Ethics Committee (ADU-HADYEK, approval number: 64583101/2023/96), this study was carried out at the Animal Experiments Laboratory, Faculty of Medicine, Aydin Adnan Menderes University. The study utilized 60 female *Wistar* rats (2-3 months old, weighing 200-230 grams) obtained from the Experimental Animal Center of Aydin Adnan Menderes University.

Following weighing and marking, the rats were randomly divided into six groups of ten. The RA model was established by administering a single subcutaneous injection of 0.1 mL FCA (Sigma-Aldrich Chemical Co., Cat. No: F5881, Interlab, Istanbul, Turkey) into the left hind paw's palmar surface in rats[14]. For the Control group, a 0.1 mL saline injection was given in the same location. The CA+100 groups received a 0.1 mL saline injection in the left hind paw, then 100 mg/kg CA daily by oral gavage for 28 days. This group was included to evaluate the safety of the highest administered dose in healthy animals. The selected CA doses (30 and 100 mg/kg) were based on previous experimental studies investigating the anti-arthritic effects of CA[15,16].

Following a 0.1 mL FCA injection in the left hind paw, the FCA+DXM group was treated with daily intramuscular injections of 0.1 mg/kg dexamethasone (DXM)[14]. This group served as the positive control representing the standard corticosteroid treatment for RA. The FCA+CA30 and FCA+CA100 groups received oral CA at doses of 30 mg/kg and 100 mg/kg, respectively, once daily for 28 days. CA was administered as a commercially available standardized dietary supplement (Gotu Kola Aerial Extract, Solgar®). According to the manufacturer's specifications, each capsule contained 100 mg of standardized *C. asiatica* aerial extract, standardized to 6% triterpene glycosides (6 mg per capsule). A commercially available formulation was selected because it represents the form commonly consumed by humans as a dietary supplement, thereby enhancing the translational relevance of the study. Immediately before administration, the capsules were opened, and the powdered extract was suspended in tap water. Fresh suspensions were prepared daily, and individual doses (30 or 100 mg/kg) were calculated according to each rat's current body weight before oral gavage.

Clinical assessment

Arthritis severity was evaluated weekly throughout the experimental period based on assessments of body weight, hind paw swelling and an arthritis index[17]. On days 7, 14, 21 and 28 of the experiment, hind paws diameters were measured with a digital caliper, the arthritis index was scored, and the body weights of all rats were recorded. The arthritis index was scored for each paw on a scale of 0 to 4, as follows: 0: no swelling or hyperemia; 1: slight swelling and/or hyperemia; 2: low to moderate edema; 3: pronounced edema with limited joint usage; 4: excess edema with joint rigidity.

Histopathologic assessment

Hind paws of sacrificed rats were collected by amputation above the ankle joint and fixed in 10% formalin for at least one week. The paws underwent decalcification in a solution containing hydrochloric acid and 0.1 M ethylenediaminetetraacetic acid (EDTA)[18]. Subsequent to decalcification, the ankle joints were transected longitudinally into two equal portions, embedded in paraffin, sliced into 5 µm-thick sections, and stained with hematoxylin and eosin (H&E). The following skeletal sites underwent histological evaluations: the tibio-talar, talus-calcaneal, midfoot, and midfinger joints. A blinded specialist pathologist examined and scored the stained sections for inflammatory infiltrate, synovial hyperplasia, cartilage and bone erosions. Each histopathological parameter was scored as 0 (absent), 1 (mild), 2 (moderate), and 3 (severe)[18].

Biochemical assessment

At the end of the experiment (29th day), blood samples were taken from rats under ketamine (50 mg/kg) and xylazine (5 mg/kg) anesthesia by cardiac puncture, centrifuged (10 minutes at 1000 x g), and the separated serum were stored at -20°C for measurement of biochemical analyses. Commercially available kits for Total Oxidant Status (TOS, Rel Assay, Gaziantep, Turkey; sensitivity <10 pg/ml)[19], Total Antioxidant Status (TAS, Rel Assay, Gaziantep, Turkey; sensitivity <10 pg/ml)[20], Receptor Activator Nuclear Kappa B Ligand (RANKL, Rat, Elisa, 96

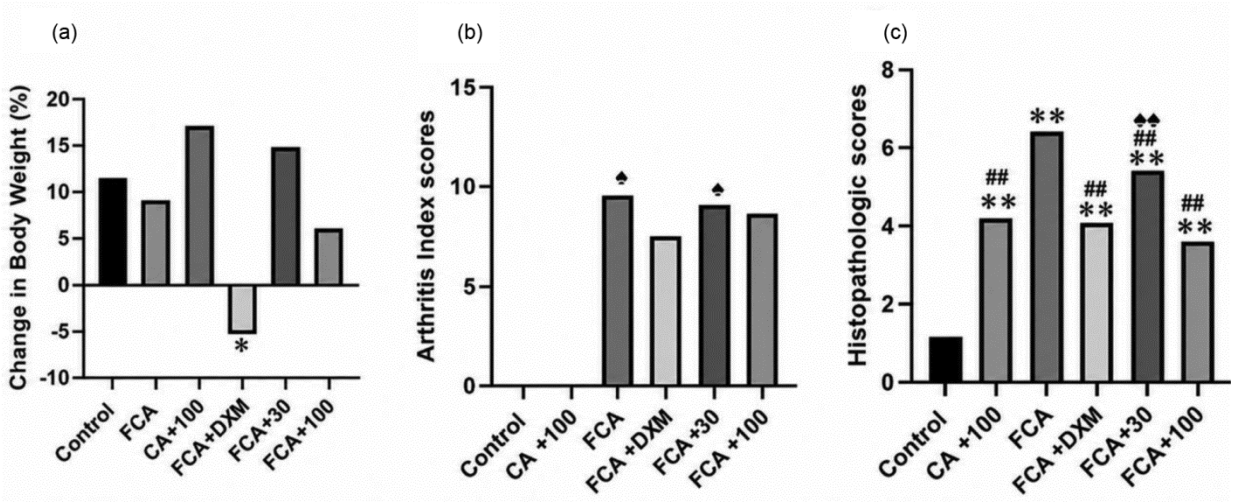

Fig. 1 — The effect of *Centella asiatica* (CA) (30/100 mg/kg) and dexamethasone (DXM) on Freund's Complete Adjuvant (FCA)-induced rheumatoid arthritis (RA). **(a)** Body weight changes (%). **(b)** Arthritis index scores. **(c)** Histopathologic Scores. The data are expressed as means ± SD, **P*<0.05 and ***P*<0.001 vs. Control group; $^{\#}$*P*<0.05 and $^{\#\#}$*P*<0.001 vs. FCA group; $^{\spadesuit}$*P*<0.05 and $^{\spadesuit\spadesuit}$*P*<0.001 vs. FCA+DXM group, n=10.

Test, BT-Lab, Zhejiang Province, China) and Osteoprotegerin (OPG, Rat, Elisa, 96 Test, BT-Lab, Zhejiang Province, China) were used according to the instructions of the manufacturer. Plates were read at 450 nm wave length by a spectrophotometer (MultiscanGo, Thermo Fisher Scientific Inc, USA).

Statistical Analysis

To compare the different groups, a One-Way ANOVA was used to perform statistical analysis on each independent variable. Descriptive analysis results were found for all parameters associated with each of the groups. Levene's test was used to assess the homogeneity of variance. Based on the outcomes of Levene's test for homogeneity of variance, the Bonferroni correction was applied to data that met the equal variance assumption, while Tamhane's test was used for data that did not. Data are presented as mean ± standard error of the mean, and a *P*-value of less than 0.05 was considered statistically significant (*P*<0.05).

Results

Changing of body weight

The impact of CA on energy metabolism was investigated by recording the body weight of all rats on a weekly basis. Weekly recordings of rat body weights were used to investigate the effect of CA on energy metabolism. The FCA+DXM group exhibited a tendency towards decreased body weight (Fig 1a). Statistical analysis revealed that the percentage change in body weight from day 0 to day 28 was significantly lower in the FCA+DXM group compared to the other groups (*P*<0.05). However, the data showed no significant difference in weekly body weight changes between the CA treatment groups and the Control group (*P*>0.05).

Arthritis index scores

Among the FCA-applied groups, the FCA+DXM group had the lowest arthritis index score (sum of scores for FCA-injected carpal and tarsal joints). Arthritis index scores of the CA+100 groups and the Control group were zero. Arthritis index scores were significantly different between the FCA group (9.6±1.07) and the FCA+DXM group (7.5±0.70) (*P*<0.05). The scores of the FCA+CA100 group (8.7±0.82) did not show a significant difference compared to the FCA+DXM group (*P*>0.05), but they were significantly different from the FCA+CA30 group (9.1±0.56) (*P*<0.05). This indicates that the higher dose of CA was more effective in reducing arthritis index scores than the lower dose (Fig 1b).

Paw diameter measurements

Left paw diameters of the FCA-injected groups increased significantly compared to the Control group (*P*<0.05) (Table 1). On day 7 of the RA model, the left paw diameters were statistically significantly decreased in the FCA+DXM and FCA+CA100 groups compared to the FCA group (*P*<0.05). There was no significant difference in left paw diameter measurements found between the FCA+DXM group

Table 1 — Measurement results of paw diameters (mm)

Groups	0. day Mean±SD	7. day Mean±SD	14. day Mean±SD	21. day Mean±SD	28. day Mean±SD
Control Left	6.38 ± 0.54	6.50 ± 0.29	6.49 ± 0.15	6.22 ± 0.39	6.12 ± 0.31
Right	6.41 ± 0.24	6.55 ± 0.20	6.27 ± 0.43	6.25 ± 0.23	6.05 ± 0.47
CA+100					
Left	6.11 ± 0.26	6.51 ± 0.33	6.55 ± 0.28	6.75 ± 0.36	6.70 ± 0.45
Right	6.08 ± 0.17	6.38±0.17	6.50 ± 0.35	6.66 ± 0.48	6.72 ± 0.32
FCA					
Left	6.37 ± 0.24	8.41 ± 0.49*	7.62 ± 0.54*	7.56 ± 0.35*	7.48 ± 0.44*
Right	6.26 ± 0.28	6.45 ± 0.40	6.27 ± 0.38	6.11±0.30	6.27 ± 0.35
FCA+DXM					
Left	6.09 ± 0.16	7.52 ± 0.54*#	7.12 ± 0.56	7.11 ± 0.43*	7.19 ± 0.39*
Right	6.08 ± 0.22	6.38 ± 0.34	6.29 ± 0.36	6.32 ± 0.47	6.47 ± 0.36
FCA+CA30					
Left	6.19 ± 0.14	7.73 ± 0.51*	7.75 ± 0.63*	7.72 ± 0.63*	7.81 ± 0.53*
Right	6.16 ± 0.11	6.38 ± 0.17	6.71±0.40	6.62 ± 0.33	6.75 ± 0.43*
FCA+CA100					
Left	6.17 ± 0.26	7.56 ± 0.53*#	7.69 ± 0.71*	7.56 ± 0.54*	7.56 ± 0.34*
Right	6.12 ± 0.19	6.45 ± 0.44	6.71 ± 0.24	6.71 ± 0.23	6.67 ± 0.31

Effect of *Centella asiatica* (CA) (30/100 mg/kg) and dexamethasone (DXM) on hind paw diameter (mm) in Freund's complete adjuvant-induced rheumatoid arthritis (RA). The data are expressed as means ± SD, **P*<0.05 vs. Control group; #*P*<0.05 vs. FCA group.

and the FCA+CA30 and FCA+CA100 groups (*P*>0.05)

Histopathological assessment

In the histopathological evaluation, inflammatory infiltration, synovial hyperplasia, cartilage and bone erosions were each scored (0-3) for each sample, and a total score was obtained. Histopathological examination of rat paws revealed inflammation in all groups except the Control group (Fig 1c). For left paw samples, the FCA+DXM group showed a statistically significant difference compared to the FCA+CA30 group (*P*<0.001), but not compared to the FCA+CA100 group (*P*>0.05). The FCA+CA100 group demonstrated the most favorable histopathological recovery (Fig 2). Compared to the FCA+DXM group, right paw histological scores were significantly higher in the FCA+CA30 group and significantly lower in the FCA+CA100 group (*P*<0.001).

Biochemical findings

There were no statistically significant differences in plasma TAS and TOS levels among the groups (*P*>0.05) (Table 2). There were no statistically significant differences in plasma RANKL and OPG levels among the groups (*P*>0.05) (Table 2).

Discussion

RA, a chronic systemic inflammatory connective tissue disease, typically involves inflammation of various joints, which over time leads to clinical symptoms such as pain, swelling, stiffness, and deformity. Current RA treatments focus on reducing clinical symptoms using disease-modifying anti-rheumatic drugs (DMARDs), nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, and biological agents[21,22]. Traditional pharmaceutical treatments for chronic diseases, including RA, are associated with adverse effects and high cost. Several factors are contributing to the increasing interest in herbal supplements. These include a greater public awareness of drug side effects, the belief in the safety and widespread availability of natural ingredients, their lower cost, cultural acceptance, and the escalating prices of traditional medicine[23-25]. Research has demonstrated that madecassoside, a component of CA a widely used herbal supplement, possesses anti-inflammatory and antioxidant properties[25,26]. As such, it is incorporated into various pharmaceutical formulations designed for the treatment of localized damage to the connective tissue[27-32]. Its effects on systemic well-being and the therapeutic benefits of CA (commercially available in capsule, tablet, or standard powder form) have made it a widely used herbal supplement[33-35]. However, information regarding its effects related to this use is still quite limited.

Current RA treatments often have side effects, creating a continuous need for better-tolerated medications. This need drives researchers to develop safer pharmacological agents. Therefore, in this study, the effect of CA treatment on clinical, histological,

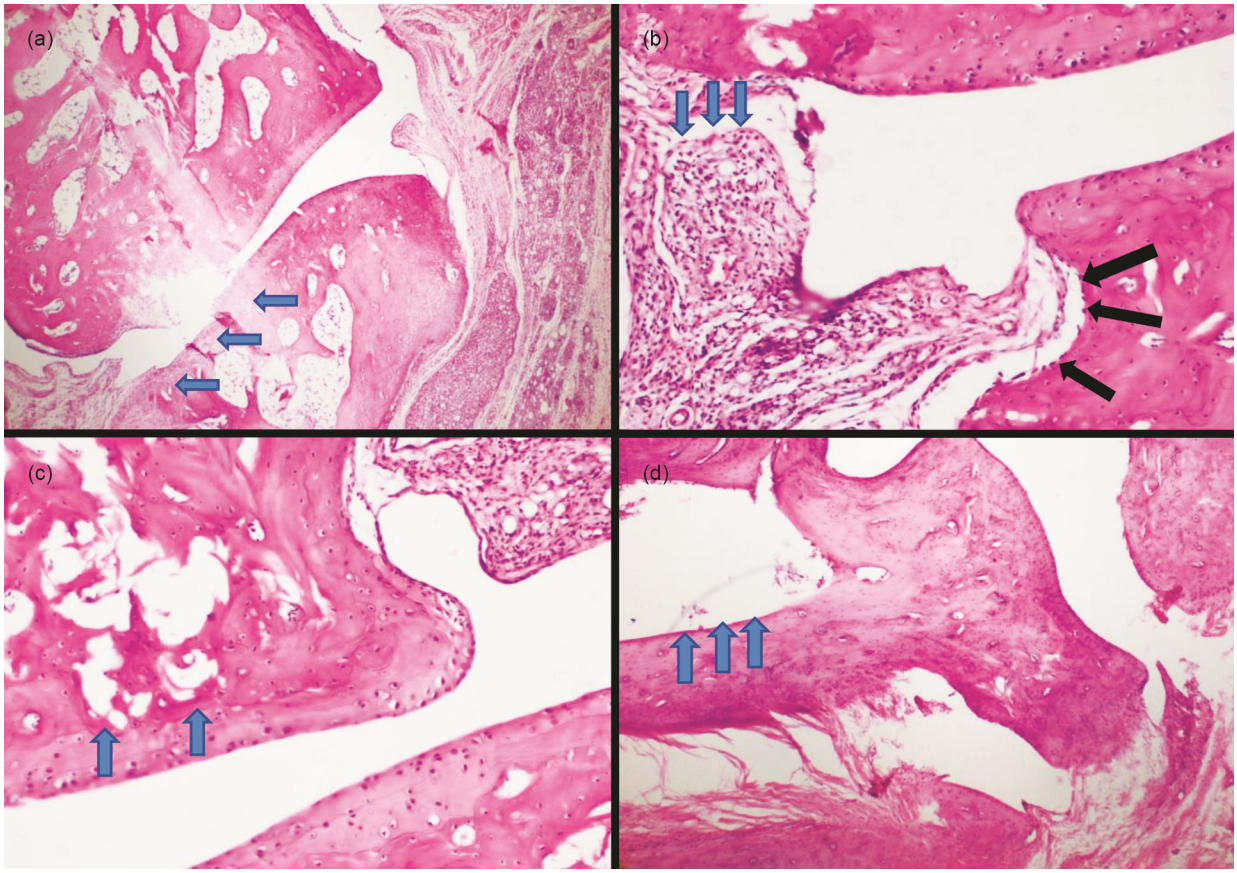

Fig. 2 — The effect of *Centella asiatica* (CA) (30/100 mg/kg) and dexamethasone (DXM) on Freund's Complete Adjuvant (FCA)-induced rheumatoid arthritis (RA). Hematoxylin & Eosin (H&E) staining was used to assess the histopathological changes in the left ankle joints of all groups. **(a)** FCA + CA100 group: Regenerated joint structures exhibiting mild signs of cartilage degeneration (original magnification 10 ×). **(b)** FCA group: Advanced bone resorption (black arrow) characterized by near-complete loss of cortical bone and the presence of numerous osteoclasts, as well as pannus formation (blue arrow) (original magnification 20 ×). **(c)** FCA + DXM group: Small resorption areas observed in the trabecular region of the distal tibia. (original magnification 20 ×). **(d)** FCA + CA30 group: Prominent resorption in the medullary bone without full-thickness defects in the cortical bone. (original magnification 10 ×).

Table 2 — Biochemical analysis results

	TAS (mmol Trolox Eq/L)	TOS (μmol H_2O_2 Eq/L)	RANKL (pg/mL)	OPG (ng/mL)
Control	1.15 ± 0.11	20.78 ± 12.72	27.05 ± 10.77	863.30 ± 485.16
CA+100	1.16 ± 0.14	27.12 ± 8.50	22.98 ± 6.92	647.09 ± 376.48
FCA	1.21 ± 0.11	21.25 ± 12.27	23.29 ± 16.98	570.34 ± 196.41
FCA+DXM	1.15 ± 0.24	42.07 ± 26.98	17.98 ± 7.02	513.54 ± 263.47
FCA+CA30	1.27 ± 0.15	25.72 ± 14.22	26.09 ± 14.41	793.97 ± 487.09
FCA+CA100	1.31 ± 0.17	46.00 ± 23.93	17.21 ± 10.02	427.14 ± 126.79

Effect of *Centella asiatica* (CA) (30/100 mg/kg) and dexamethasone (DXM) on serum total antioxidant status/total oxidant status (TAS/TOS), receptor activator of nuclear factor kappa-B ligand (RANKL), and osteoprotegerin (OPG) levels in Freund's complete adjuvant (FCA)-induced rheumatoid arthritis (RA). The data are expressed as means ± SD.

and biochemical parameters was evaluated using an FCA-induced RA rat model. As reported in the literature review, CA was orally administered at low dose (30 mg/kg) and high dose (100 mg/kg) to explore its effects on arthritis[15,16].

In the present study, no significant difference in weight change was observed among the low-dose CA, high-dose CA treatment groups, and the Control group. Similarly, a study examining the effectiveness of CA extract (ECa 233) on chronic pain found no significant difference in body weight after 28 days of treatment across the sham, control, and low- and high-dose CA (30 mg/kg and 100 mg/kg) groups[16]. This finding indicated that neither the treatment nor the

induced condition significantly interfered with the animals' feeding or general health. Additionally, another investigation noted that oral administration of 30 mg/kg CA in arthritic rats demonstrated a therapeutic effect and was also able to prevent disease-related weight loss[36]. Consequently, these findings suggest that CA administration did not significantly alter body weight change or energy metabolism, thereby indicating the maintenance of systemic stability, and holds the potential to attenuate inflammation-induced weight loss.

The current study revealed that the FCA+DXM group exhibited a statistically significant reduction in the percentage of body weight change compared to the other groups. This finding supports the tendency of DXM treatment to induce weight loss in rats. In line with this, results of the experimental study investigating the effects of Madecassoside (Madec) and DXM on Adjuvant-Induced Arthritis revealed that while body weight loss was significantly reduced in the Madec-treated group, DXM failed to alleviate this weight loss[37]. Corticosteroids, as the gold standard treatment, may have prevented weight gain in the FCA+DXM group, likely resulting from the attenuation of the severity of inflammation. Furthermore, the weight loss observed in the DXM-treated group may be attributable to its potent anti-inflammatory effects, which could have led to improved rat motility and increased energy expenditure.

In our FCA-induced arthritis model, CA treatment at a dose of 100 mg/kg demonstrated efficacy comparable to that of DXM, a standard treatment. This was evident in the reduction of arthritis severity, measured by paw diameter and arthritis index scores, and the alleviation of histopathological findings. Specifically, both the FCA+DXM and FCA+CA100 groups showed a significant decrease in paw diameter measurements after 7 days of administration compared to the FCA control group. This suggests that in the early stages of RA, a high dose of CA exhibits an effect similar to DXM in reducing joint inflammation-induced edema in acute term. Wei-Guang *et al.*[37], confirmed that both Madec, an active ingredient of CA, and DXM treatments significantly reduced arthritis scores and paw volumes, and they noted that Madec demonstrated a significant therapeutic effect in reversing histological lesions.

Histopathological evaluations in the FCA-induced arthritis model clearly demonstrated characteristic features of RA, including synovial hyperplasia, inflammatory cell infiltration, and cartilage and bone erosion[37]. Experimental studies in RA rodent models generally agree that CA ameliorates joint pathology, improving histopathology and significantly attenuating clinical signs (paw edema and arthritis index scores), supporting its therapeutic potential[36-39].

Importantly, these pathological alterations were markedly alleviated in both the FCA+CA100 and FCA+DXM groups, demonstrating the structural preservation effect of CA. Our findings suggest that a high dose of CA (100 mg/kg) exerts anti-inflammatory effects by preserving joint tissue integrity. In clinical practice, better functional outcomes and a slower rate of structural damage are closely associated with histopathological improvement, such as attenuated synovial hyperplasia and osteoclastic erosion. Recent European Alliance of Associations for Rheumatology (EULAR) updates confirm that suppressing inflammation prevents radiographic progression and preserves long-term function[1,21].

In contrast, only limited improvement was observed in the FCA+CA30 group, suggesting a dose-dependent therapeutic response. Consistent with our findings, Sharma *et al.*[40], reported that a methanolic fraction of CA attenuated synovial inflammation and protected cartilage architecture in a collagen-induced arthritis model. Similarly, in a type II collagen-induced arthritis model, madecassoside suppressed the NF-κB/MMP-13 axis, significantly reducing proinflammatory cytokines and joint damage at the histological level[38,39]. The effective suppression of synovial inflammation and promotion of histopathological recovery by high-dose CA treatment in this study is consistent with the established wound-healing properties of madecassoside. Furthermore, the beneficial effects of CA have been attributed to its modulatory role on NF-κB signaling pathways across various pathologies, including other inflammation models, diabetic wound healing, and the dextran sulfate sodium (DSS)-induced ulcerative colitis model[41,42].

The OPG and RANKL system is recognized as a crucial signaling pathway that regulates bone tissue metabolism. This system is a key pathological determinant of the bone erosion observed in RA[43]. Previous studies have confirmed its activity using limited models of FCA-induced arthritis; however, no literature to date has evaluated the role of CA on this

specific pathway. Various methodologies have been employed to study this system. Samarpita *et al.*[44], for instance, utilized Western blot analysis to quantify RANKL and OPG in knee synovial tissue collected on the 21st day of FCA-induced arthritis. Similarly, Wu *et al.*[43] detected both RANKL and OPG protein and mRNA expression levels using Western blotting and qRT-PCR, respectively, within pathogenetic sites, also collected on the 21st day of FCA-induced arthritis.

We did not observe evidence for CA's modulatory effect on the NF-κB signaling pathway, given the lack of significant change in the measured RANKL/OPG ratio. Despite clinical and histological improvements, no significant changes were observed in systemic biochemical parameters such as TAS/TOS and RANKL/OPG at week 4 (day 28). This result leads to several potential interpretations. Firstly, it suggests that CA's therapeutic effects may be primarily observed in the acute phase of the model, rather than the chronic phase of arthritis. Secondly, this may indicate either that the experimental FCA model did not sustain a widespread systemic inflammatory response by day 28 or that the anti-inflammatory effects of CA are primarily localized and not systemic. Thirdly, these findings suggest that CA's mechanism of action is not solely dependent on its antioxidant power.

A limitation of the current study is its sole reliance on measuring total oxidant and antioxidant capacity; a more comprehensive understanding of the underlying mechanisms would necessitate detailed analyses of individual oxidative/antioxidative species and a broader panel of cytokine measurements. The administered CA doses did not induce any detectable toxicity throughout the study, confirming their safety within the experimental parameters. Furthermore, by including a comprehensive evaluation of clinical, histological, and systemic biochemical markers, this research makes a valuable contribution to the limited literature examining the therapeutic effects of CA on RA.

Conclusion

Results from the present study demonstrate that in this dose and route CA is a safe supplement on aforementioned parameters and has a remarkable anti-arthritic effects, comparable to DXM, the current standard treatment for RA. In the experimental RA model, a high dose (100 mg/kg) of CA protected the joint against inflammatory effects and cartilage degeneration, showing no significant side effects (toxicity). This approach may help decrease the requirement for corticosteroids and DMARDs in patients, or serve as a supportive therapy for those who have developed side effects to standard treatments. Given these promising preclinical findings, clinical studies are warranted as a next step to determine the efficacy and safety profile of CA in RA.

Ethical statement

This study was approved by the Aydin Adnan Menderes University Animal Experiments Local Ethics Committee (Approval number: 64583101/2023/96).

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Conflict of interest

The authors declare that they have no competing interests.

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