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ORIGINAL ARTICLE

Anti-arthritic Effect of Neferine on Adjuvant-Induced Experimental Arthritis in Rats via Nuclear Factor Kappa B Signaling Pathway

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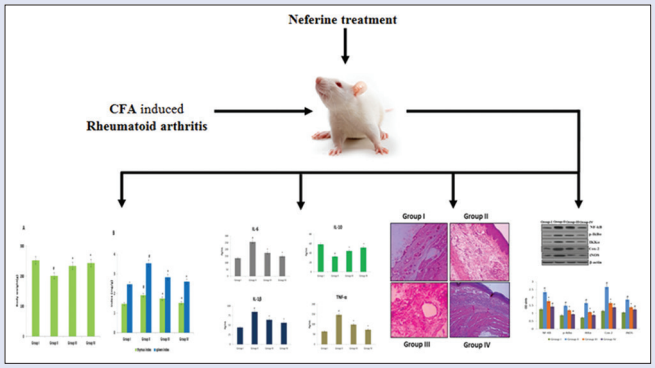
ABSTRACT

Background: Inflammation plays an important role in the progression of arthritis. The imbalance between pro-inflammatory and anti-inflammatory mediators regulates the induction and progression of arthritis. Nuclear factor kappa B (NF-κB) protein is ubiquitously present in all the cells. It controls the activation of immune system and regulates the inflammatory responses. Therefore, targeting NF-κB signaling pathway may be an effective strategy in treating arthritis. Neferine, a bisbenzylisoquinoline alkaloid, is present in the seeds of *Nelumbo nucifera*. **Materials and Methods:** In this study, arthritis was induced in rats with complete Freud's adjuvant (CFA, Group 2) and treated with neferine (Group 3) and diclofenac sodium (Group 4), respectively. The impact of neferine on arthritis was assessed by measuring the weight of organ, arthritis score index, hematological indices, and cytokines levels. **Results:** Hepatic enzymes were measured to assess the toxicity of neferine. The oxidative stress induced by CFA and the antioxidant property of neferine were estimated with biochemical assay, and their impact on the synovial tissue was confirmed with hematoxylin and eosin staining. To confirm the anti-inflammatory activity of neferine, the inflamed synovial tissue of normal and investigational animals was inspected through immunoblotting of NF-κB signaling proteins. **Conclusion:** Our overall results confirm that neferine effectively scavenged the oxidative stress induced by CFA and inhibited the NF-κB signaling, thereby alleviating the severity of arthritis. Histological analysis of the synovial tissue and arthritic score of arthritis-induced and neferine-treated rats authentically prove the potency of anti-arthritic drug in rat model.

Key words: Anti-arthritic drug, arthritis, complete Freud's adjuvant-induced arthritis rat model, cytokines, neferine, nuclear factor kappa B signaling

SUMMARY

- Inflammation plays a key role in the induction of arthritis, both osteoarthritis and rheumatoid arthritis leading to the formation of pannus formation, destruction of cartilage tissue, and excessive infiltration of immune cells
- Neferine, a potent phytochemical isolated from the traditional Chinese medicinal plant *Nelumbo nucifera*, scavenged the reactive oxygen species, thereby preventing the stimulation of nuclear factor kappa B transcription factor and subsequent pro-inflammatory cytokines.



Abbreviations used: CFA: Complete Freund's adjuvant; RBC: Red blood cells; WBC: White blood cells; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

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INTRODUCTION

Arthritis is a chronic autoimmune disorder that affects nearly 0.5%–1% population, and the annual incidence rate of arthritis increases by 5–50 cases per one lakh population.[1,2] Prolonged arthritis leads to the destruction of cartilage that eventually leads to permanent disability in patients with arthritis.[3,4] It also affects the major organs such as lungs and heart that may result in comorbidities. Arthritis primarily decreases the quality of life of patients and also increases the global disease burden on medication expenditure.[5,6] Inflammation plays a crucial role in the pathophysiology of arthritis that primarily damages the synovial joint tissue, cartilage, and small joints in wrist and feet.[7-9]

Inflammation is responsible for the induction of arthritis, as well as osteoarthritis and rheumatoid arthritis (RA), leading to the formation of pannus, destruction of cartilage tissue, and excessive infiltration of immune cells. The activated immune cells, synoviocytes, and chondrocytes secrete cytokines, tumor necrosis factor alpha (TNF-α), and interleukin (IL)-1, causing degradation of the cartilage and bone.[10-12] Nuclear factor kappa B (NF-κB), present in most of the cells, regulates various diseases such as autoimmune diseases, inflammatory diseases, and cancer.[13] Previous studies have demonstrated that the

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expression pattern of NF-κB increases in RA.[14,15] NF-κB activates more than 150 genes especially those that encode proteins, such as cytokines, growth factors, and cell adhesion molecules, which play an important role in the pathophysiology of RA.[16] Under normal physiological conditions, nitric oxide (NO) has been demonstrated to mediate various T-cell functions, whereas the excessive production of NO may lead to the dysfunction of T-cells. Thus, NO-mediated tissue damage is a great concern in various rheumatic diseases, e.g., RA.[17] A previous study suggests that cyclooxygenase-2 (COX-2) is considered as the imperative regulator on the pathological progression of the RA.[18]

Arthritis is mostly treated with steroid medications which suppress the symptoms induced due to the inflammation. Nonsteroidal anti-inflammatory drugs, disease-modifying antirheumatic drugs, and drugs inhibiting TNF-α and CD20 have been shown to inhibit the root cause of arthritis.[19] Even though these drugs are boon to patients with arthritis, they have numerous side effects. Conventional medicines may be an effective alternative for arthritis as they do not render any side effects during long-term medication. Phytochemicals, secondary metabolites of the plants, play a key role in alleviating numerous diseases. One such phytochemical is alkaloids which possess various pharmacological properties such as anticancer, antibacterial, antidiabetic, analgesic, and neuroprotectant.[20,21] Neferine, a bisbenzylisoquinoline alkaloid, isolated from the seeds of *Nelumbo nucifera*, exhibits an anti-inflammatory,[22] anticancer,[23] neuroprotective, and antidepressant[24] properties. Therefore, this study was planned to assess the impact of neferine against arthritis in rat model.

Various immune models using animals have been used to mimic arthritis. Among such models, two are routinely used: collagen-induced arthritis and adjuvant-induced arthritis in rats.[25-27] Collagen-induced arthritis in rats shows rapid onset of arthritis, whereas adjuvant-induced arthritis exhibits many clinical features similar to human arthritis and is widely used to assess the efficacy of Anti-arthritic drugs.[28] In this study, we estimated the Anti-arthritic potency of phytochemical neferine, an alkaloid in completed Freud's adjuvant (CFA) which stimulated arthritis in rats.

MATERIALS AND METHODS

Chemicals

Neferine and CFA were bought from Sigma-Aldrich, USA. Cytokines' enzyme-linked immunosorbent assay (ELISA) test kits were bought from MyBioSource, USA. Primary antibodies were acquired from Cell Signaling Technology, USA. Enzyme chemiluminescence kit was acquired from Millipore, USA. All other chemicals were of diagnostic grade.

Animals

Twenty-four healthy adult male Wistar rats (150–200 g) were acquired from the institutional animal house subsequent to approval sanctioned by the institutional animal ethics committee. Rats were adapted to the laboratory conditions for 3 days and supplemented with rat pellet diet, and sterile drinking water was provided *ad libitum*.[29] The cages were changed once in 3 days and bedding was changed daily. The study protocols were approved by the institutional animal ethical committee (2019–2011), and we took utmost care and concern to eliminate animal sufferings.

Induction of arthritis

Arthritis was induced by intradermally injecting 0.1 mL of CFA (F5881, Sigma-Aldrich) consisting of 1 mg of heat-killed *Mycobacterium tuberculosis* (H37Ra, ATCC 25177) mixed with 0.85 mL of paraffin oil and 0.15 mL mannide monooleate into the right hind foot pad of rats. The hind paw volume was estimated on days 1, 5, 10, 15, 20, and 25 using a plethysmometer.

Experimental design

After the acclimatization period, the rats were arbitrarily alienated into four groups with six rats in each cage. Group 1 rats (control group) were treated with dimethyl sulfoxide (DMSO); Group 2 rats were injected with CFA; Group 3 rats were induced with CFA on day 1 and were administered with 20 mg/kg/day of neferine[30] through gavage feeding from days 2 to 25; Group 4 rats were induced with CFA on day 1 and were administered with positive control (5 mg/kg/day diclofenac sodium) through gavage feeding from days 2 to 25. Both neferine and diclofenac sodium were dissolved in DMSO.

On day 26, rats were euthanized through cervical decapitation, and the blood was collected for biochemical analysis while hind paw tissue was collected for histopathological analysis.

Body weight and organ index

The body weight of each rat from all groups was monitored regularly, and the average weight of each rat was calculated. On day 26, rats were euthanized and the thymus and spleen were carefully dissected out, and the organs were immediately rinsed with saline, dried on the tissue paper, and weighed. The mean average organ weight for each group of animals was calculated and represented as milligrams per gram of the thymus, spleen wet weight, and bodyweight.

Arthritis score index

The arthritis score index was calculated using the method of Hawkins *et al.*[31] Briefly, the hind paw volume of both control and experimental rats was measured daily up to day 25. Then, the arthritis score was recorded as follows: 0 - Normal, 1 - Mild with mild redness and swelling of the ankle, 2 - Slight swelling of the ankle, 3 - Severe redness and complete swelling of the whole paw, 4 - Maximum inflammation of both limbs and joints.

Hematological indices

The control and experimental rat blood were subjected to hematological indices such as total red blood cell (RBC) and white blood cell (WBC) count, and hemoglobin content was determined using Coulter CB-9000, Chariot, India.

Estimation of pro-inflammatory cytokines

Arthritis is induced by the pro-inflammatory cytokines secreted by the activated macrophages. The levels of TNF-α (MBS355371), IL-6 (MBS490361), IL-10 (MBS2700945), and IL-1β (MBS774854) in samples were investigated using ELISA test kits (MyBioSource, USA). The assays were conducted based on the manufacturer. Samples were added to the anti-TNF-, IL-, IL-1, and IL-1β-coated ELISA plates. The intensity of the color developed is directly proportional to the cytokine levels. The absorbance of the reaction mixture was read at 450 nm, and the levels were calculated based on the standard curve plotted with known concentrations of the standard provided in the respective kits.

Biochemical indices

Estimation of hepatic enzymes

In this study, we analyzed the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) based

on the methods described by Reitman and Frankel,[32] and serum alkaline phosphatase (ALP) was examined based on the protocol described by Kind and King.[33]

Estimation of oxidative stress

Antioxidant potency of neferine against the oxidative stress induced by the CFA was estimated via detecting the statuses of lipid peroxide, superoxide dismutase, catalase, and reduced glutathione. Lipid peroxides were measured based on the protocol described by Slater and Sawyer,[34] and the values are presented as nmol of malondialdehyde generated per milligram of protein. The activity of superoxide dismutase and catalase was estimated using the protocol described by Misra and Fridovich[35] and Aebi.[36] The content of reduced glutathione was estimated based on the method described by Ellman,[37] and the values are expressed as unit per milligram of protein.

Histopathological analysis

The right hind paw was excised from the normal and investigational rats and fixed with 10% formalin solution. The formalin-fixed tissue sample was hydrated and dehydrated with xylene and ethanol, respectively. The tissue was then embedded with paraffin and sliced into thin slices of about 5 μ using microtome. The sections were fixed on to the albumin-coated slides, deparaffinized, and stained with hematoxylin and eosin. The stained slides were examined under the optical microscope and photographed. The images were analyzed for histopathological changes using ImageJ software.

Immunoblotting

The hind paw tissues were collected from the inflammatory site of control and investigational rats. The tissue samples were subjected to homogenization with RIPA buffer. Tissue homogenate was spun at 12,000 rpm for 15 min at 4°C, and the supernatant was collected and the protein was estimated using Bradford reagent. Briefly, 40 μg of protein samples was electrophoresed using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 100 mV for 1 h. The electrophoresed samples were blotted on to the polyvinylidenefluoride (PVDF) membrane and then blocked with 5% blocking buffer for 2 h at 4°C. After incubation period, the PVDF membranes were rinsed with Tris buffer and then incubated with primary polyclonal rat antibodies: COX-2, inducible nitric oxide synthase (iNOS), NF-κB, phosphorylation of IκB kinase (P-IκB)-α, and IκB kinase (IKK)-α at a dilution of 1:1000 overnight at 4°C. The primary antibodies from the membranes were removed using stripping buffer, then cleaned with Tris buffer, and then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. The membranes were then examined for protein bands using enzyme-based chemiluminescence assay kit (Millipore, USA). The membranes were stripped and incubated with internal control β-actin protein.

Statistical analysis

Results are reported as mean ± standard deviation (SD) of six rats in every group. All experiments were conducted in triplicates, and data were examined statistically via one-way analysis of variance. Subsequently, Student's Newman–Keuls test with the aid of SPSS software. $P < 0.05$ was regarded as statistically significant.

RESULTS

Effect of neferine on body weight and organ index of arthritis-induced rats

Figure 1a depicts the body weight of control and experimental rats. According to the results, the bodyweight of rats induced with arthritis was drastically decreased when compared with normal rats. However, neferine-treated rats did not show significant reduction in bodyweight similar to that of arthritic rats. Compared to the bodyweight, the weight of spleen and thymus was drastically augmented in the arthritic rats. The neferine-treated rats showed significantly decreased spleen and thymus weight compared to that of standard drug diclofenac sodium-treated rats [Figure 1b].

Effect of neferine on arthritis score index

The hind paw volume of arthritic rats gradually augmented from day 5 to 25, while neferine-treated rats showed increase in the hind paw volume till day 10, whereas it started to decrease from day 15 [Figure 2a]. Figure 2b represents the arthritis score index in the control and investigational rats. Compared to the arthritic rats, the neferine- and positive control-treated rats significantly showed decrease in arthritis score at the end of the treatment period.

Effect of neferine on hematological indices

Table 1 depicts the RBC and WBC counts and hemoglobin content estimated in the control and experimental rats. The RBC count was significantly reduced in arthritic rats ($3.09 \pm 0.93 \times 10^6$ cells/μL) compared to control animals ($7.14 \pm 0.19 \times 10^6$ cells/μL), whereas neferine- ($5.65 \pm 0.33 \times 10^6$ cells/μL) and diclofenac sodium-treated rats ($6.86 \pm 0.49 \times 10^6$ cells/μL) significantly increased the counts of RBC compared to the control animals. Hemoglobin content was also decreased in rats induced with arthritis (5.17 ± 0.048 g/dL) compared to control animals (12.29 ± 1.13 g/dL), whereas neferine- and diclofenac sodium-treated rats significantly increased the levels of hemoglobin (9.37 ± 0.41 g/dL and 11.72 ± 1.03 g/dL, respectively). The WBC count was significantly increased in the rats induced with arthritis ($19.65 \pm 1.40 \times 10^3$ cells/μL) compared to the control animals ($12.36 \pm 0.91 \times 10^3$ cells/μL), neferine-treated rats ($15.03 \pm 1.38 \times 10^3$ cells/μL), and diclofenac sodium-treated rats ($13.47 \pm 1.02 \times 10^3$ cells/μL).

Effect of neferine on inflammatory cytokines

Inflammatory cytokines play a key role in the induction and progression of arthritis. Therefore, the levels of pro-inflammatory cytokines, namely TNF-α [Figure 3a], IL-6 [Figure 3b], IL-10 [Figure 3c], and IL-1β [Figure 3d], were measured in the serum of experimental rats. The levels of TNFα, IL-6, and IL-1β were markedly augmented in the rats induced

Table 1: Effect of neferine on hematological indices of complete Freund's adjuvant-treated arthritis-induced rats

Groups	RBC ($\times 10^6$/μl)	WBC ($\times 10^3$/μl)	Hb (g/dl)
Group I	7.14±0.19	12.36±0.91	12.29±1.13
Group II	3.09±0.93#	19.65±1.40#	5.17±0.48#
Group III	5.65±0.33*	15.03±1.38*	9.37±0.41*
Group IV	6.86±0.49*	13.47±1.02*	11.72±1.93*

Blood was collected after treatment period and subjected to hematological indices analysis using Coulter CB-9000 instrument. Values were statistically analyzed and expressed as mean±SD for six independent observations of each group. #$P<0.05$ when compared with the control group; *$P<0.05$ when compared with arthritis-induced group. SD: Standard deviation; RBC: Red blood cell; WBC: White blood cell

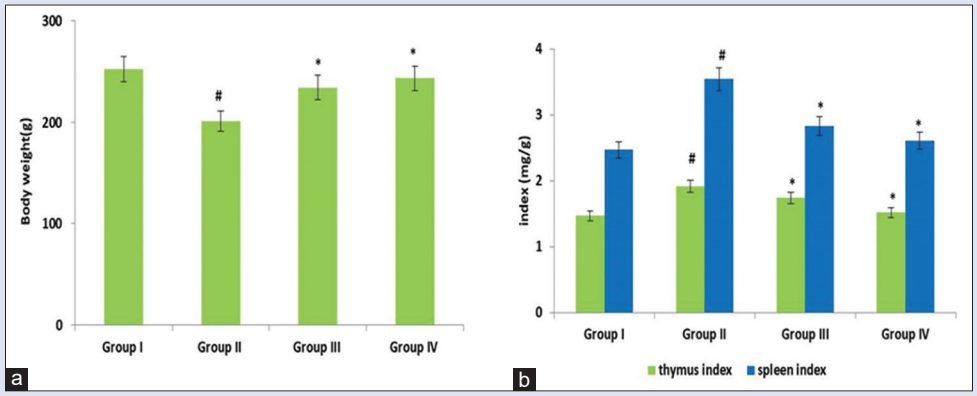

Figure 1: Effect of neferine on body weight and organ index of complete Freud's adjuvant-treated arthritis-induced rats. The rat's weight was monitored regularly and recorded for every 3 days up to the end of the treatment period (a). Immune organs, spleen and thymus, were dissected from control and experimental rats, and the relative organ weight were calculated (b). Values were statistically analyzed and expressed as means ± standard deviation for six independent observations of each group. $^{\#}P < 0.05$ when compared with the control group and $^{*}P < 0.05$ when compared with arthritis-induced group

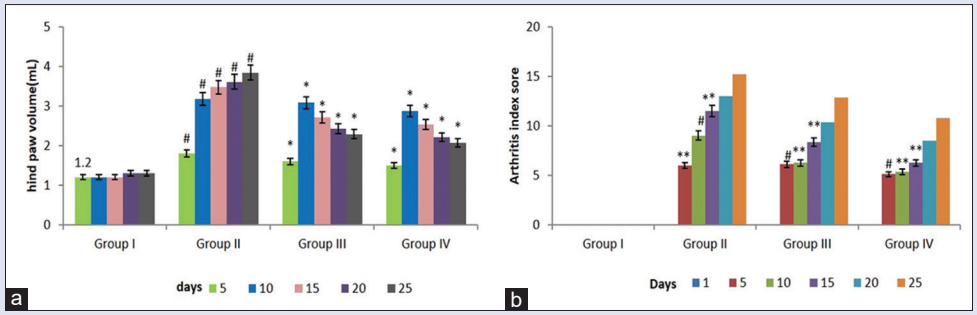

Figure 2: Anti-inflammatory effect of neferine on hind paw volume and arthritis score index of complete Freud's adjuvant-treated arthritis-induced rats. The hind paw volume was measured every 5 days till the end of the treatment period (a). Arthritis score index was calculated based on the hind paw volume (b). Values were statistically analyzed and expressed as means ± standard deviation for six independent observations of each group. $^{\#}P < 0.05$ when compared with the control group and $^{*}P < 0.05$ when compared with arthritis-induced group

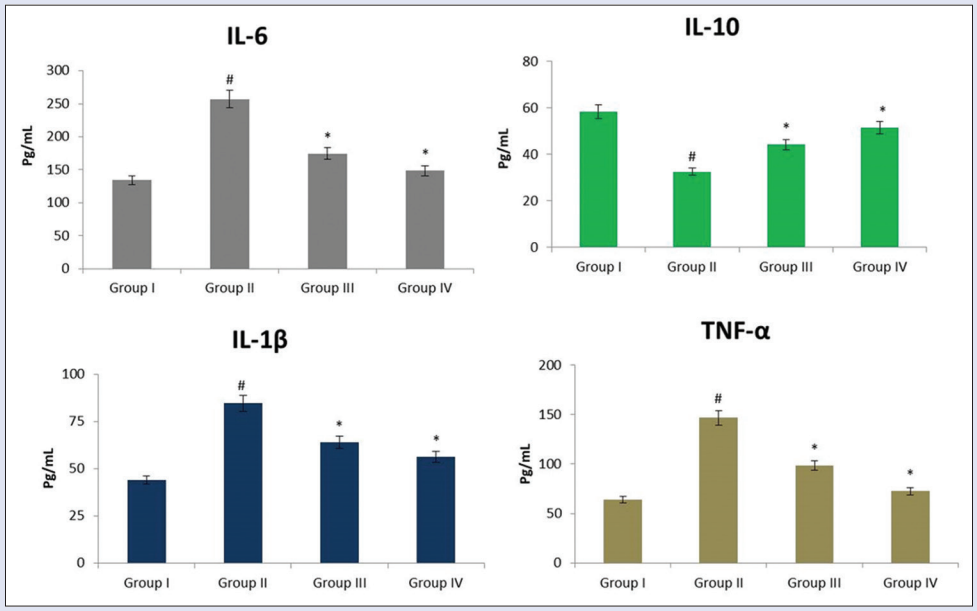

Figure 3: Anti-inflammatory effect of neferine on inflammatory cytokines of complete Freud's adjuvant-treated arthritis-induced rats. Blood samples were collected from control and experimental rats after the animal scarification at 25th day and the level of interleukin-6, interleukin-1β, tumor necrosis factor-alpha, and interleukin-10 in the serum were analyzed. Values were statistically analyzed and expressed as means ± standard deviation for six independent observations of each group. $^{\#}P < 0.05$ when compared with the control group and $^{*}P < 0.05$ when compared with arthritis-induced group

with arthritis when compared to control rats, whereas the level of IL-10 was significantly reduced in rats induced with arthritis. Neferine- and diclofenac sodium-treated rats showed significantly similar levels of cytokines.

Effect of neferine on liver function marker enzymes

Figure 4 shows the impact of neferine on hepatic enzymes, namely serum glutamate oxalate aminotransferase (SGOT), serum glutamate pyruvate aminotransferase (SGPT), and serum ALP in arthritis-induced rats. The levels of these enzymes were significantly augmented in arthritis-induced rats than that of normal rats, whereas their levels in neferine- and diclofenac sodium-treated rats were found to be similar.

Effect of neferine on complete Freud's adjuvant-induced oxidative stress

The amount of lipid peroxidation and the level of antioxidants were measured to assess the effect of neferine against CFA-induced oxidative

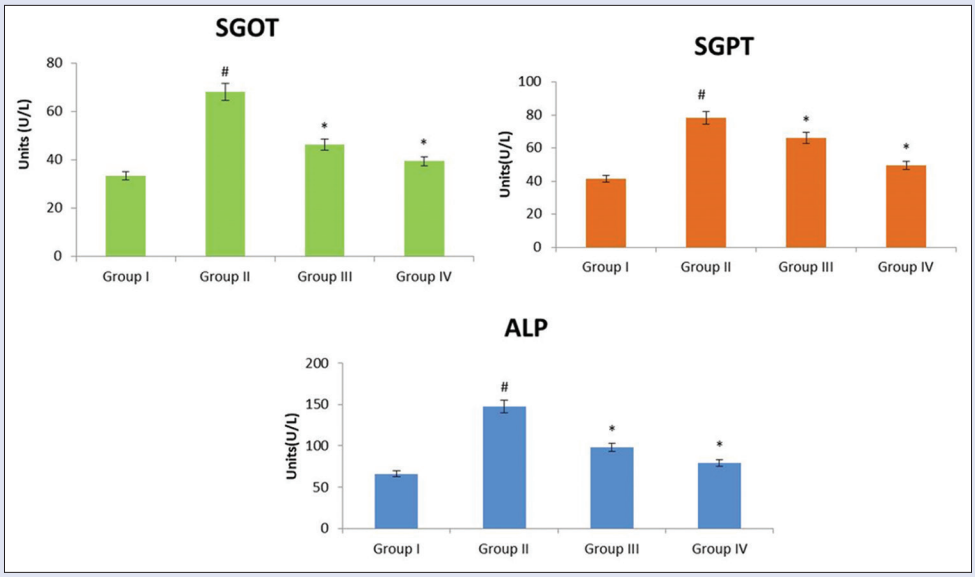

Figure 4: Anti-inflammatory effect of neferine on liver functioning enzymes of complete Freud's adjuvant-treated arthritis-induced rats. Blood samples were collected from control and experimental rats after the animal scarification at 25th day and the levels of serum glutamate oxalate aminotransferase, serum glutamate pyruvate aminotransferase, and alkaline phosphatase in the serum were analyzed. Values were statistically analyzed and expressed as means ± standard deviation for six independent observation of each group. $^{\#}P < 0.05$ when compared with the control group and $^{*}P < 0.05$ when compared with arthritis-induced group

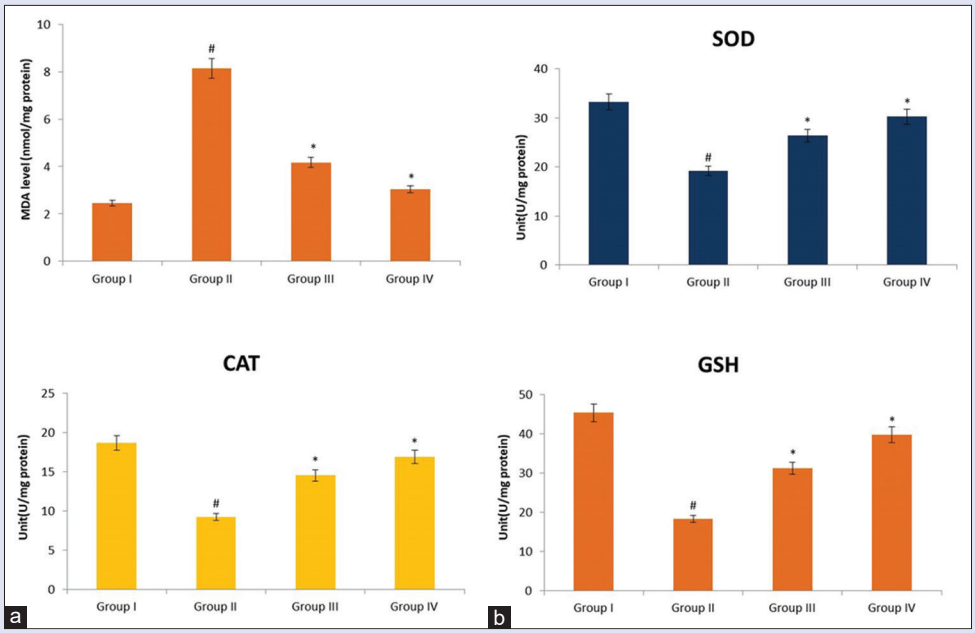

Figure 5: Anti-inflammatory effect of neferine on oxidative stress induction and antioxidant status in complete Freud's adjuvant-treated arthritis-induced rats. The control and experimental rat hind paw tissue samples were subjected to oxidative stress lipid peroxidation (a) and antioxidant status (b). Values were statistically analyzed and expressed as means ± standard deviation for six independent observations of each group. $^{\#}P < 0.05$ when compared with the control group and $^{*}P < 0.05$ when compared with arthritis-induced group

stress [Figure 5]. Arthritis-induced rats showed significantly augmented levels of lipid peroxidation and the activity of superoxide dismutase, catalase, and reduced glutathione were reduced compared to the control animals. The neferine- and diclofenac sodium-treated rats showed significantly increased levels of antioxidants [Figure 5b] and decreased levels of lipid peroxidation [Figure 5a] compared to the CFA-induced arthritic rats.

Effect of neferine on ankle joint histomorphology of arthritis-induced rats

CFA-induced arthritis in animals showed massive histopathological changes such as excessive infiltration of inflammatory cells, synovial hyperplasia, synovial cell layer disruption, increased vasculation, and edema [Figure 6b] compared to the control rats with normal synovial cell membrane and vasculation [Figure 6a]. Compared to the arthritis-induced rats, the neferine- and diclofenac sodium-treated rats showed reduced levels of infiltration of inflammatory cells and synovial cell membrane disruption is minimal [Figure 6c and d].

Effect of neferine on inflammatory molecules

Figure 7 shows the levels of NF-κB, P-IκBα, and IKKα proteins that are involved in canonical NF-κB signaling pathway was remarkably augmented in arthritis-induced rats, whereas it is significantly reduced in neferine- and diclofenac sodium-treated rats. Next, iNOS generates NO that is involved in various physiological and pathophysiological processes such as inflammation. In this study, NO was increased in arthritis-induced animals compared to neferine- and diclofenac sodium-treated rats. COX-2, which is responsible for pain and induction of inflammation, was significantly decreased in neferine- and diclofenac sodium-treated rats compared to the rats induced with arthritis.

DISCUSSION

Arthritis, a chronic autoimmune disorder, was mimicked in animals with various models such as adjuvant-, collagen-, antigen-induced arthritis models.[38] Among these, adjuvant-induced arthritis animal model is one of the broadly utilized arthritic models to investigate the remedial potency of Anti-arthritic drugs and also to study the pathogenicity of arthritis.[28] The induction of inflammation occurs after 2 weeks of adjuvant therapy and then subsequently resolves after 3 weeks.[39] Adjuvant develops rapid, easily measurable polyarthritis and causes degradation of cartilage and cellular reflux, which was observed in patients with arthritis.[40] In this study, we administered CFA and assessed the efficacy of phytochemical neferine against the induction of arthritis. CFA effectively induced inflammation which was evidenced with our hind paw volume measurement which increased drastically from day 10 of the treatment period. However, neferine-treated rats reduced the inflammation, thereby decreasing the paw volume. The body weight was of CFA-induced rats decreased, whereas the relative organ weight of the spleen and thymus was increased. This may be due to the inflammatory response induced by CFA which activated the autoimmunity, thereby decreasing the body weight of CFA-induced rats. Neferine inhibited the inflammatory response of CFA which is evidenced with the decreased arthritis score index in neferine-treated rats.

One of the most frequent problems encountered by patients with arthritis is anemia, which is due to the deregulated iron storage in the reticuloendothelial system and synovial tissue.[41] The decreased life span of RBCs, decreased rate of erythropoietin production, and decreased erythroid cell proliferation also play a key role in the pathogenesis of anemia.[42] Anti-arthritic drugs cause blood

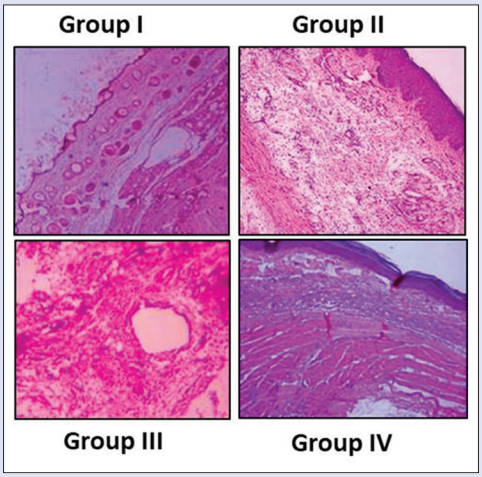

Figure 6: Anti-arthritic effect of neferine on ankle joint histomorphology of complete Freud's adjuvant-treated arthritis-induced rats. The control and experimental rat ankle joint muscle tissue were processed for histological analysis and sectioned into slices of 5 μ thickness. The sectioned slides were stained with hematoxylin and eosin stains. The stained slides were viewed under light microscope and photographed. The experiments were performed in triplicates

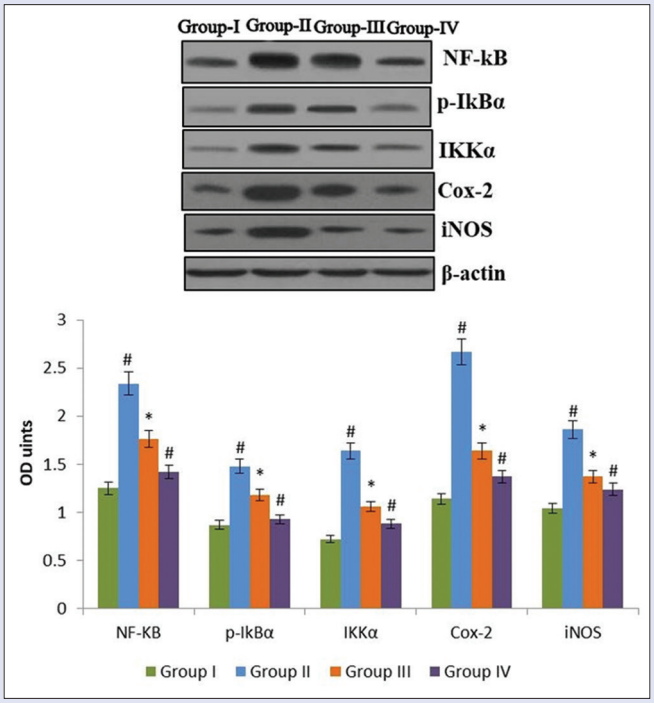

Figure 7: Anti-arthritic effect of neferine on inflammatory signaling molecules in complete Freud's adjuvant-treated arthritis-induced rats. The ankle joint tissue collected from inflamed site was lysed with RIPA buffer, centrifuged at 1200 rpm for 15 min and the supernatant was subjected to protein estimation. 40 μg of total protein from control and experimental rat samples were subjected to electrophoresis and immunoblotting analysis with specific proteins cyclooxygenase-2, inducible nitric oxide synthase, nuclear factor kappa B, phosphorylation of IκB kinase α, and IκB kinase α signaling protein. The protein bands were visualized using enzyme chemiluminescence kit and representative images were depicted. $^{\#}P < 0.05$ when compared with the control group and $^{*}P < 0.05$ when compared with arthritis-induced group

loss through the gastrointestinal tract.[43] In this study, neferine significantly increased the hemoglobin levels and RBC count in the arthritis-induced rats, which shows that it does not impart any side effects that are observed in currently prescribed anti-arthritic drugs. The WBC count was significantly reduced in the neferine-treated rats which may be due to the additional effect of neferine against CFA-induced autoimmunity. Extra-articular manifestation was reported in 40% of the patients with arthritis; one of the common manifestations observed among 6%–74% of patients with RA is hepatic damage.[44,45] Elevated levels of ALP, gamma-glutamyl peptidase, and aminotransferases have recorded in patients with RA.[46] Therefore, in this study, we assessed the potency of neferine in preventing damage to the liver. The statuses of SGOT, SGPT, and ALP were significantly decreased in the neferine-treated rats, which shows that neferine protected the liver from damage.

Inflammation of the synovial tissue, a hallmark pathogenesis reported in patients with arthritis, occurs due to the increased level of oxidative stress.[47] Reactive oxygen species trigger various inflammatory signaling pathways that lead to an increased proliferation of the inflammatory cells, thereby damaging the cartilage tissue in patients with arthritis. CFA increased the level of lipid peroxidation which may be due to the oxidative stress stimulated by the CFA which in turn stimulated lipolysis and LDL oxidation.[48] Our results correlate with those reported by a previous study.[49] Neferine treatment significantly decreased the amount of lipid peroxidation in rats induced with arthritis, which may be due to the antioxidant property of alkaloid neferine. Superoxide dismutase, a metalloprotein, and catalase, a hemoprotein, scavenge the free radicals, thereby preventing the cells from oxidative damage.[50] Reduced glutathione levels decrease in arthritic conditions,[51] whereas neferine appreciably augmented the levels of antioxidants, which confirms the potency of neferine against CFA-induced oxidative stress.

Deregulation of inflammatory responses causes increased accumulation of pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6. Reports suggest that the excessive production of pro-inflammatory mediators secreted in the synovial tissue targets the distant organ, leading to the extra-articular manifestations of arthritis such as insulin resistance, pro-thombotic effects, and hepatitis[52] TNF-α is the major inducer cytokine which regulates the equilibrium among pro-inflammatory and anti-inflammatory mediators in the synovial tissue of patients with arthritis.[53] Arthritis treatment was recently focused on targeting the inhibition of TNF-α protein using monoclonal antibodies.[54] In this study, neferine treatment had significantly decreased the levels of TNF-α and other pro-inflammatory cytokines such as IL-6 and IL-1β, whereas it elevated the levels of anti-inflammatory cytokine IL-10.

Constitutive activation of NF-κB has been reported in the synovial tissue of patients with arthritis which in turn triggers the synthesis of pro-inflammatory cytokine.[55] Experimental arthritis models induced with adjuvants, pristine, lipopolysaccharides, and collagen also express increased levels of activated NF-κB protein.[56] NF-κB transcription factor triggers the pro-inflammatory cytokines to activate the matrix metalloproteinase causing degradation of cartilages.[57] IKK masks the activation of NF-κB and P-IκB leads to the ubiquitin-dependent IκB degradation, thereby promoting the nuclear translocation of NF-κB. Activated NF-κB further triggers numerous other inflammatory proteins, apoptotic proteins, and cytokine receptors.[58] The P-IκB is regulated by the kinases IKKα and β, which phosphorylates the RelA/p65 subunit.[59] In this study, neferine decreased the expression IKKα and pIκBα, which in turn diminished the expression of NF-κB transcription factor in the synovial tissue of arthritis-induced rats. It also diminished the expression of iNOS and COX-2 that are responsible for the inflammation in cartilage tissues and also protected chondrocytes from apoptosis.[60] Excessive infiltration of immune cells into the synovium causing pannus formation has been reported in patients with arthritis.[61] Our histopathological analysis confirms that neferine significantly decreased the penetration of immune cells into the synovium, synovial hyperplasia, and vasculation, thereby preventing the synovial tissue disruption induced by the CFA treatment.

CONCLUSION

CFA-induced arthritis model is a sensitive model which closely resembles the pathogenesis of human arthritis such as increased levels of oxidative stress, pro-inflammatory cytokines, hepatic enzymes, and NF-κB signaling and decreased levels of antioxidants. Neferine, a potent phytochemical, isolated from the traditional Chinese medicinal plant *N. nucifera*, scavenged the reactive oxygen species, thereby preventing the stimulation of NF-κB transcription factor and subsequent pro-inflammatory cytokines. It also prevented the arthritis-induced rats from hepatic damage, and our histopathological analysis confirmed that neferine effectively suppressed the pathogenicity induced by CFA. Overall, our results prove that neferine is a potent anti-arthritis drug with no side effects and may be prescribed for patients with arthritis with further trials.

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Conflicts of interest

There are no conflicts of interest.

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