

Original Article



Attenuation of Collagen Induced Arthritis by *Centella asiatica* Methanol Fraction via Modulation of Cytokines and Oxidative Stress

Shikha Sharma¹, Ritu Gupta², and Sonu Chand Thakur^{1, #}

1. Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, New Delhi-110025, India; 2. Lab Oncology, All India Institute of Medical Sciences, New Delhi-110029, India

Abstract

Objective To investigate the anti-inflammatory, antioxidant and anti-arthritis effects of *Centella asiatica* methanolfraction (CaME) on collagen-induced arthritis (CIA), an animal model of rheumatoid arthritis.

Methods Arthritis was induced in female wistar rats by immunization with porcine type II collagen. The CIA rats were treated orally with CaME (50, 150, and 250 mg/kg/day) for 15 d (beginning on day 21 of the experimental period). The clinical, histological, biochemical, and immunological parameters were assessed.

Results CaME treatment (150 and 250 mg/kg) significantly attenuated the severity of CIA and reduced the synovial inflammation, cartilage erosion, and bone erosion as evident from both histological and radiographic data. The escalated plasma levels of pro-inflammatory cytokines TNF- α , IL-1 β , IL-6, and IL-12 alongwith nitric oxide in CIA rats decreased significantly on CaME treatment. The serum levels of type-II collagen antibody were significantly lower in rats of CaME (150 and 250 mg/kg) treated group than those in the arthritic group. Furthermore, by inhibiting the above mediators, CaME also contributed towards the reversal of the disturbed antioxidant levels and peroxidative damage.

Conclusion Our results clearly indicate that oral administration of CaME suppresses joint inflammation, cytokine expression as well as antioxidant imbalance, thereby contributing to an amelioration of arthritis severity in CIA rats.

Key words: *Centella asiatica*; Collagen-Induced arthritis; Inflammation; Oxidative stress; Antioxidants; Anti-Inflammatory

Biomed Environ Sci, 2014; 27(12): 926-938

doi: 10.3967/bes2014.133

ISSN: 0895-3988

www.besjournal.com (full text)

CN: 11-2816/Q

Copyright ©2014 by China CDC

INTRODUCTION

Natural products have served as the source of the most active ingredients of medicines since time immemorial, and now more than 80% of the pharmaceutical products are either derived from natural products or inspired

by a natural compound^[1]. The marked anti-inflammatory effects produced by certain natural agents add an important adjunct to drug discovery. Inflammation is a process central to many pathological conditions and is mediated by a variety of soluble factors and cellular signaling events acting in a complex network. Rheumatoid arthritis (RA) is a

[#]Correspondence should be addressed to Sonu Chand Thakur, Tel: 91-11-26981717, Fax: 91-11-26981717, E-mail: sonuchandthakur@gmail.com

Biographical note of the first author: Shikha Sharma, female, born in 1985, PhD candidate, main research interest is to evaluate the potential anti-inflammatory, antioxidant and anti-arthritis activities of plant extracts and isolated compounds in cell free, cell culture, and animal model system.

chronic, systemic immune-mediated disease characterized by inflammation of the multiple synovial joints with concomitant destruction of joint tissues. Production of TNF- α and interleukins play a critical role in the pathogenetic mechanisms of RA through their contribution to articular degeneration. These cytokines induce and activate inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), resulting in the production of PGE₂ and NO^[2]. Activation of macrophages leads to the ingress of large numbers of polymorphonuclear cells (PMNs) into the synovial tissue and synovial fluid. Recent investigations have revealed that uncontrolled release of free radicals also participates in the pathogenesis of articular cartilage degradation by stimulating inflammatory pathways and damage lipids, proteins, or DNA^[3]. Type II collagen-induced arthritis (CIA) is a T-cell dependent animal model of RA in which rats develop experimental arthritis after immunization with heterologous type II collagen^[4]. This model is widely used to address questions of disease pathogenesis, to validate therapeutic targets and to evaluate potential therapeutic compounds^[5]. Similar to RA, the up-regulation of pro-inflammatory cytokines (TNF- α and IL-1 β), nitric oxide, prostanoids, and leukotrienes is also associated with CIA. Besides, both CIA and RA share many clinical, pathological, and histological features. Hence, the modulation of these products provides a target for controlling inflammatory diseases. Currently prescribed anti-arthritis drug regimen; nonsteroidal anti-inflammatory drugs (NSAIDs) and disease modifying antirheumatic drugs (DMARDs), can effectively manage the pain and symptoms of the disease, though with rather discouraging profile of side effects^[6]. So, there is a pressing need for the development of novel anti-inflammatory therapeutic agents for RA that can prevent the progression of disease and confer safe prolonged treatment. With respect to this, natural plant antioxidants that are considered to be promising and safe source might serve as leads for the development of novel drugs.

Centella asiatica, commonly known as Gotu Kola, belonging to the family Mackinlayaceae, is a species native to Asia. It is a perennial herb and several therapeutic properties are attributed to it in the Indian system of medicine for the treatment of several disorders, such as insanity, asthma, leprosy, ulcers, and eczema and for wound healing^[7]. In addition, numerous scientific reports have documented its antiviral, anti-inflammatory, diuretic, nerve stimulant, adaptogenic, anti-stress, and anxiolytic properties^[8-9]. Steroids have also been

isolated from the plant for the treatment of leprosy. *C. asiatica* is also used to re-vitalize the brain and nervous system, increase attention span, and concentration^[10]. Madecassoside (MA), a major pentacyclic triterpenoid saponin component of *C. asiatica*, has been described to have wound healing, anti-apoptosis, antioxidant, and anti-inflammatory activities. It has also shown to be effective against collagen-induced arthritis in mice^[11-12]. However, as far as literature survey is concerned, no studies have been conducted so far to investigate the combined antioxidant and anti-inflammatory effects of *C. asiatica* methanol fraction both *in vitro* and *in vivo*.

Therefore, our aims in this study were multiple. Firstly, we wanted to investigate the antioxidant and free radical scavenging activities of different fractions from *C. asiatica* whole plant extract in different cell-free model systems. Secondly, we wanted to check the most active fraction in CIA rat model for the suppression of arthritic symptoms via downregulation of cytokines and nitric oxide and finally, to assess the lipid peroxidation and protein oxidation status in plasma and joints of all the experimental rats. Dexamethasone was used in positive control group in this study. So, the present work defined in the study will be of great interest to define the usefulness of this plant as a dietary supplement as well as in therapeutic usage.

MATERIALS AND METHODS

Collection of Plant Material and Extract Preparation

The whole plant of *C. asiatica* was procured from local dealer, Delhi, India and authenticated by Dr. H. B. Singh from National Institute of Science Communication and Information Resources, Delhi, India. A voucher specimen was deposited in the herbarium; code number: 1532/130.

Preparation of the Extracts

The plant was air-dried under shade at ambient temperature (25 °C) and ground to powder form. The powdered plant was exhaustively extracted thrice with methanol at room temperature (RT) for 72 h in an orbital shaker, with a mass to volume ratio of 1:10 (g/mL). The supernatant was then passed through Whatman filter paper No.1, concentrated under reduced pressure in rotary evaporator, lyophilized and weighed to determine the total extractable components (EC). The crude methanol extract was successively fractionated with different solvents in the order of increasing polarity. The

solvents used were hexane, chloroform, ethyl acetate, methanol, and water. Extraction from each solvent was done exhaustively and the residue was air dried overnight to be used for next solvents. Finally, the combined fractions for each solvent were filtered, dried under vacuum using a rotary evaporator and kept at 4 °C until required. The yield of evaporated dried extracts based on dry weight basis was also calculated.

Determination of Total Phenolic, Flavonoid, and Proanthocyanidin Content

Total phenolic content of the *C. asiatica* fractions were determined by the modified Folin-Ciocalteu method^[13]. Total flavonoid content was determined by the aluminium chloride colorimetric method^[14]. Method reported by Afolayan et al.^[15] was used to determine the total proanthocyanidin content.

Determination of in vitro Antioxidant Activity

All the fractions from *C. asiatica* were evaluated for antioxidant capability using various *in vitro* assay systems. In all the experiments, BHT was taken as reference antioxidant.

The superoxide anion radical scavenging activity of CaME was measured by the method of Kumari and Kakka^[16] with minor modifications and that of hydrogen peroxide radicals was determined using modifications of the method of Ogunlana & Ogunlana^[17]. The nitric oxide radical scavenging activity was estimated using the method of Temraz and El-tantawy^[18]. Radical scavenging activity by antioxidants present in the *C. asiatica* fractions was evaluated using DPPH radicals and carried out according to the procedure of Sanja et al.^[19]. Variable concentrations of fractions from *C. asiatica* on inhibition of lipid peroxidation induced by iron-ascorbate system in young rat liver cytosolic fraction were assessed according to the method of Qu et al.^[20]. The total antioxidant capability of the fractions from *C. asiatica* was evaluated by the phosphomolybdenum method according to the procedure described by Prieto et al.^[21].

Animal Experiments

This study was conducted in Central Animal Facility, All India Institute of Medical Sciences (AIIMS), New Delhi, India and the prior permission for experiment on animals was taken from Institutional Animals Ethics Committee at AIIMS.

Female wistar strain rats ($n=36$, 6-8 weeks old) were procured from the Central Animal Facility and housed separately in cages, maintained under 12 h: 12 h light and dark cycles and food and water provided *ad libitum*. The environmental temperature and humidity were maintained at 25 ± 2 °C and $55\pm 5\%$ respectively. After 5 days acclimatization period, the rats were randomly divided to six groups ($n=5$). Group 1 was non-immunized untreated control given saline only and used as sham group. The other five groups were a CIA+vehicle group, a positive control group (CIA+Dexamethasone) and three CaME-administered groups (CIA+CaME at 50, 150, and 250 mg/kg).

Induction of Arthritis

CIA was induced as previously described by Sahu et al.^[22]. Briefly, porcine immunization-grade native collagen type II (Chondrex) was dissolved in 0.05 mol/L acetic acid at a concentration of 2 mg/mL by stirring overnight at 4 °C. Emulsion was prepared by homogenizing CII with FCA (1:1) at 4 °C. Each rat was immunized with 200 µg of emulsified CII, intradermally at multiple sites on the back and base of the tail followed by a booster dose with the same antigen preparation on day 7. On day 21, respective dosages of CaME and dexamethasone (1 mg/kg) were orally administered once daily for 15 d.

Arthritis Assessment-arthritic Score and Index Alongwith Body Weight

The rats were evaluated daily for the clinical signs of arthritis. After physical examination, each paw was scored macroscopically every 3 d. Based on the disease severity, number of limbs affected, redness/swelling of the joints, each paw was scored macroscopically every 3 d. This scoring system assigns numerical values to the digits and paws of each of the hind limbs as follows: 0=normal; 1=swelling or redness confined to 1 digit; 2=swelling or redness in 2 or more digits; 3=swelling or redness in entire paw; 4=severe arthritis of the entire paw and digits with difficulty in walking. The mean arthritis score for each rat was calculated by dividing the total scores for the group by the number of animals in the group.

Hind paw edema of the rats was monitored every 3rd day, using a dial thickness gauge. The arthritic index (AI) and CIA inhibition were calculated. Body weight was also measured every 3rd day.

Radiological and Histological Analysis

On day 36, the animals were euthanized under light anesthesia. The knee joint was excised postmortem, trimmed off skin and muscular parts, and fixed in 10% buffered formalin. Afterwards, the joints were placed in decalcifying solution for almost 10 d, embedded in paraffin, sectioned (10 μ) and stained with hematoxylin and eosin. The sections were observed under light microscope at 10 \times magnification. For radiographic analysis, the fixed hind limbs were imaged on high speed radiographic film.

Measurement of Cytokines and Nitric Oxide Concentration in Plasma and Joints

The plasma levels of TNF- α , IL-1 β , IL-6, and IL-12 were evaluated using the respective rat specific ELISA kits as per the manufacturer's protocol (Peprotech Inc., USA).

NO was measured by incubating 100 μ L of the plasma with 100 μ L of the Griess reagent (Sigma Inc., USA) as per manufacturer's protocol. The absorbance was measured at 540 nm using ELISA microplate reader (Biorad, USA) and nitrite concentration was determined with a standard curve of sodium nitrite (NaNO₂).

Serum Anti-CII Antibody Determination

The serum antibodies against CII were quantitated by ELISA using HRP-labeled goat anti-rat IgG (Chondrex Inc., USA) as per the manufacturer's protocol. Serum was isolated from the blood of all the experimental rats, 42 d post-CII primary immunization.

Determination of Levels of Oxidative Stress Parameters

The antioxidant enzymes were measured in joints and plasma.

Preparation of Tissue Homogenate

The dissected joints were washed in cold saline solution and homogenized in the buffer (50 mmol/L Tris-HCl, pH 7.4 containing PI cocktail) using a Teflon homogenizer. The homogenate was centrifuged at 2000 rpm for 10 min, to remove debris. The supernatant was centrifuged at 16,000 rpm for 30 min at 4 °C. The cytosolic fraction after discarding the floating lipid layer was used for assaying the malondialdehyde and protein carbonyl content.

Homogenates were kept at -80 °C prior to the biochemical assays. Protein concentration was estimated by Bradford reagent (Biorad, USA) using BSA as the standard. The level of thiobarbituric acid reactive substances as a measure of malondialdehyde (MDA) and protein carbonyl (PCO) content as a measure of protein oxidation were estimated by the method of Liu et al.^[12].

Statistical Analysis

Experimental results are mean \pm SD of three parallel measurements. The IC₅₀ values are calculated from linear regression analysis and % inhibition using the formula:

$$\% \text{Inhibition} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})}{\text{Absorbance}_{\text{control}}} \quad (1)$$

For the *in vivo* studies, *n* represents the number of rats. Results for the groups are expressed as the mean \pm standard deviation of *n*=5 animals per experimental group. The effects of CaME and dexamethasone treatment were analyzed by ANOVA. When a significant *P*-value was obtained, the Tukey's multiple comparisons test was employed to determine differences between the groups. Student's *t*-test and non-parametric Mann-Whitney U test were also used to analyze biochemical data using GraphPad Prism version 6.00 (GraphPad Prism Inc., San Diego, CA). The level of significance was accepted at *P*<0.05.

RESULTS

Extractable Total Phenolics, Flavonoids, and Proanthocyanidin Content

The results given in Table 1 demonstrate that the method using methanol as a solvent was more efficient in extracting phenolic, flavonoid, and proanthocyanidin compounds from the *C. asiatica* followed by aqueous and ethyl acetate. However, the extraction with chloroform and hexane resulted in lower yields of phenolics and flavonoids.

In-vitro Antioxidant Activity

Superoxide Anion Radical Scavenging Activity

The fractions from *C. asiatica* exhibited high superoxide radical inhibitions at relatively lower concentrations of 5-50 μ g/mL (Figure 1A). Methanol fraction (92.36 \pm 0.56%) exhibited strongest scavenging effect at almost all the concentrations studied, followed by the ethyl acetate (82.67 \pm 1.04%) fraction. BHT (60.13 \pm 0.46%)

showed the lowest inhibitory activity throughout. IC_{50} values in terms of scavenging abilities on superoxide radicals were comparable ($P>0.05$) for methanol ($4.18\pm 0.2 \mu\text{g/mL}$), ethyl acetate ($5.3\pm 0.73 \mu\text{g/mL}$), and aqueous ($5.4\pm 0.3 \mu\text{g/mL}$) fractions. These values were much lower in comparison to that obtained for the standard, BHT ($315.01\pm 6.51 \mu\text{g/mL}$). The results were found to be statistically significant ($P<0.0001$).

Hydrogen Peroxide Radical Scavenging Activity

As shown in Figure 1B, the abilities of methanol ($88.38\pm 0.71\%$), aqueous ($88.23\pm 0.28\%$), and ethyl acetate ($81.82\pm 0.65\%$) fractions to inhibit hydrogen peroxide radicals were significantly

($P<0.0001$) lower than BHT ($99.11\pm 0.14\%$) at $500 \mu\text{g/mL}$ concentration. There was no statistically significant difference ($P>0.05$) between the radical scavenging activities of aqueous and methanol fractions in the concentration range of $100\text{--}500 \mu\text{g/mL}$. In contrast, chloroform and hexane fractions showed the lowest and comparable scavenging activity throughout (data not shown). Significant differences ($P<0.0001$) were also observed in the IC_{50} values calculated for different fractions of *C. asiatica* and BHT, which followed the order: ethyl acetate ($20.18\pm 3.69 \mu\text{g/mL}$) < BHT ($29.04\pm 1.69 \mu\text{g/mL}$) = methanol ($29.87\pm 0.96 \mu\text{g/mL}$) < aqueous ($41.11\pm 2.21 \mu\text{g/mL}$).

Table 1. Total Extractable Components (EC), Total Phenolic, Flavonoid, and Proanthocyanidin Contents in Aqueous, Methanol, Ethyl Acetate, Chloroform, and Hexane Fractions from *C. asiatica* Whole Plant Methanol Extract

Extracts	EC	Phenolic Content*	Flavonoids Content**	Proanthocyanidin Content***
Aqueous	10.43	83.56 ± 0.087	64.95 ± 0.01	41.15 ± 0.01
Methanol	12.32	101.9 ± 0.083	144.76 ± 0.136	86.41 ± 0.02
Ethyl acetate	5.396	58.63 ± 0.012	83.23 ± 0.078	61.39 ± 0.005
Chloroform	2.175	6.07 ± 0.05	ND	74.15 ± 0.005
Hexane	1.243	31.24 ± 0.079	ND	47.41 ± 0.018

Note. Values were the means \pm SD of three replicates. * Expressed as mg gallic acid equivalents/g dry weight of plant extract; ** Expressed as mg rutin equivalents/g dry weight of plant extract; *** Expressed as mg catechin equivalents/g dry weight of plant extract.

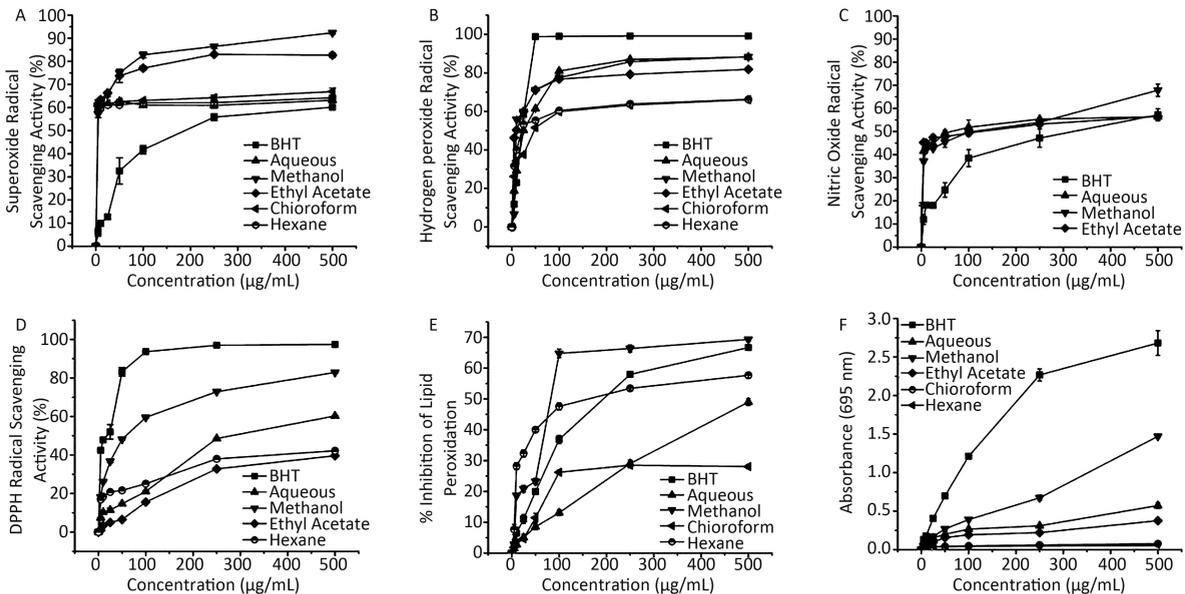


Figure 1. Free radical scavenging activity of CaME on A) Superoxide radicals, B) Hydrogen peroxide radicals, C) Nitric oxide radicals, D) DPPH radicals. Lipid peroxidation inhibition potential of CaME is shown in (E) and total antioxidant capacity is shown in (F). For comparison, BHT was considered as standard antioxidant. Values are mean \pm SD of three parallel measurements.

Nitric Oxide Radical Scavenging Activity The results presented in Figure 1C showed that methanol, ethyl acetate, and aqueous extracts from *C. asiatica* markedly decreased the generation of nitrite ions in a concentration-dependent manner from its artificial source sodium nitroprusside. At the same point, the activity of methanol fraction (67.87%±2.64%) was superior ($P<0.0001$) to that of BHT (57.22%±2.65%). Chloroform extract showed negligible nitric oxide radical scavenging activity (data not shown). Among the extracts, the IC₅₀ value calculated for methanol (166.48 ± 16.86 µg/mL) was lowest followed by the ethyl acetate (169.88±20.73 µg/mL) and did not show any significant difference ($P>0.05$) from that of the aqueous (176.91±44.62 µg/mL) fraction. The chloroform fraction showed the highest IC₅₀ value.

DPPH Radical Scavenging Activity Figure 1D shows that the scavenging effect of aqueous, methanol, ethyl acetate, and hexane extracts on DPPH radicals increased in a dose-dependent manner and were 48.58%±0.29%, 72.89%±0.13%, 32.79%±0.21%, and 37.98%±0.17%, respectively at a concentration of 250 µg/mL, indicating that the methanol fraction was most effective followed by the aqueous fraction. After addition of respective fractions at a concentration of 500 µg/mL, the scavenging activity increased to 60.26%±0.22%, 82.87%±0.25%, 39.63%±0.11%, and 42.2%±0.35%, respectively. Ethyl acetate and hexane fractions showed moderate and almost similar scavenging activities while chloroform extract was considerably less effective as a DPPH radical scavenger ($P<0.0001$). No definitive pattern was inferred from the chloroform extract. All the extracts showed less DPPH radical scavenging activity as compared with the BHT ($P<0.0001$). It was seen that methanol extract (69.03±1.41 µg/mL) exhibited strongest antioxidant activity as shown by its lowest IC₅₀ value, followed by the aqueous (317.61±8.34 µg/mL), ethyl acetate (347.18±5.94 µg/mL), and hexane (416.57±25.68 µg/mL) extracts. These values were significantly ($P<0.0001$) higher from the IC₅₀ value obtained for BHT (11.16±0.91 µg/mL), indicating that none of the extract was as effective as the reference antioxidant.

Suppression of Lipid Peroxidation in Rat Liver Cytosolic Fraction Methanol fraction showed a sharp increase in activity of 3 fold from 25-50 µg/mL and thereafter almost levelled off with further increase in concentration (Figure 1E). Surprisingly, the hexane fraction exhibited a good lipid peroxidation inhibitory activity of 57.68%±0.94% at

the concentration of 500 µg/mL. The order of potency of the fractions and standard at the highest concentration was: methanol (69.33%±0.71%)>BHT (66.67%±0.68%)>hexane (57.68%±0.95%)>aqueous (48.98%±1.1%)>chloroform (28.06%±0.71%). Among the fractions, methanol exhibited strongest inhibition of MDA formation at concentrations of approximately greater than >40 µg/mL. However, when compared to the standard, methanol fraction exhibited stronger inhibitory activity throughout. No deducible pattern was found regarding the lipid peroxidation inhibitory activity of ethyl acetate extract (data not shown). IC₅₀ values for the inhibition of lipid peroxidation were 80.41±0.87, 95.86±2.28, and 413.21±69.73 µg/mL for methanol, hexane, and aqueous fractions respectively. The methanol fraction exhibited significantly ($P<0.0001$) lower IC₅₀ value in comparison with BHT (298.86±6.39 µg/mL).

Total Antioxidant Capability The total antioxidant capability of different fractions from *C. asiatica* assayed at different concentrations (0-500 µg/mL) is presented as optical density in Figure 1F. This assay exhibited the following sequence of total antioxidant capability ($P<0.0001$) in terms of optical density: methanol (1.47±0.02) > aqueous (0.57±0.04) > ethyl acetate (0.37±0.02). BHT (2.68±0.16) showed the highest activity throughout. The chloroform and hexane extracts exhibited the least activity.

Since this phosphomolybdenum method is quantitative, the total antioxidant capability was also expressed as the number of equivalents of L-ascorbic acid. Total antioxidant capability equivalent to L-ascorbic acid was 770.58, 204.6, and 108.8 µg/mg plant material for methanol, aqueous, and ethyl acetate extracts, respectively.

Effect of Oral CaME Treatment on Kinetics of the Clinical Progression of CIA

The onset of symptoms of arthritis in the immunized rats were observed within 9-12 d after challenge. Periarticular erythema, edema, redness, and a reduction of paw function were observed as clear signs of the evolution of the inflammation. The severity gradually increased as a function of time and reached a maximum plateau of the peak of CIA response from day 18 to 32 (mean AI and arthritic score being 121.02 and 8, respectively) and started decreasing gradually thereafter (Figure 2B). The CaME treatments (50, 150, and 250 mg/kg) to the respective groups were started from day 21 of the experimental period. There was a significant

dose-dependent reduction of the mean arthritis scores and index of the rats treated with CaME compared with those of the vehicle-treated rats (Figure 1). Towards the end of the experiment, the CIA inhibitions for the three dosages of CaME were calculated to be 20.92% (50 mg/kg), 94.67% (150 mg/kg), and 91.65% (250 mg/kg). The result of the treatment with CaME (150 and 250 mg/kg) was at par with that of the dexamethasone, which showed an inhibition of 90.27%.

As shown in Figure 2D, treatment with CaME (150 and 250 mg/kg) and dexamethasone proved to be beneficial in the CIA associated weight loss in comparison to the vehicle treated arthritic rats. This suggests that CaME is not toxic at the given dosages. Besides, CaME was able to positively affect the weight gain of CIA-immunized rats in a dose-dependent manner. The unimmunized control rats showed a gradual gain of body weight with time.

Histopathology and Radiographic Analysis of CIA Rats Treated with CaME

The radiographic images of hind-limbs of CIA rats demonstrated severe soft tissue swelling, edema, joint, and bone erosion alongwith osteophyte formation. The representative roentgenograms of hind-limbs of CIA rats are shown in Figure 3 (A-B). The rats of CIA + vehicle group showed joint damage

in the form of joint space narrowing, and bone erosion. In contrast, the rats treated with CaME (150 mg/kg) and dexamethasone ($P<0.0001$) brought about significant reduction in the severity of all of these clinical indices thereby exerting a protective effect in the amelioration of CIA.

Figure 3(E-H) shows the representative joint histopathology of the experimental groups. The tibio-femoral joints of vehicle-treated CIA rats showed noticeable pathological changes of severe arthritis like synovial hyperplasia, massive mixed inflammatory cell infiltrate as well as extensive pannus formation. The orally administered CaME (150 and 250 mg/kg) inhibited these histopathological events in a dose-dependent manner, thus inducing a substantial recovery in the joints of CIA rats. No significant effect was observed in the group of rats treated with 50 mg/kg of CaME (data not shown). Only the representative photographs of CaME (150 mg/kg) treatment group are shown here. The rats of dexamethasone-treated group also revealed pronounced protection against joint destruction, cartilage, and bone erosion.

Effect of Therapeutic Treatment with CaME on Cytokine Levels in Plasma and Joints of CIA rats

In order to determine the effects of CaME on the production of pro-inflammatory cytokines closely

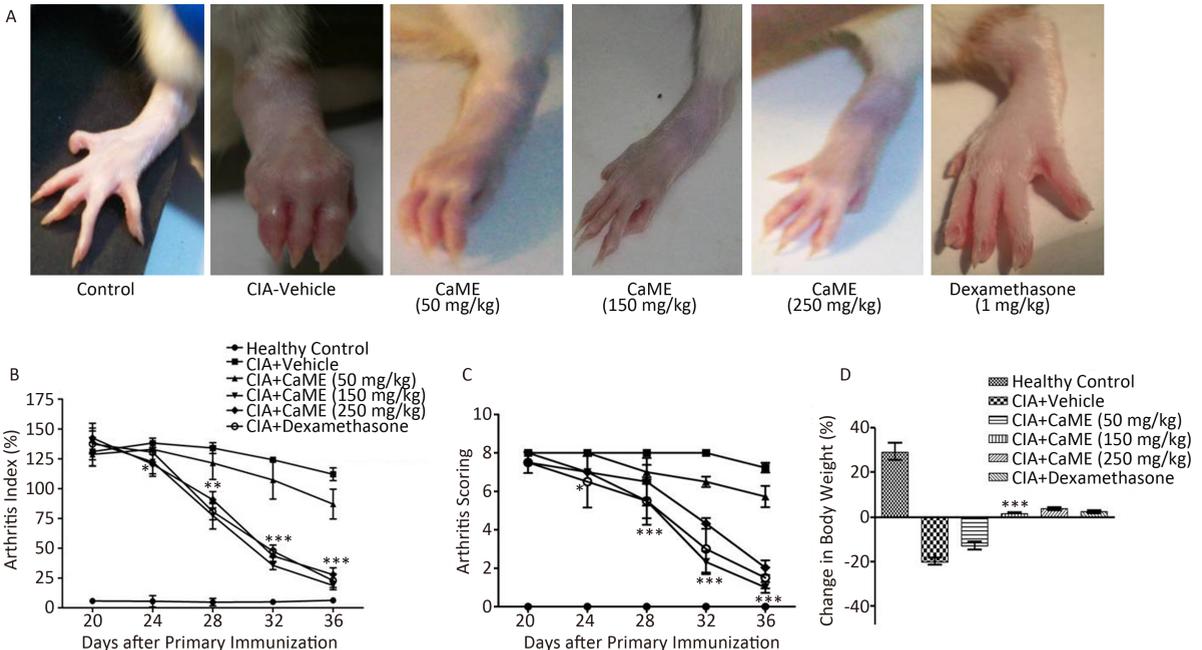


Figure 2. Effect of CaME on collagen-induced arthritis. (A) Representative photographs of rat hind paws, (B) arthritic index, (C) arthritic score and (D) change in body weight. Values are the mean \pm SD of 5 animals per group. $P<0.05$, $P<0.01$, and $P<0.001$, versus CIA+vehicle group, using student's *t*-test.

associated with joint inflammation and arthritis progression, we measured the plasma and joint levels of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6, and IL-12) in the vehicle or CaME-treated CIA rats on day 36. Consistent with joint swelling and macroscopic scoring, plasma and joint levels of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6, and IL-12) were increased significantly in the vehicle-treated CIA rats relative to non arthritic control rats, but the increase was inhibited in the CIA rats treated with CaME at a dosage of either 150 mg/kg ($P<0.01$) or 250 mg/kg ($P<0.01$) (Figure 4). No significant difference ($P>0.05$) was found between the two higher dosages (150 and 250 mg/kg). However, at the lowest dosage (50 mg/kg), the decline was not significant ($P>0.05$) in the plasma level of TNF- α , IL-1 β , and IL-6 except the plasma level of IL-12 ($P<0.05$). The effect of dexamethasone treatment on the levels of these cytokines followed the same trend as that of the morphological parameters.

Inhibition of Nitric Oxide and Anti-Collagen II Antibody in CIA Rats Treated with CaME

Results shown in Figure 5A revealed that inflammatory mediator, such as nitric oxide, was increased by 2.65 and 2.87 fold in plasma and joints of collagen-induced arthritic rats, respectively. This elevated nitric oxide production was not inhibited by treatment with CaME (50 mg/kg). Notably, a significant ($P<0.01$) inhibition of 2.91 and 2.15 fold in plasma and joints of CaME (150 mg/kg) treated arthritic rats was observed. No significant difference ($P>0.05$) was observed between the middle and higher dosage groups.

In CIA, the effect of CaME on the humoral immunologic component was assessed by measuring the anti-CII antibody titer. Serum anti-CII antibody titer significantly increased in the CIA rats as measured on day 36 (Figure 5B). In the group of rats treated with CaME (250 mg/kg), the anti-CII antibody

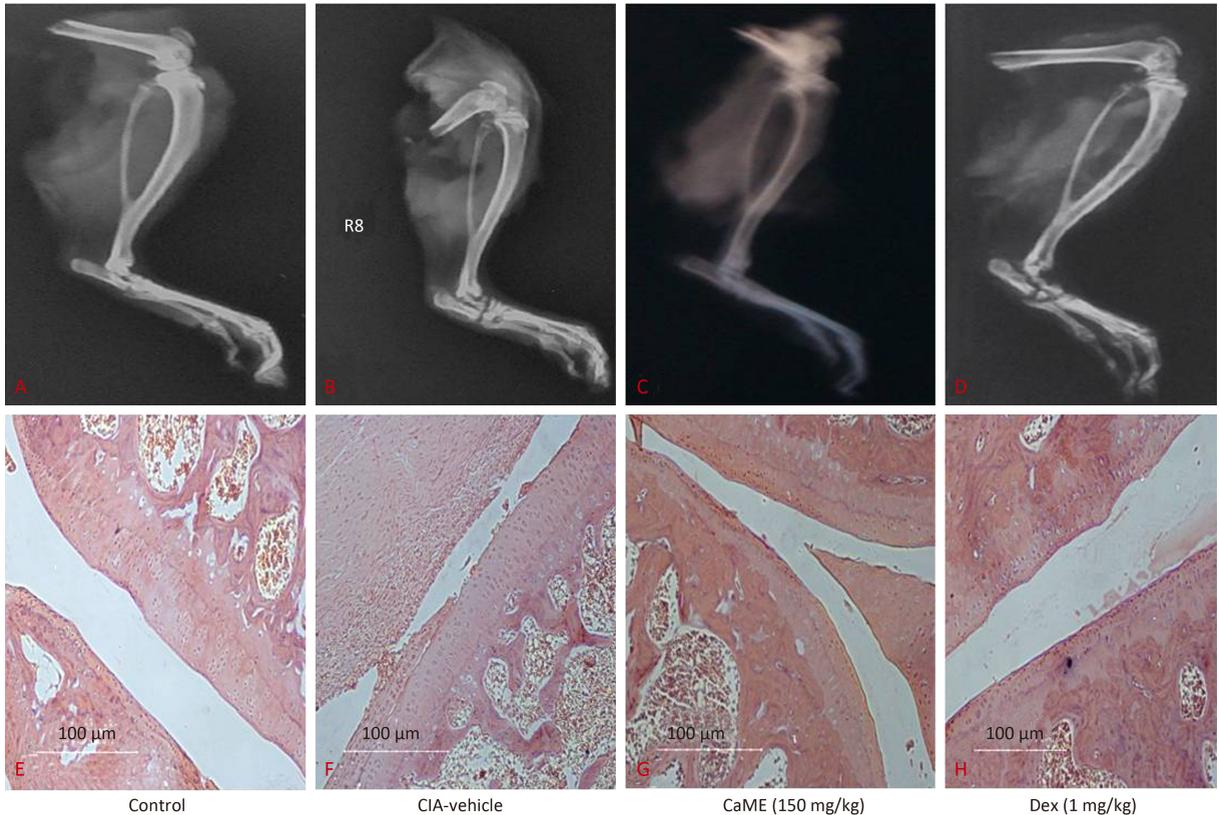


Figure 3. Effect of CaME on collagen-induced arthritis. Figure (A-D) represents representative roentgenograms and figure (E-H) represents photomicrographs of hematoxylin/eosin-stained section of rat joints from each experimental group. All these photographs are representative of $n=5$ animals per experimental group.

level was found to be significantly suppressed ($P < 0.05$). No significant difference was anti-CII antibody titers were found in the serum of control rats.

Antioxidant Effects of CaME in the Plasma and Joints of Arthritic Rats

Figure 6 shows the effects of different dosages of CaME on lipid peroxidation and protein oxidation in plasma and joints of normal and arthritic rats. The results showed that the level of malondialdehyde and protein carbonyl content was significantly higher

than those in the control group. The administration of CaME at doses of 150 and 250 mg/kg tended to bring the level of malondialdehyde and protein carbonyl content significantly toward normal values, while normal rats did not exhibit any significant alterations in these parameters during the experiment. No significant differences ($P > 0.05$) in the plasma and joints malondialdehyde and protein carbonyl content were observed in rats treated with CaME (50 mg/kg), when compared to arthritic rats. Both the dosages of CaME (150 and 250 mg/kg) were found to be similar effective with dexamethasone.

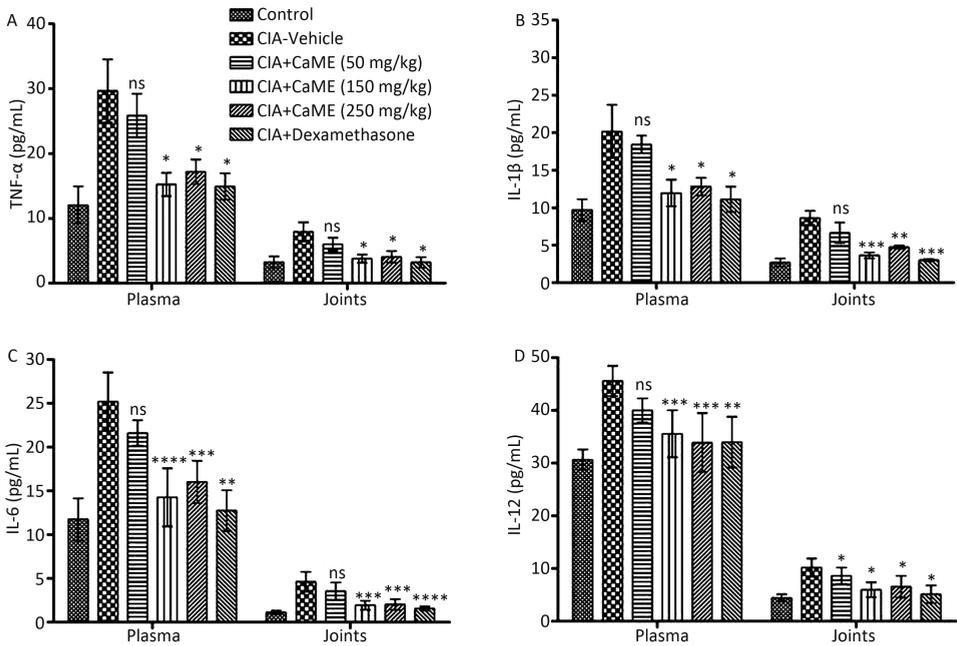


Figure 4. Effect of CaME and dexamethasone treatment on pro-inflammatory cytokines level in the plasma and joint. A) TNF- α , B) IL-1 β , C) IL-6, and D) IL-12. Results are expressed as the mean \pm SD of $n=5$ animals per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ versus CIA+vehicle group, using one-way ANOVA followed by Tukey's post test.

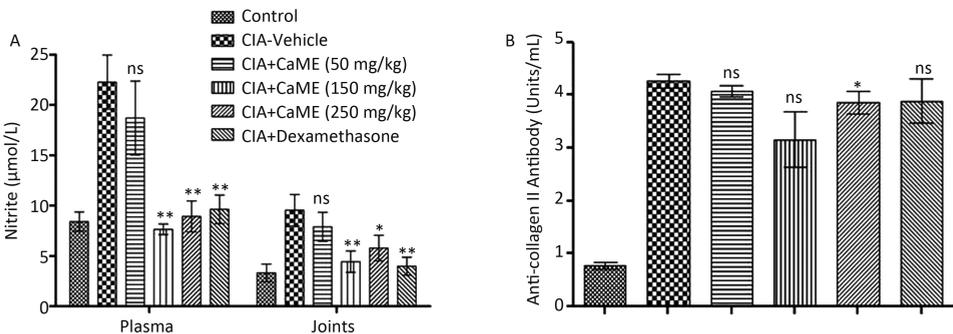


Figure 5. Efficacy of CaME and dexamethasone treatment on the plasma level of nitric oxide (A) and serum level of anti-collagen II antibody (B). * $P < 0.05$, ** $P < 0.01$, versus CIA+vehicle group, using one-way ANOVA followed by Tukey's post test.

DISCUSSION

Rheumatoid arthritis is a chronic, systemic inflammatory disorder that may cause progressive joint damage. It involves hyperplasia of synovial tissues and structural damage to cartilage, bone, and ligaments mediated by the toxic substances released in the synovium^[23]. The cornerstone therapy for inflammatory and immune disorders is non-steroidal anti-inflammatory drugs (NSAIDs) and disease modifying anti-rheumatic drugs (DMARDs). The limitations of these therapies are their well-known toxicity and variation in clinical efficacy. Hence, there is a need to explore the efficacy of various natural products against inflammation and particularly arthritis. In lieu of the fact that *C. asiatica* plant is already used in folklore medicine for the treatment of inflammatory disorders and the earlier reports on its anti-inflammatory and antioxidant property, it is worthwhile to assess the merit of free radical scavenging property and the anti-arthritic efficacy of CaME in both cell free system and an animal model of rheumatoid arthritis.

Prior to the *in vivo* evaluation, the antioxidant activities in cell free system of CaME including scavenging activity on superoxide, hydrogen peroxide, nitric oxide and, DPPH radicals along with inhibition of lipid peroxidation were investigated. Superoxide radicals are considered as the primary reactive oxygen species (ROS). These radicals by interacting with other molecules generate secondary ROS like hydroxyl, hydrogen peroxide, and singlet oxygen either directly or prevalently through enzyme or metal catalyzed processes^[24]. The higher percentage inhibition of superoxide radicals by CaME compared with the reference drug, BHT, suggests a marked and higher antioxidant activity. Similarly,

CaME is an effective scavenger of hydrogen peroxide radicals (no significant difference between the IC₅₀ values) at different magnitudes of potency. The protective role of CaME against both superoxide and hydrogen peroxide radicals can prevent oxidative damage in lipids, proteins, and DNA. Such observable effects may be related to its phenolic content, which have direct quenching effects on free radicals^[25]. Furthermore, CaME noticeably decreased the amount of nitrite generated by the decomposition of sodium nitroprusside and was found to be superior than BHT. The observed reduction of nitric oxide may be due to the antioxidant properties of CaME, which compete with oxygen to react with nitric oxide, thereby inhibiting the generation of peroxynitrite^[26].

The reducing power of a plant extract may serve as a significant indicator of its potential antioxidant activity. The concentration-dependent, high reducing power of CaME reflects its ability to reduce the transition state of iron and consequently, the rate at which superoxide and hydroperoxyl radicals are generated from the metal^[27]. A strong relationship between the total phenolic content and reducing activity in plant extracts has been reported. Therefore, the reducing power of the extract may be attributed to its phenolic content.

The scavenging activity of *C. asiatica* extracts on DPPH radicals was studied by exploring the ability of antioxidants present in the extracts to reduce the stable DPPH radical (purple) to the non-radical form DPPH-H (yellow). Although CaME was not found to be as efficient as BHT, but its activity was highest amongst all the other fractions. This can be due to the fact that hydrogen-donating compounds are more likely to be present in polar solvents. From this significantly high DPPH radical scavenging activity of

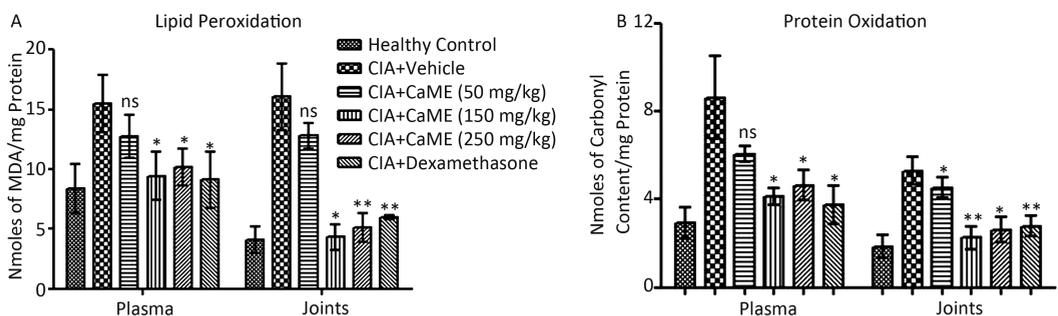


Figure 6. Effect of CaME on oxidative tissue damages in CIA rats. Levels of A) malondialdehyde, and B) protein carbonyl content were measured in the plasma and joints of rats from all the experimental groups. Data is expressed as the mean \pm SD of $n=5$ animals per group. * $P<0.05$, ** $P<0.01$, versus CIA+vehicle group, using one-way ANOVA followed by Tukey's post test.

could directly react with and quench DPPH radicals through its proton donating ability. Furthermore, the transition metal Fe^{2+} has been shown to induce oxyradical production and lipid peroxidation, that cause damage to cellular lipids, nucleic acids, proteins and carbohydrates leading to cellular impairment^[28]. Therefore, we have also studied the inhibitory effect of CaME on iron-ascorbate induced lipid peroxidation in rat liver cytosolic fraction. CaME was observed to be a better inhibitor of lipid peroxidation in comparison with BHT and this observable effect might be due to its direct scavenging effect on superoxide, hydrogen peroxide, and nitric oxide radicals alongwith the reduction of ferricyanide complex to ferrous form.

In the present study, we also made an attempt to further characterize the anti-arthritis effects and mechanisms underlying the activity of orally administered CaME. We focused on CIA-induced pro-inflammatory cytokines, nitric oxide, and oxidative damages via the immune responses of T cells and macrophages. Since CIA is the closest experimental model used to investigate the pathogenesis of RA and identification of potential therapeutic targets^[29-30], the *in vivo* studies for the demonstration of anti-arthritis effects of CaME were carried out using CIA rats. Our present data shows that among the various dosages of CaME screened, the 150 and 250 mg/kg dosages inhibited edema formation significantly and subsequently alleviated the arthritic symptoms. There was a significant reduction in arthritis severity compared with that of vehicle-treated CIA rats. To the best of our knowledge, this is the first report demonstrating the anti-arthritis effects of CaME. A significant reduction of the inflammation (articular index and score) in the hind paws of the immunized rats was observed in the treatment groups of CaME (150 and 250 mg/kg) and dexamethasone when compared to their untreated counterparts, suggesting the anti-arthritis potential of CaME at two dosages. Major reduction of paw swelling observed from day 25 onwards might be due to immunological protection rendered by CaME. The CIA inhibition by CaME at the dosage of 150 mg/kg (94.67%) was found to be similar to that by dexamethasone (90.27%). CaME (150 and 250 mg/kg) treatment reversed the loss of body weights alongwith disease progression as opposed to the untreated CIA groups, which showed a drastic loss of body weight. The group of rats treated with dexamethasone also showed weight loss during the experimental period. This clearly shows the lower

toxicity and recuperative effects of CaME at the given dosages. CaME treatment also dramatically alleviated other pathological symptoms of joints of CIA rats, including infiltration of inflammatory cells into the joint cavity, synovial hyperplasia, pannus formation, and erosion of bone and cartilage as evidenced by histopathological and radiographic data. This study is comparable to previous studies, which showed that *C. asiatica* fractions and madecosside, main effective component of *C. asiatica* when given to arthritic rats, showed no toxicity alongwith the diminution of arthritic symptoms^[11-12]. Based on these findings, we therefore, support a view that the therapeutic effectiveness of CaME might be due to the presence of phenolic and flavonoid contents in it. Studies have suggested a strong relationship between antioxidant activity of plant extracts and their phenolic content, probably due to their redox properties, which allows them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers^[31]. Some phenolics, flavonoids, and the active constituents of *C. asiatica* being asiatic acid, madecosside and asiaticoside type A and B have been reported to possess significant anti-inflammatory, antioxidant, and wound-healing properties. Besides, studies clearly show that *C. asiatica* extracts contain significantly greater amounts of madecosside, that has a substantial role in preventing mice CIA^[11-12]. So, it can be speculated that the combined action of these asiaticosides, multiple phenolic and flavonoid compounds present in CaME might be responsible for its beneficial effects for RA remedy.

One of the most effective ways to screen plant fractions that might be of use in inhibiting the inflammatory arthritis is to look for their ability in modulating cytokines and antioxidant enzymes. Several areas of investigation have shown that in both RA and CIA, the primary site of inflammation is the synovial tissue and the inflamed synovium consists of diverse cell populations, including B cells, T cells, macrophages, synovial fibroblasts (SF), endothelial cells, and plasma cells, all of which can produce abundance of cytokines^[32]. $\text{TNF-}\alpha$ is also released by superoxide radicals (via mechanisms not yet fully defined). $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ play central role in the maintenance of chronic inflammation and tissue damage during the progression of both CIA and RA. $\text{TNF-}\alpha$, being the primary agent in the inflammatory process, is involved in differentiation and proliferation of T and B cells, and bone resorption^[4], whereas interleukin- 1β is responsible for the destruction of cartilage and bone^[33]. $\text{TNF-}\alpha$ is

also involved in leukocyte recruitment to the articulations and regulates nitric-oxide synthase-2 and cyclooxygenase-2 expression in the synovial tissue and cartilage of arthritic rats. The induction of iNOS, in turn leads to the production of nitric oxide both in synovial tissue and blood mononuclear cells. Recent *in vivo* studies have shown that apart from TNF- α and IL-1 β , there is another pro-inflammatory cytokine, IL-6 that also perpetuates this chronic inflammatory process by mediating joint destruction, leukocyte recruitment, apoptosis, and T cell activation. So, in the endeavor to demonstrate whether administration of CaME suppresses the immune-modulated pathologic process in CIA, we investigated its effect on the level of certain pro-inflammatory cytokines and nitric oxide in plasma and joints of all experimental rats. We confirm here that the onset of CIA was accompanied with a substantial increase in the levels of TNF- α , IL-1 β , IL-6, and IL-12 in both plasma and joints of rats. Interestingly, in our study, we found that treatment with CaME significantly decreased the elevated level of cytokines TNF- α , IL-1 β , IL-6, and IL-12. Besides, the abnormally elevated level of nitric oxide in vehicle-treated CIA rats was found to be restored to basal values in the CaME-treated rats. This might be a secondary effect due to the reduced formation of endogenous TNF- α and IL-1 β . It is therefore proposed that CaME, being an important negative regulator of these pro-inflammatory cytokines related to RA might deactivate the inflammatory response of infiltrating and proliferating synovial cells. Inhibition of neutrophil infiltration may be another mechanism by which CaME achieves its anti-inflammatory effect. Histopathological study also revealed that severe edema formation and elevated level of cellular infiltration in arthritic rats were decreased on treatment with CaME, which supported above results. Hence, it is conceivable to suggest that part of the beneficial anti-inflammatory and cartilage/bone protective effects of Rg3 may be mediated through the inhibition of TNF- α and IL- β . In line with the findings of Choi and Kim^[29], we have also obtained lower serum titers of type-II collagen antibody in CaME-treated rats compared to those of the control rats. It has been reported that type-II-collagen-specific IgG efficiently fixes complement factors and binds to cartilage, leading to joint damage.

There is mounting evidence which shows that oxygen metabolism plays an important role in the progression of arthritis. Oxygen free radicals have been implicated as mediators of joint tissue and

cartilage damage in RA by changing the antioxidant systems of various tissues. An accelerated production of ROS and RNS can damage protein, lipids, nucleic acids, and matrix components, thereby amplifying the synovial inflammatory- proliferative-response. Lipid peroxidation can reduce membrane fluidity, inactivate membrane-bound proteins, and decompose into cytotoxic aldehydes such as malondialdehyde or hydroxynonenal^[34] whereas the oxidative damage to proteins is reflected by increase in levels of protein carbonyls. In our study also, the induction of arthritis in rats significantly increased malondialdehyde and protein carbonyl content in plasma and joints. The CaME treatment significantly abrogated these effects. This shows that lipid peroxidation and protein oxidation are closely associated with arthritis and CaME treatment effectively prevented the oxidation damage. The inhibition of free radicals in the inflamed joints may be due to the reduced cell influx in arthritic joints such as the PMNs infiltration. The *in vitro* lipid peroxidation inhibitory activity of CaME (as discussed above) in rat liver homogenate is also in line with the above findings.

CONCLUSION

The results obtained in the present work indicate the remarkable anti-arthritis activity present in CaME, which is comparable with the standard anti-inflammatory drug, dexamethasone. To the best of our knowledge, this is the first schematic report demonstrating here that CaME is able to scavenge superoxide, hydrogen peroxide, nitric oxide, and DPPH radicals, has high reducing power, total antioxidant capability, and conferred protection against biological macromolecular damage. Afterwards, CaME (150 and 250 mg/kg) exhibited high anti-inflammatory and antioxidant activities both *in vitro* and *in vivo*. The oral administration of CaME inhibited CIA progression by reducing the production of pro inflammatory cytokines, nitric oxide, and oxidative stress without any toxicity. The direct oxygen free-radical scavenging activity of CaME might also contribute to its *in vivo* antioxidant activity. Therefore, in light of the above findings, CaME should be considered as a new source of anti-arthritis agent/natural antioxidant for clinical application/dietary needs.

ACKNOWLEDGEMENT

We wish to acknowledge the financial assistance

received from University Grants Commission to undertake the present study.

Received: April 8, 2014;

Accepted: August 11, 2014

REFERENCES

- Raghav SK, Gupta B, Shrivastava A, et al. Inhibition of lipopolysaccharide-inducible nitric oxide synthase and IL-1 β through suppression of NF-kappaB activation by 3-(1'-1'-dimethyl-allyl)-6-hydroxy-7-methoxy-coumarin isolated from *Ruta graveolens* L. Eur J Pharmacol, 2007; 560, 69-80.
- Kim KR, Park KK, Chun KS, et al. Honokiol inhibits the progression of collagen-induced arthritis by reducing levels of pro-inflammatory cytokines and matrix metalloproteinases and blocking oxidative tissue damage. J Pharmacol Sci, 2010; 114, 69-78.
- Karatas F, Ozates I, Canatan H, et al. Antioxidant status and lipid peroxidation in patients with rheumatoid arthritis. Indian J Med Res, 2003; 118, 178-81.
- Rioja I, Bush KA, Buckton JB, et al. Joint cytokine quantification in two rodent arthritis models: kinetics of expression, correlation of mRNA and protein levels and response to prednisolone treatment. Clin Exp Immunol, 2004; 137, 65-73.
- Williams RO, Inglis JJ, Simelyte E, et al. Analysing the effect of novel therapies on cytokine expression in experimental arthritis. Int J Exp Pathol, 2005; 86, 267-78.
- Yeom MJ, Lee HC, Kim GH, et al. Anti-arthritis Effects of *Ephedra sinica* STAPF Herb-Acupuncture: Inhibition of lipopolysaccharide-induced inflammation and adjuvant-Induced polyarthritis. J Pharmacol Sci, 2006; 100, 41-50.
- Handa SS, Deepak M, Mangal AK. Indian Herbal Pharmacopoeia, Indian Drug Manufacture. 1988. Mumbai and Regional Res. Lab; Jammu-Tawi, India Centella asiatica, 47-55.
- Winston D, Maimes S. Adaptogens: Herbs for strength, stamina, and stress relief. Healing Arts Press, 2007; 226-7.
- Bradwejn J, Zhou Y, Koszycki D, et al. A double-blind, placebo-controlled study on the effects of Gotu Kola (*Centella asiatica*) on acoustic startle response in healthy subjects. J Clin Psychopharmacol, 2000; 20, 680-4.
- Brinkhaus B, Lindner M, Schuppan D, et al. Chemical, pharmacological and clinical profile of the East Asian medical plant *Centella asiatica*. Phytomedicine, 2000; 7, 427-48.
- Li H, Gong X, Zhang L, et al. Madecassoside attenuates inflammatory response on collagen-induced arthritis in DBA/1 mice. Phytomedicine, 2009; 16, 538-46.
- Liu M, Dai Y, Yao X, et al. Anti-rheumatoid arthritic effect of madecassoside on type II collagen-induced arthritis in mice. Int Immunopharmacol, 2008; 8, 1561-6.
- Wolfe K, Wu X, Liu RH. Antioxidant activity of apple peels. J Agr Food Chem, 2003; 51, 609-14.
- Lin JY, Tang CY. Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation. Food Chem, 2007; 101, 140-7.
- Afolayan AJ, Aboyade OM, Sofidiya MO. Total phenolic content and free radical scavenging activity of *Malva parviflora* L (Malvaceae). J Biol Sci, 2008; 8, 945-9.
- Kumari A, Kakkar P. Screening of antioxidant potential of selected barks of Indian medicinal plants by multiple *in vitro* assays. Biomed Environ Sci, 2008; 21, 24-9.
- Ogunlana OE, Ogunlana OO. *In vitro* assessment of the free radical scavenging activity of *Psidium guajava*. Research Journal of Agriculture and Biological Sciences, 2008; 4, 666-71.
- Temraz A, El-tantawy WH. Characterization of antioxidant activity of extract from *Artemisia vulgaris*. Pak J Pharm Sci, 2008; 21, 321-6.
- Sanja SD, Sheth NR, Patel NK, et al. Characterization and evaluation of antioxidant activity of *Portulaca oleracea*. Int J Pharm Pharm Sci, 2009; 1, 74-84.
- Qu ZQ, Zhou Y, Zeng YS, et al. Pretreatment with *Rhodiola Rosea* Extract Reduces Cognitive Impairment Induced by Intracerebroventricular Streptozotocin in Rats: Implication of Anti-oxidative and Neuroprotective Effects. Biomed Environ Sci, 2009; 22, 318-26.
- Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal Biochem, 1999; 269, 337-41.
- Sahu D, Saroha A, Roy S, et al. Suramin ameliorates collagen-induced arthritis. Int immunopharmacol, 2012; 12, 288-93.
- Choi EM, Kim YH. A preliminary study of the effects of an extract of *Ligularia fischeri* leaves on type II collagen-induced arthritis in DBA/1J mice. Food Chem Toxicol, 2008; 46, 375-9.
- Bauerová K, Bezek A. Role of reactive oxygen and nitrogen species in etiopathogenesis of rheumatoid arthritis. Gen Physiol Biophys, 1999; 11, 15-20.
- Thuong PT, Su ND, Ngoc TM, et al. Antioxidant activity and principles of Vietnam bitter tea *Ilex kudingcha*. Food Chem, 2009; 113, 139-45.
- Kumaran A, karunakaran RJ. Antioxidant and free radical scavenging activity of an aqueous extract of *Coleus aromaticus*. Food Chem, 2006; 97, 109-14.
- Rizzo AM, Berselli P, Zava S, et al. Endogenous antioxidants and radical scavengers. Adv Exp Med Biol, 2010; 698, 52-67.
- Mathew S, Abraham TE. Studies on the antioxidant activities of cinnamon (*Cinnamomum verum*) bark extracts, through various *in vitro* models. Food Chem, 2006; 94, 520-8.
- Choi EM, Kim YH. A preliminary study of the effects of an extract of *Ligularia fischeri* leaves on type II collagen-induced arthritis in DBA/1J mice. Food Chem Toxicol, 2008; 46, 375-9.
- Hitchon CA, El-Gabalawy HS. Oxidation in rheumatoid arthritis. Arthritis Res Ther, 2004; 6, 265-78.
- Samak G, Shenoy RP, Manjunatha SM, et al. Superoxide and hydroxyl radical scavenging actions of botanical extracts of *Wagatea spicata*. Food Chem, 2009; 115, 631-4.
- Fox DA, Gizinski A, Morgan R, et al. Cell-cell interactions in rheumatoid arthritis synovium. Rheum Dis Clin North Am, 2010; 36, 311-23.
- Cuzzocrea S, McDonald MC, Mota-Filipe H, et al. Beneficial effects of tempol, a membrane permeable radical scavenger, in a rodent model of collagen-induced arthritis. Arthritis Rheum, 2000; 43, 320-8.
- Sanocka D, Kurpisz M. Reactive oxygen species and sperm cells. Reprod Biol Endocrinol, 2004; 23, 2-12.