Evaluation of Anti-inflammatory Effect of Careya arborea in CFA Induced Chronic Inflammation

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ABSTRACT

The objective of the study was to explore the anti-inflammatory effect of methanolic extract of stem bark of Careya arborea Roxb, a plant used locally in India for various painful inflammatory conditions; using chronic inflammatory model of Complete Freund’s Adjuvant (CFA) induced chronic inflammation in rats. The anti-inflammatory activities of methanol extract of C. arborea (MECA) at doses of 100 and 200 mg/kg, p.o. were investigated in CFA induced inflammation using Indomethacin (5 mg/kg, p.o.) as reference drug. Inflammation was induced by injecting 0.1 ml of CFA containing 5 mg/ml of heat killed Mycobacterium tuberculosis into the sub plantar region of the left hind paw. Treatment with the extract and standard was started on the day of induction of inflamogens and continued up to 28 days. The effect of MECA on the production of nitric oxide, myeloperoxidase, gamma glutamyl transferase, malondialdehyde and C-reactive protein were determined. Oral administration of MECA (100 and 200 mg/kg) significantly reduced paw volume and tibio-tarsal joint diameter (p < 0.001) when compared with CFA control. The score of arthritic index in groups received methanolic extract (100 and 200 mg/kg) and Indomethacin (5 mg/kg) treatment decreased significantly when compared with CFA control (p < 0.01, p < 0.001 and p < 0.001, respectively). Moreover, the levels of nitric oxide, myeloperoxidase, gamma glutamyl transferase, malondialdehyde and C-reactive protein were significantly down-regulated after administration of methanolic extract of C. arborea. The biochemical findings were further supported by the radiographic changes. In conclusion, our findings suggest that methanolic extract of C. arborea stem bark exhibited potent anti-inflammatory effects in CFA induced chronic inflammation.

Keywords: Careya arborea, Chronic inflammation, Malondialdehyde, Myeloperoxidase, Nitric oxide, γ-glutamyl transferase.

INTRODUCTION

Arthritis, an autoimmune disorder, is a chronic inflammatory disease which manifests itself in multiple joints of the body. The inflammatory process primarily affects the lining of the joints (synovial membrane), but can also affect other organs. The autoimmune antibodies form immune-complexes with self-antigens in local joints, triggering the activation of complement and R signaling pathway, which leads to local inflammation and proliferation of synovium. The inflamed synovium leads to erosions of the cartilage and bone, and sometimes to joint deformity. In addition, neutrophils and macrophages recruited to the local joints secrete TNF-α and IL-1, which contribute to cartilage and bone tumor necrosis factor- destruction. The modern drugs both steroidal and non-steroidal anti-inflammatory drugs are used for the amelioration of the symptoms of the disease, however they offer only temporary relief and also produce variable side effects. Pro-inflammatory mediators such as nitric oxide, as well as pro-inflammatory enzymes such as myeloperoxidase, γ-glutamyl transferase, are involved in the inflammatory response and they are the future target of studies on drugs that have potential anti-inflammatory properties.

C. arborea Roxb. (Lecythidaceae) has multiple applications in traditional medicine because it exhibits analgesic, antibacterial, anti-inflammatory, anti-ulcer, and hepatoprotective effects. It is a large tree found throughout India in deciduous forests and grassland. The plant has been extensively investigated and a number of chemical constituents from the stem barks, leaves and seeds of the plant have previously been reported which includes triterpenoids, flavonoids, sterols, coumarin, saponins, and tannins. Stem bark of C. arborea is traditionally used in tumors, inflammation, anthelmintics, bronchitis, epileptic fits, astringents, antidote to snake-venom, skin disease, diarrhea, dysentery with bloody stools, dyspepsia, toothache and ear pain. The leaves are useful in ulcers. Pharmacological activity and mode of action of the plant in inflammation have yet to be established. C. arborea methanolic extract was previously tested for anti-inflammatory activity using carrageenan induced paw edema. However, C. arborea was not tested using chronic inflammatory models to prove its efficacy. The scientific studies are vital to work out the actual efficacy and to explore their scope for future use if they come out to be really effective. The present study was focused to prove the therapeutic potential of C. arborea as an anti-arthritis agent against Complete Freund's adjuvant (CFA) induced inflammation. Further, the possible mechanisms of the anti-inflammatory effects of C. arborea was investigated with special focus on the formation of some important inflammatory mediators such as of nitric oxide, myeloperoxidase, gamma glutamyl transferase, malondialdehyde and C-reactive protein.
MATERIALS AND METHODS

Plant material
The stem bark of *C. arborea* was collected Kanyakumari district, Tamil Nadu, India, during the month of April, 2011. The plant was identified by Mr. V. Chellandurai, Research officer (Botanist), FMR, AYUSH, India and the specimen was deposited in the Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Jamia Hamdard, New Delhi-110062, India.

Drugs and chemicals
All HPLC grade solvents were purchased from Merck (Darmstadt, Germany). Complete Freund’s Adjuvant from Chondrex, Inc. (USA). Indomethacin, thiobarbituric acid, trichloroacetic acid, sulphanilamide, N-1-naphthylethylene diaminedihydrchloride, hexadecyl trimethylammonium bromide, o-dianisidine hydrochloride, γ-glutamyl-p-nitroanilide and other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rat CRP ELISA assay kit was purchased from Immunology Consultants Laboratory, Inc. (Portland, Oregon, USA).

Preparation of plant extract
The dried powdered material was extracted with 2L methanol for 8h at 40°C by using the Soxhlet apparatus. The solvent was removed at the reduced pressure with the help of rotary vacuum evaporator to yield dark brown residue (67.35 g, 22.25%). The solid methanolic extract of *C. arborea* (MECA) was stored in refrigerator and reconstituted later for the various studies.

Animals
Male albino Wistar rats (180-220 g) were used for the study. They were housed in polypropylene cages under standard laboratory conditions (12 h light-12 h dark, 21 ± 2°C). The animals were fed in a standard pellet diet and water ad libitum. The experimental study was approved by the Institutional Animal Ethical Committee (754/CPCSEA, 2011) and the care of laboratory animal was taken as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Induction of complete Freund’s adjuvant (CFA) induced arthritis
Arthritis was induced by injecting (subcutaneous) a 0.1 ml of CFA containing 5 mg/ml of heat killed *Mycobacterium tuberculosis* into the sub plantar region of the rat left hind paw [19]. The animals were divided into five groups, each consisting of six animals and received following treatment;

- **Group I**: Normal control rats (1 ml/kg, p.o)
- **Group II**: CFA Control rats (0.1 ml of CFA+ 1 ml/kg normal saline, p.o)
- **Group III**: Indomethacin treated rats (0.1 ml of CFA + 5 mg/kg Indomethacin, p.o)

- **Group IV**: MECA treated rats (0.1 ml of CFA + 100 mg/kg MECA, p.o)
- **Group V**: MECA treated rats (0.1 ml of CFA + 200 mg/kg MECA, p.o)

Treatments were given to the animals 30 minutes before the administration of CFA and continued till 28th day.

The progression of Complete Freund’s adjuvant induced arthritis was evaluated by measuring the clinical parameters. The swelling in the hind paw from the ankle was measured before induction of arthritis and periodically on 7, 14, 12 and 28th day after CFA injection using plethysmometer (Ugo Basile, Comerio VA, Italy) and Percent inhibition was calculated using the following formula.

\[
\frac{V_e - V_t}{V_e} \times 100
\]

Where \(V_e\) and \(V_t\) represent the mean increase in paw volume in CFA control and treated groups, respectively.

The joint diameter was measured in millimeters with the help of vernier calipers and change in joint diameter was calculated. The percentage of inhibition of the edema volume and joint diameter of the injected paw was measured using the formula described above. Secondary lesions and immunologically mediated changes were characterized by inflammation on the injected and non injected sites (hind legs, forepaws, ears, nose and tail) and nodules formation on the days 7, 14, 21 and 28 according to the method of Schorlemmer.

Estimation of nitric oxide (NO) level in paw tissue of CFA induced inflamed rats
NO was measured by means of the Griess method [21]. Briefly 10% paw tissue was homogenized in ice-cold PBS (pH 7.4) and centrifuged at 3000 rpm for 10 min at 4°C. The resulting supernatant was used to estimate NO. 50 µl of each experimental sample was added to a 96 well plate in duplicate. Similarly 50 µl of standard sodium nitrite (50, 25, 12.5, 6.25, 3.125 and 1.56 µM/ml) was placed in different well, in duplicate. After this 50 µl of standard sodium nitrite solution (1% Sulphanilamide in 5% phosphoric acid) was dispensed to all the wells containing experimental samples and nitrite standards.

The plate was incubated for 5-10 min at room temperature, protected from light. Following this 50 µl of the NED (0.1% N-1-Naphthylethylendiamine dihydrochloride in distill water) solution was dispensed in all wells. The plate was incubated again for 5-10 min at room temperature, protected from light. Absorbance was measured within 30 min in an ELISA plate reader with a filter of 520 nm.
Estimation of myeloperoxidase (MPO) level in paw tissue of CFA induced inflamed rats
Tissue neutrophil infiltration was quantified by measuring the MPO activity using a Spectrophotometric method as proposed by Bradley. Briefly, 10% paw tissue was homogenized in homogenizing solution containing 50 mM potassium phosphate buffer (pH 6.0) with 0.5% hexadecyl trimethylammonium bromide and 5 mM EDTA. The homogenate was sonicated and centrifuged at 15000 g for 15 min at 4°C. The supernatant was mixed in a ratio of 1:15 with assay buffer comprising 100 mM potassium phosphate buffer (pH 6.0), 0.167 mg o-dianisidine ml⁻¹ and 0.0005% hydrogen peroxide. MPO activity was assayed by measuring the change in A460 from 0 min to 4 min over intervals of 30 s. MPO level was calculated by using an absorption coefficient of o-dianisidine 11.3 mM⁻¹ cm⁻¹ at 460 nm and was reported as µM/ml.

Estimation of γ-glutamyl transferase (GGT) activity in paw tissue of CFA induced inflamed rats
The activity of cellular γ-glutamyltransferase (GGT) in hind paw joint tissue homogenate was measured by the method of Orlowski & Meister as modified by Ondrejckova et al. Samples were homogenized in a buffer at 1:9 w/v (buffer composition: 2.6 mM Na₂HPO₄; 50 mM NaH₂PO₄; 15 mM EDTA; 68 mM NaCl; pH 8.1) for 1 min at 4°C. Substrates (8.7 mM γ-glutamyl-p-nitroanilide, 44 mM methionine) were added to 300 µl of plasma, then mixed and incubated in a water bath at 90°C for 30 min. The reaction was stopped by dipping the test tubes into ice for 10 min. Samples were centrifuged at 3 000 rpm. The supernatant was removed and absorbance measured at 406 nm. Reaction mixtures in the absence of either the substrate or acceptor were used as reference samples and it was expressed as nM 4-nitroaniline/min/g of tissue.

Estimation of malondialdehyde (MDA) in plasma of CFA induced inflamed rats
Lipid peroxidation in plasma was measured spectrophotometrically by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA). The amount of 1.5ml of 0.67% thiobarbituric acid, 1.5 ml of 20% trichloroacetic acid, 700 µl of phosphate buffer (pH 7.4) were added to 300 µl of plasma, then mixed and incubated in a water bath at 90°C for 30 min. The reaction was stopped by dipping the test tubes into ice for 10 min. Samples were centrifuged at 3 000 rpm. The supernatant was removed and absorbance measured at 535 nm in a 1 cm cuvette. The level of MDA was calculated based on the absorbance coefficient of TBA-MDA complex (ε = 1.56 x 105cm⁻¹M⁻¹) and it was expressed as nM/ml.

Estimation of C - reactive protein (CRP) in plasma of CFA induced inflamed rats
Plasma concentration of CRP was determined by Rat CRP ELISA (Immunochemistry Consultants Laboratory, Inc., Portland, OR, USA). Measurements were performed according to the manufacturer’s protocol.

Radiography
Wistar rats were sacrificed on 28th day of Freund’s complete adjuvant administration and legs are removed and placed on formalin containing plastic bag. This plastic bag was kept at a distance of 100 cm from the X-ray source, the radiographic analysis of normal and arthritic rat hind paws was performed by X-ray machine with a 300-mA exposition for 0.01 s.

Statistical analysis
All the results were expressed as mean ± standard error mean (SEM). Data were analyzed using one-way ANOVA followed by Tukey post test (GraphPad InStat 3 software). Values of p < 0.05 were considered as statistically significant.

RESULTS
Effect of MECA on CFA induced inflammation
The paw volume of CFA control was significantly increased whereas significant (p < 0.001) decrease in paw volume was observed in treatment groups as compared to CFA control group (Figure 1). Administration of MECA at the dose of 100 and 200 mg/kg to arthritic rats reduced paw volume significantly (p < 0.001) and exhibited 35.51% and 65.74% of inhibition, respectively as compared to CFA control group at the end of the study period.

Figure 1: Effect of MECA and indomethacin on paw volume of CFA induced inflammation
Each value is Mean ± S.E.M (n = 6). * p < 0.05, ** p < 0.01, *** p < 0.001. ns = Non significant when compared to untreated CFA control.

Indomethacin (5 mg/kg) caused profound inhibition of the tibio-tarsal joint diameter (62.67%) compared with CFA control animals. MECA (100 and 200 mg/kg) significantly (p < 0.001) inhibited this inflammatory response at the end of the study period. The percent of inhibition was 38.03% and 59.92%, respectively (Figure 2).

Signs and symptoms of arthritis appeared within 3 days after the injection of complete Freund’s adjuvant. The peak of edema, redness and stiffness in movement was on day 21. Score of arthritis showed in Figure 3. Oral administration of MECA at doses of 100 and 200 mg/kg
for 28 days decreased the score of arthritis significantly (p < 0.01 and p < 0.001, respectively).

The activity of γ-glutamyl transferase (GGT) was significantly increased in CFA induced inflamed rats paw tissue as compared with normal control (p < 0.01). MECA (100 and 200 mg/kg) significantly decreased the GGT activity in inflamed paw tissues as compared with CFA control (p < 0.01 and p < 0.001, respectively) when compared with diseased control group.

Markers of redox imbalance in plasma (MDA) were significantly increased (p < 0.001) by CFA administration as compared to normal control (Figure 6B). MECA (100 and 200 mg/kg) treatment significantly decreased (p < 0.01 and p < 0.001, respectively) the level of MDA in CFA rats when compared with CFA control.

As shown in the results (Figure 6B), plasma level of CRP was significantly (P<0.001) elevated in CFA control and treated animals as compared to normal control group. While treating with MECA (100 and 200 mg/kg) and Indomethacin (5 mg/kg), there was a significant (P<0.001, P<0.001 and P<0.001, respectively) reduction in CRP levels as compared to diseased control group.
Rheumatoid arthritis is an autoimmune disorder, the immunologically mediated complete Freund’s adjuvant induced arthritic model of chronic inflammation is considered as the best available experimental model of rheumatoid arthritis. Complete Freund’s adjuvant-induced arthritis is a model of chronic polyarthritis with features that resemble rheumatoid arthritis. The determination of paw swelling is apparently simple, sensitive and quick procedure for evaluating the degree of inflammation and assessing of therapeutic effects of drugs. In our study the methanolic extract of C. arborea exhibited a significant anti-arthritic activity in a dose dependent manner. Meca suppressed the chronic phase of inflammation significantly when compared with the CFA control group. A similar pattern was observed in the animals treated with Indomethacin at a dose of 5 mg/kg. In the present study, we showed that MECA could significantly inhibit the progression of the arthritis in treated animals. However, standard drug and methanolic extract significantly suppressed the signs and symptoms of rheumatoid arthritis such as difficulty in movement and edema in chronic phase which may be due to the suppression of inflammatory mediator released due to induction of Freund’s adjuvant. Moreover, the mean scores for each group of drug treated animals were compared with that of CFA control animals. In CFA control group, arthritic index was significantly higher compared to normal control group while MECA and Indomethacin treated groups showed significantly fewer score as compared to model control group. These results indicate the anti-inflammatory effect and immunosuppressant properties of test drug against adjuvant-induced arthritis.

NO is a major product and its production is controlled by the nitric oxide synthases (NOS), which include iNOS, eNOS and nNOS. Most importantly, iNOS is highly expressed in macrophages; its activation leads to organ destruction in inflammatory and autoimmune diseases. The inducible isom, inducible NOS (iNOS) when activated is expressed for a longer period of time. It is activated by a variety of factors such as the cytokines, microbial products (e.g., endotoxin), immune complexes, and others. NO is reported to induce the production of cytokines such as TNF-α, IL-1β and IFN-γ in arthritis patients. The inhibition of nitric oxide production is considered to be a promising approach to the treatment of various diseases including inflammation and cancer. Therefore, in the present study the reduction of NO production might be due to down regulation of iNOS by MECA.

At the site of inflammation, myeloperoxidase (MPO) level in the hind paw joint homogenate of arthritic rats was approx. 4-times higher than in normal control. This finding is of importance as MPO is the most abundant enzyme in neutrophils. It is a marker of oxidative stress and reactive oxygen species. MPO can deplete the NO synthesis. NO is considered to be a promising approach to the treatment of various diseases including inflammation and cancer. Therefore, in the present study the reduction of NO production might be due to down regulation of iNOS by MECA.

GTT is an important component of inflammatory processes since its activity is closely connected with the overall antioxidant status of the organism. In earlier study increased activity of GTT was observed in adjuvant...
arthritic rat paw tissue.\textsuperscript{35-39} We found that the activity of GGT in paw tissue was approx. 3.5 times higher in arthritic animals than in normal control. The increased activity of GGT in CFA control might be due to elevation of systemic oxidative stress. MECA was significantly effective in suppressing the increased activity of GGT in the paw tissue, as expected from its antioxidant potential.\textsuperscript{37}

A potential marker of lipid peroxidation is MDA assessed as an adduct with TBA. Clinical studies have shown increased plasmatic levels of MDA in patients with rheumatoid arthritis.\textsuperscript{39,40} In animal models of arthritis, the level of MDA was elevated in the plasma of arthritic animals.\textsuperscript{38,45,46} Treatment with MECA (200 mg/kg) was more effective in decreasing the plasma level of MDA than standard Indomethacin and it decreased to the normal control level on day 28. From the results obtained, it is anticipated that methanolic extract of C. arborea is the more efficient scavenger than standard Indomethacin.

C-reactive protein is an acute phase protein. Since C-reactive protein levels in the blood rise more quickly after the inflammatory or infective process begins, due to a rise in the plasma concentration of IL-6, which is produced predominantly by macrophages.\textsuperscript{47} CRP factor is a diagnostic index of bacterial infection, chronic rheumatoid arthritis, suppurative arthritis, gout, malignant tumor and rheumatoid fever.\textsuperscript{48} CRP binds to phosphocholine on microbes, which is thought to assist in complement binding to foreign and damaged cells and enhances phagocytosis by macrophages, which express a receptor for CRP. A CRP factor is also believed to play another important role in innate immunity, as an early defense system against infections.

Present study showed significant elevation in CRP levels in CFA control animals as compared to normal control group while treatment with MECA (100 and 200 mg/kg) significantly lowered CRP levels as compared to CFA control group. This indicates that the plant extract play an important role in immunity and defense system, thus justifying its protective effect.

The present study has indicated that the methanolic extract of Careya arborea stem bark has a better effect on controlling CFA induced inflammation. The methanolic extract of C. arborea stem bark had shown significant reduction in paw edema, joint thickness and arthritic index when compared with CFA control group. The higher dose of MECA treated rats showed better protective effect in reducing pro-inflammatory and oxidative mediators. This depicts the anti-arthritic activity of the methanolic extract of C. arborea stem bark.

CONCLUSION

In conclusion, the results of the present study indicated the dose dependent anti-arthritic activity of the plant C. arborea which might be due to the anti-oxidative and immunomodulatory activity of C. arborea. However, the precise molecular mechanism by which MECA exerts its protective effect against inflammation remains to be established. Nevertheless, detailed mechanistic action on immunomodulation and oxidative stress by MECA requires further study.

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REFERENCES


