Original Article

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Pharmacological evaluation of the antirheumatic activity of *Callicarpa macrophylla* leaves extracts by the estimation of hepatic, renal, and inflammatory markers

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ABSTRACT

Background: *Callicarpa macrophylla* is used in traditional medicine to treat many disorders including analgesic, antipyretic and anti-inflammatory. The aim of this study is to investigate the ethanolic and ethylacetate extracts of *C. macrophylla* leaves against rheumatoid arthritis (RA). **Materials and Methods:** Acute toxicity was studied *in vivo* to determine the toxic doses of the ethanolic and ethyl acetate extracts. Anti-rheumatic activity was also evaluated *in vivo* using formaldehyde and Complete Freund's Adjuvant models in Wistar rats. After that paw volume was evaluated by the digital Vernier caliper and blood sample was taken by retro-orbital plexus route for the estimation of hematological marker and plasma was used for the estimation of liver, kidney, and inflammatory markers. Further, the histopathology of the joint tissue was done by sacrificing the under experimented animals. **Results:** At 200 and 400 mg/kg, the ethanol and ethyl acetate extracts were the most active against RA. There was dose dependent reduction in paw volume in both arthritic models as compared to the arthritic control. The results of biological markers and hematological parameters were restored which were altered due to the progression of arthritis. All these results were further supported with histopathology of joint tissue where the extracts performed a reversal in inflammation and hyperplasia of synovium. The results of this study concluded that *C. macrophylla* ethanol extracts have better anti-arthritic activity in comparison with ethyl acetate extracts. **Conclusion:** The results of the present study support the traditional use of the leaves of *C. macrophylla* and may possibly serve as prospective material for further development of safe new pharmacoactive antirheumatic agents.

Keywords: Antirheumatic activity, *Callicarpa macrophylla,* Complete Freund's Adjuvant-induced arthritis, formaldehyde, rheumatoid arthritis

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease of synovial joints which can cause progressive and permanent disability by movement of inflammatory cells into the synovial tissue. The risk factors for RA include age, gender, genetics, and environmental exposure (cigarette smoking, air pollutants, and occupational patterns). Sometime RA can also affect the other vital organs such as dermal, retinal, cardiovascular, renal, and pulmonary effects. Clinically the development of RA is characterized by the presence of arthralgia, edema, and redness of joints. The prevalence of RA varies between 0.4% and 1% based on the ratio of women to man was 3:1 and the in United States and North European countries population generally suffered from this disease due to their living habits.[1-3] Women faced twice the progression of severe disease symptoms due to genetic predisposition, immunologic, lifestyle patterns, and environmental factors. The progression of the RA is mainly due to the release of

inflammatory cytokine mediators in the blood such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), C-reactive protein (CRP), T-cell receptor, and human leukocyte antigen. If RA is left untreated, the patient may experience joint, tissue and cartilage damage, results in severe disability, decreased quality of life, the onset of co-morbidities, and premature mortality.[4]

The treatment of RA is achieved by different drugs such as disease modifying anti-rheumatic drugs (DMARDs) includes methotrexate, non-steroidal anti-inflammatory drugs (NSAIDs), lefluonomide/teriflunomide, sulfasalazine, chloroquine/hydroxychloroquine, and corticosteroids. Recently biological DMARDs such as monoclonal antibodies targeting TNF-receptor, B-lymphocyte antigen (CD20), IL-6 receptor, receptor activator of nuclear factor kappa-B ligand (RANKL), and Janus kinase/signal transducers and activators of transcription (JAK) have been tested in the treatment of RA.[5] The pharmacological effects of RA using DMARDs are mainly limited by the safety factor as usage of most of these agents is associated with severe adverse events. The major side effects observed with NSAIDS were patients suffered from a gastrointestinal disturbance such as ulcer development, gastrointestinal bleeding, and also impacts on cardiovascular events. Further, sometimes RA recurrence could be observed in patients on DMARDs treatment due to incomplete remission.[6,7] At present, there is no permanent solution for RA, whereas most of the drugs available in the market were giving symptomatic relief. Hence, the search of effective natural drugs in the therapeutic management of RA would be the need of last hour.

The oxygen metabolism played an important role in the pathogenesis of RA. It had been noticed clinically as well as in experimental studies that significant elevation in reactive oxygen species (ROS) was observed in RA suffering individuals which subsequently results in increase in inflammatory markers rise that leads to damage of synovial membrane.[8-10] The several antioxidants had shown therapeutic adjuvant potential in the treatment of RA preclinical studies. However, the best part for antioxidants was the safety associated with these natural originating agents.[11,12] Thereby the plant extracts have the significant unexplored potential for the treatment of RA because plant sources are rich in antioxidants.

In this study, we have explored the plant *Callicarpa macrophylla* to evaluate anti-arthritic activity in animal models, popularly known as Priyangu, which was previously evaluated for antioxidant activity.[13] *C. macrophylla* is a traditional plant used in regulation of circulatory, digestive, endocrine, respiratory, and skeletal systems as well as to treat infectious diseases. The oil obtained from seeds of *C. macrophylla* had shown analgesic, anti-inflammatory, and anti-pyretic effects such as that of ibuprofen, paracetamol, and indomethacin.[14] Further, the aqueous and ethanolic extracts of *C. macrophylla* roots and leaves had shown analgesic and anti-inflammatory potentials in tail immersion test and carrageenan paw edema models.[15,16] In this study, we have explored the pharmacological anti-rheumatic effect of both extracts (ethanol and ethyl-acetate) of *C. macrophylla* leaves in arthritis-induced animals.

MATERIALS AND METHODS

Plant Extraction

Dried leaves of *C. macrophylla* (Verbenaceae) were purchased from a commercial herb market in Pune and authenticated by Dr. G.S. Kritikar, Head Pharmacognosist, Samanthak enterprises, Pune (Voucher specimen number SE/AC/2019/05). All the chemicals, reagents, and kits used in this study were of analytical grades and the plasma parameters were performed using commercially available kits from Accurex. The inflammatory markers were also estimated by the kit of Sigma-Aldrich procured from commercial suppliers.

Extract Preparation

The dried leaves of *C. macrophylla* (1 Kg) were powdered. The crude drug was first defatted with petroleum ether and then consequently residues were extracted successively with ethyl acetate and ethanol solvent in Soxhlet apparatus. The filtrate was evaporated using rotary evaporator to obtain the extract used for pharmacological evaluation.[17] Callicarpa macrophylla the yield of the extracts were 8.56% (crithmum maritimum [CM] ethyl acetate extract) and 7.24% (CM ethanolic extract).

Preliminary Phytochemical Screening

The preliminary phyotochemical screening of active plant constituents in the ethyl acetate and ethanol extracts was done according to the procedure described by Kokate *et al*., 2007.

Experimental Animals

The Albino Wistar rats were obtained from the Department of Pharmacology, Pinnacle Biomedical Research Institute, Bhopal after obtaining Institutional Animal Ethics Committee approval with reference number (PBRI/IAEC/2019/12-21/009). Animals were kept at controlled temperature (22 ± 2°C) and relative humid (55 ± 5%) conditions. Animals were acclimatized and quarantined for 1 week before initiation of experimental work. The food and water were maintained *ad libitum* to rats throughout the experimental period.

Acute toxicity studies

Acute toxicity study was performed as per OECD Guidelines 423 to calculate the therapeutic dose. The dose levels of the extracts used were 5, 50, 300, and 2000mg/kg body weight. The rats were overlooked for behavioral alterations for the first four hours after the dose administration and then examined day-to-day for 14 days.

Pharmacological evaluation of anti-rheumatic Activity

Formaldehyde-induced Rat Paw edema Model

The animals were divided into seven groups, each group containing six rats. The Group 1 served as normal control received only normal saline solution orally. The Group 2 was arthritic control where animals received 0.1 mL of formaldehyde was injected in the right hind paw of experimental rat. The Group 3 served as a standard group received indomethacin (10 mg/kg) in arthritis-induced animals. The Groups 4 and 5 animals received ethyl acetate extract of *C. macrophylla* leaves at 200 and 400 mg/kg. The Groups 6 and 7 received the ethanolic extract of *C. macrophylla* leaves at 200 and 400 mg/kg of dosage.

The plant extracts was administered orally to the animal 30 min before the injection of formaldehyde and continued till 10th day. Paw volume was evaluated on day 0, 2, 4, 6, 8, and 10 h after formaldehyde administration using a digital Vernier caliper.[17]

Complete Freund's adjuvant (CFA)-induced Rat Paw model

Animals were divided into seven groups, each group containing six rats. The Group 1 served as normal control received only saline. The Group 2 was arthritic control where animals received 0.1 mL of formaldehyde in the right hind paw of rat. The Group 3 was a standard group received indomethacin (10 mg/kg) in arthritic animals. The Groups 4 and 5 animals received ethyl acetate extract of *C. macrophylla* leaves at 200 and 400 mg/kg. The Groups 6 and 7 received the ethanolic extract of *C. macrophylla* leaves at 200 and 400 mg/kg of dosage. The plant extracts (both ethyl acetate and ethanolic) was administered orally to the animal 30 min before the administration of CFA and continued till 21th day. Paw volume was evaluated on 4th h, 24th h, 2, 4, 8, 12, 16, and 21 days after CFA administration using digital Vernier caliper.

Plasma parameters

After 21st day of CFA injection, blood samples were collected into heparinized (purple colored) vials by puncturing into the retro-orbital plexus using capillary tube after anesthesia animals and analyzed for red blood corpuscles (RBCs), white blood cells (WBCs), hemoglobin (Hb), and erythrocyte sedimentation rate (ESR). The 0.5 mL of blood from each group was collected and centrifuged to obtain clear plasma. This plasma samples were used for the estimation of liver and kidney injury markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin, creatinine, blood urea nitrogen (BUN), and uric acid. All the estimations were performed using commercially available kits from Accurex.[17]

Histopathological studies

After sacrificing the animals, knee joint tissue was collected and fixed in 10% buffered formalin, decalcified using 10% EDTA for 30 days at 4°C, processed in graded alcohol and xylene and embedded in paraffin. Sections measuring 5 μ were taken on the pre-coated slides and stained with hematoxylin and eosin (H&E) reagent. The severity and extent of damage were observed in each group under the compound microscope and photographs were taken at different magnification.[17]

Estimation of inflammatory markers

The inflammatory markers such as TNF-α, IL-6, CRP, and RF were measured in plasma samples using kits from Sigma-Aldrich, USA.[17]

Statistical Analysis

The data were reported as mean ± SEM. The significance of difference between multiple groups was evaluated using one-way analysis of variance (ANOVA) followed by Bonferroni's test using GraphPad prism (version 7) software. A value of $P < 0.05$ was considered significant.

RESULTS

The phytochemical screening of the extract showed the presence of anti-oxidant compounds in the form of flavonoids and phenolic compounds.

Paw Volume in Formaldehyde and CFA-induced Arthritis

In formaldehyde-induced arthritic animals, there was a significant increase ($P < 0.001$) in paw volume observed at all-time points such as days 0, 2, 4, 6, 8, and 10. Positive control or standard (Indomethacin) showed time-dependent significant decline in paw volume comparison to arthritic control animals at all-time points. The dose- and time-dependent significant decline in paw volume by CME at 200 and 400 mg/kg was shown from the day 2 to day 10. CMEA showed a significant decline in paw volume only after days 6 at a dose of 200 and 400 mg/kg [Table 1 and Figure 1]. In CFA-induced arthritic animals, there was a significant increase in paw volume observed at all-time points such as 4 h, and 24 h, days 2, 4, 8, 12, 16, and 21 d. Positive control or standard (Indomethacin) showed a significant decline in paw volume in comparison to arthritic control animals after day 4 till day 21. CME at both the doses caused dose- and time-dependent significant decline in paw volume in comparison to arthritic control animals from day 2 to day 21. On the other hand, CMEA at the dose of 200 and 400 mg/kg showed significant decline in paw volume from day 12 and day 16, respectively, till day 21 [Table 2 and Figure 2]. Results from both of these animal models clearly showed superiority of the ethanol extract than ethyl acetate extract.

Liver and Kidney Markers in CFA-induced Arthritis

The significant increase ($P < 0.001$) in liver (AST, ALT, ALP, and bilirubin) and kidney (creatinine, BUN, and uric acid) markers was observed in CFA-induced arthritic animals. The positive control and the standard (Indomethacin) showed a significant decline ($P < 0.001$) in all markers of liver and kidney tests in comparison to arthritic control animals. The CME and CMEA at 200 mg/kg dose showed significant improvement in only AST and bilirubin in comparison with arthritic control animals. The CME at 400 mg/kg dose demonstrated a significant decline in liver and kidney function marker except for creatinine and the CMEA at 400 mg/kg produced significant declines in all liver and kidney markers except ALT, creatinine, and BUN [Table 3 and Figures 3 and 4].

Hematological Markers in CFA-induced Arthritis: A significant decrease in Hb levels, RBCs, and WBCs was observed in CFA-induced arthritic animals, whereas ESR was significantly increased. Positive control or standard (Indomethacin) showed a reversal in Hb, RBCs, WBCs, and ESR when measured on day 21. CME and CMEA at 400 mg/kg dose showed significant improvement in Hb, RBCs, WBCs, and ESR whereas in CME and CMEA group at 200 mg/kg only ESR was significantly reduced [Table 4 and Figure 5].

Inflammatory Markers in CFA-induced Arthritis

The increased inflammation is associated with a rise in pro-inflammatory markers in plasma of animals. We observed a significant increase in TNF-α, IL-6, CRP, and RF level in arthritic control animals. CME at 400 mg/kg dose showed a significant reduction of all these inflammatory markers when compared with arthritic control animals [Table 5 and Figure 6].

Table 1: Effects of different plant extract on paw volume of formaldehyde-induced arthritic animals

Treatment groups	Day of treatment					
	0 day	2 day	4 day	6 day	8 day	10 day
Normal Control	6.98±0.43	6.97±0.42	6.97±0.48	6.95±0.40	6.96±0.39	6.96±0.40
Arthritic control	8.47±0.36**	9.66±0.66**	12.56±0.84***	13.94±0.77***	14.52±1.46***	16.70±1.38***
Standard	7.76±0.72^{NS}	8.18±1.17$^{\#}$	8.00±1.34$^{\#\#}$	6.77±1.20$^{\#\#}$	6.06±0.54$^{\#\#}$	5.65±0.46$^{\#\#}$
CMEA (200mg/kg)	9.00±0.43 ^{NS}	10.08±0.34^{NS}	13.13±0.43^{NS}	12.18±0.33$^{\#\#}$	11.75±0.44$^{\#\#}$	11.17±0.43$^{\#\#}$
CMEA 400mg/kg	8.17±0.20^{NS}	9.94±0.41^{NS}	12.56±0.75^{NS}	11.76±0.62$^{\#\#}$	10.86±0.42$^{\#\#}$	9.98±0.41$^{\#\#}$
CME (200 mg/kg)	8.16±0.46^{NS}	8.09±0.44$^{\#\#}$	8.01±0.43$^{\#\#}$	7.87±0.40$^{\#\#}$	7.61±0.37$^{\#\#}$	7.44±0.37$^{\#\#}$
CME (400 mg/kg)	8.29±0.52^{NS}	8.22±0.52$^{\#\#}$	8.05±0.51$^{\#\#}$	7.69±0.44$^{\#\#}$	7.27±0.37$^{\#\#}$	6.14±0.33$^{\#\#}$

All the values are expressed in mean±SEM and $^{**}P<0.01$, $^{***}P<0.001$ versus normal control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ versus arthritic control group, NS- not significant. Data were analyzed by one-way ANOVA followed by Bonferroni test ($n=6$). CMEA: *Callicarpa macrophylla* ethyl acetate extract, CME: *Callicarpa macrophylla* ethanolic extract

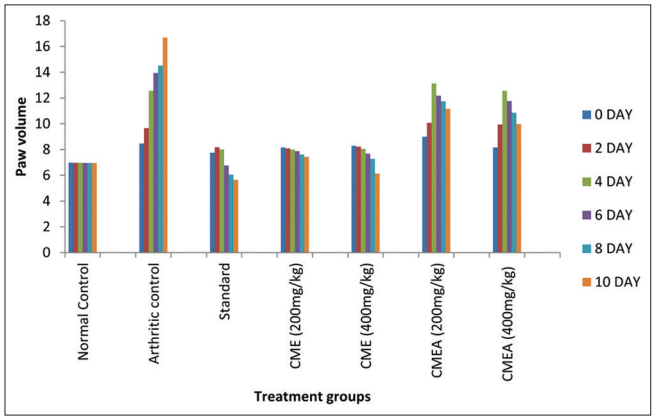

Figure 1: Paw volume by formaldehyde induced arthritis

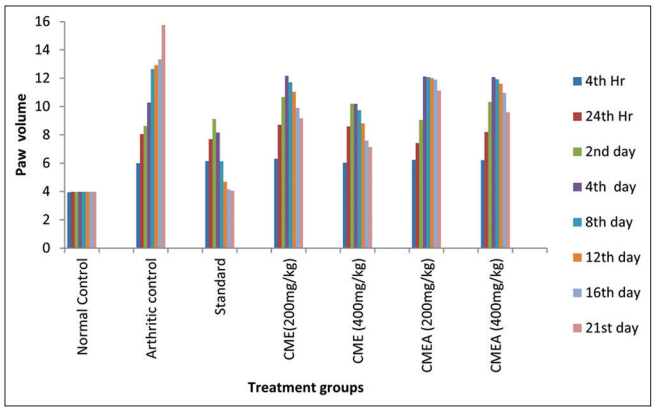

Figure 2: Paw volume by complete Freund's induced arthritis

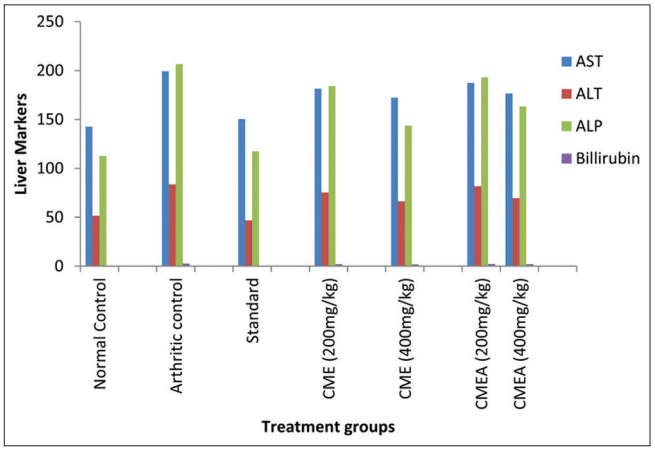

Figure 3: Effects of the extracts on liver markers

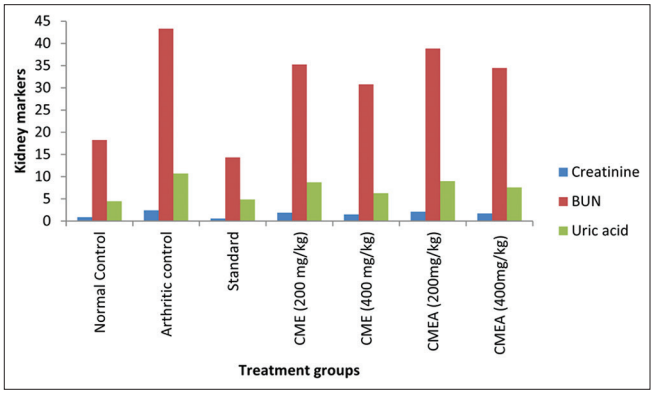

Figure 4: Effects of the extracts on kidney markers

Histopathological Studies of Joint Tissue

The Group 1 (normal control) showed normal joint space, normal adjacent soft tissue, and cartilage. The Group 2 (arthritic control) showed marked synovial hyperplasia, inflammatory cell infiltration, and cartilaginous bone destruction. The Group 3 (standard group) showed a good reduction in inflammatory cells and less hyperplasia. Whereas in Groups 4-5, ethanolic extract (200 and 400 mg/kg) showed less reduction in inflammatory cells and hyperplasia of synovium tissue compared to Groups 5-6 which received ethylacetate (200 and 400 mg/kg) extract and showed better reduction when compared with the CFA treated group [Figure 7].

DISCUSSION

RA is a systemic auto-immune disease which is primarily manifested in joints, leading to pain, and inflammation of the joints.[18] Several synthetic and biological agents have been tried, but majority of them failed due to serious adverse events. Hence, there is a call for safe and effective medicines. There were several hearsay in the literature where plant extracts

Table 2: Effects of plant extracts on paw volume of Complete Freund's adjuvant (CFA)-induced arthritic animals

Treatment groups	Days of treatment							
	4 h	24 h	2 day	4 day	8 day	12 day	16 day	21 day
Normal Control	3.95±0.29	3.97±0.29	3.96±0.29	3.97±0.29	3.97±0.29	3.97±0.29	3.97±0.29	3.98±0.29
Arthritic control	6.00±0.16**	8.05±0.32***	8.63±0.28***	10.28±0.25***	12.65±0.65***	12.93±0.55***	13.33±0.57***	15.76±0.74***
Standard	6.15±0.46^{NS}	7.70±0.78^{NS}	9.12±0.60^{NS}	8.16±0.33$^{\#}$	6.13±0.55$^{\#\#}$	4.69±0.35$^{\#\#}$	4.15±0.24$^{\#\#}$	4.05±0.22$^{\#\#}$
CMEA (200mg/kg)	6.24±0.15^{NS}	7.43±0.47^{NS}	9.06±0.72^{NS}	12.13±0.83^{NS}	12.08±0.83^{NS}	12.02±0.81^{NS}	11.9±0.77$^{\#\#}$	11.14±0.78$^{\#\#}$
CMEA 400mg/kg	6.21±0.15^{NS}	8.20±0.46^{NS}	10.32±0.75$^{\#\#}$	12.08±0.34^{NS}	11.93±0.32^{NS}	11.61±0.30$^{\#\#}$	10.96±0.20$^{\#\#}$	9.61±0.38$^{\#\#}$
CME (200 mg/kg)	6.32±0.14 ^{NS}	8.71±0.36^{NS}	10.67±0.47$^{\#\#}$	12.17±0.85^{NS}	11.72±0.72^{NS}	11.04±0.61$^{\#\#}$	9.91±0.52$^{\#\#}$	9.17±0.77$^{\#\#}$
CME (400 mg/kg)	6.03±0.13 ^{NS}	8.59±0.52^{NS}	10.20±0.71$^{\#}$	10.19±0.45$^{\#\#}$	9.74±0.26$^{\#\#}$	8.80±0.41$^{\#\#}$	7.59±0.81$^{\#\#}$	7.14±0.61$^{\#\#}$

All the values are expressed in Mean±SEM and $^{**}P<0.01$, $^{***}P<0.001$ versus normal control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ versus arthritic control group. Data were analyzed by one-way ANOVA followed by Bonferroni test ($n=6$). CMEA: *Callicarpa macrophylla* ethyl acetate extract, CME: *Callicarpa macrophylla* ethanolic extract

Table 3: Effects of different plant extract on liver and kidney function tests

Groups	AST	ALT	ALP	Bilirubin	Creatinine	BUN	Uric acid
Normal Control	142.66±5.48	51.56±8.90	112.73±3.33	0.55±0.11	0.9±0.31	18.23±1.23	4.49±0.54
Arthritic control	199.19±5.44**	83.68±7.39**	206.52±7.42***	2.77±0.10*	2.42±1.24**	43.30±5.33*	10.70±1.79**
Standard	150.28±8.06$^{\#\#\#}$	46.85±4.84$^{\#\#\#}$	117.38±4.13$^{\#\#\#}$	0.61±0.18$^{\#\#\#}$	0.58±0.14$^{\#\#\#}$	14.33±1.98$^{\#\#\#}$	4.86±0.61$^{\#\#\#}$
CMEA (200 mg/kg)	187.53±4.91^{NS}	81.78±3.97^{NS}	193.15±4.65 ^{NS}	2.35±0.13$^{\#}$	2.10±0.15^{NS}	38.86±4.72^{NS}	9.00±0.19 ^{NS}
CMEA 400 mg/kg	176.62±6.31$^{\#}$	69.54±4.27^{NS}	163.34±6.87$^{\#}$	2.10±0.14$^{\#}$	1.73±0.12^{NS}	34.48±4.82^{NS}	7.61±0.52$^{\#}$
CME (200 mg/kg)	181.51±6.39$^{\#}$	75.43±6.68^{NS}	184.05±17.34 ^{NS}	2.06±0.10$^{\#}$	1.90±1.22^{NS}	35.27±3.13^{NS}	8.74±0.75 ^{NS}
CME (400 mg/kg)	172.38±4.58$^{\#}$	66.30±5.79$^{\#}$	143.82±6.93$^{\#}$	1.79±0.21$^{\#}$	1.51±0.36^{NS}	30.80±0.84$^{\#}$	6.27±0.31$^{\#}$

All the values are expressed in mean±SEM and $^{**}P<0.01$, $^{***}P<0.001$ versus normal control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$ versus arthritic control group. Data were analyzed by one-way ANOVA followed by a Bonferroni test ($n=6$). CMEA: *Callicarpa macrophylla* ethyl acetate extract, CME: *Callicarpa macrophylla* ethanolic extract, AST: Aspartate transaminase, ALT: Alanine transaminase, ALP: Alkaline phosphatase, BUN: Blood urea nitrogen

Table 4: Effects of different plant extracts on hematological parameters

Treatment groups	Hb	RBCs	WBCs	ESR
Normal Control	14.34±1.04	7.44±0.80	7.58±0.65	3.35±0.62
Arthritic control	10.29±0.94**	5.30±0.79*	5.99±0.34*	10.00±0.40**
Standard	13.15±1.94$^{\#\#}$	7.28±0.97$^{\#}$	7.83±0.49$^{\#}$	3.87±0.64$^{\#}$
CMEA (200 mg/kg)	10.64±0.57 ^{NS}	6.37±0.44^{NS}	7.69±0.12^{NS}	5.00±0.34$^{\#\#}$
CMEA 400 mg/kg	12.20±1.22^{NS}	6.78±0.50^{NS}	8.18±0.87$^{\#\#}$	4.11±0.64$^{\#\#}$
CME (200 mg/kg)	11.90±1.64^{NS}	6.63±0.96^{NS}	7.73±1.08^{NS}	5.27±0.50$^{\#\#}$
CME (400 mg/kg)	13.74±1.62$^{\#\#}$	7.64±1.33$^{\#\#}$	8.01±1.20$^{\#\#}$	5.08±0.33$^{\#\#}$

All the values are expressed in Mean±SEM and $^{*}P<0.05$, $^{**}P<0.01$ versus normal control group; $^{\#}P<0.05$; $^{\#\#}P<0.01$ versus arthritic control group. Data were analyzed by one-way ANOVA followed by a Bonferroni test ($n=6$). CMEA: *Callicarpa macrophylla* ethyl acetate extract, CME: *Callicarpa macrophylla* ethanolic extract, Hb: Hemoglobin, RBCs: Red blood cells, WBCs: White blood cells, ESR: Erythrocyte sedimentation rate

Table 5: Effect of different plant extract on inflammatory markers

Groups	IL-6	TNF-α	CRP mg/ml	RF IU/L
Normal Control	29.92±0.11	31.76±0.24	12.14±0.24	2.7±0.23
Arthritic control	72.17±0.81***	68.94±0.51***	25.21±0.41***	61.14±0.47***
Standard	43.48±0.28$^{\#\#}$	41.16±0.66$^{\#\#}$	18.23±0.58$^{\#\#}$	31.73±0.77$^{\#\#}$
CME (400 mg/kg)	46.80±0.18$^{\#}$	45.72±0.14$^{\#}$	14.78±0.18$^{\#}$	37.95±0.61$^{\#}$
CMEA (400mg/kg)	45.04±0.487**	44.07±0.651**	7.07±0.238**	34.75±0.606**

All the values are expressed in mean±SEM and $^{***}P<0.001$ versus normal control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ versus arthritic control group. Data were analyzed by one-way ANOVA followed by a Bonferroni test ($n=6$). CME: *Callicarpa macrophylla* ethanolic extract, CMEA: *Callicarpa macrophylla* ethyl acetate extract

have been used as a source of antioxidant to replenish depleted antioxidant level in RA.[19] These medicinal plants could be extracted for the isolation of different phytoconstituents and evaluated for pharmacological studies which could reveal a natural therapeutic agent with fewer side effects. We have investigated pharmacological effects of the ethyl acetate and ethanol extract of CM leaves in animal model of RA. The RA in animals was studied using two different animal models such as formaldehyde and CFA-induced RA models.[20] We observed a significant dose and time dependent increase in paw volume in both of these animal models in all groups except the normal group which was corresponded with the previous studies utilizing same animal models.[21-24] We observed potent dose- and time-dependent reduction in paw volume measured at different time points by the CMEA and CME extracts. Further, the ethanol extracts of plant was having better activity than ethyl acetate extract of CM leaves. There was a significant increase in liver and kidney injury markers which is matched with different studies carried out in the past on the same model.[25,26] Further, the CM showed significant reduction in these injury markers and ethanol extract was showing better protection than ethyl acetate extract. The abnormal changes in hematological parameters were observed in CFA-induced arthritic animal models, which were correlated with the previous studies carried out on the same model.[25] All these changes were ameliorated by treatment with *C. macrophylla*. The *C. macrophylla* ethanol extract was having better activity in normalizing hematological markers. Further, the inflammatory markers (TNF-α, IL-6, and RF) level increased showed that these markers play a pivotal role in pathogenesis of RA.[27] There were studies which suggested relationship between TNF-α and T-effector and T-regulator cells which were the main cause for RA.[28] Similar kind of correlation between IL-6 and RA pathophysiology had been observed in the previous reports.[29-32] All these changes were normalized by *C. macrophylla* extracts at higher dose of 400 mg/kg. The

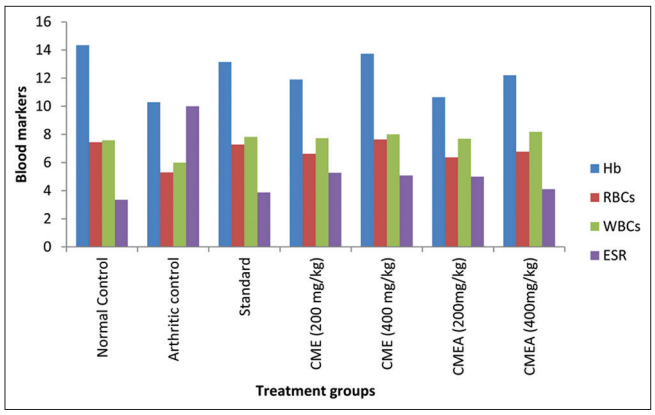

Figure 5: Effects of the extracts on blood markers

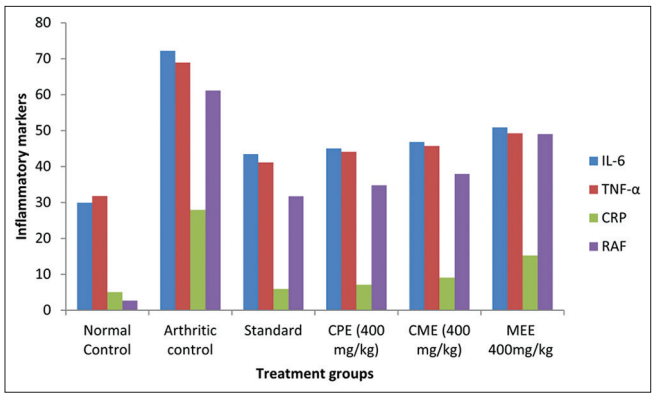

Figure 6: Effects of the extracts on inflammatory markers

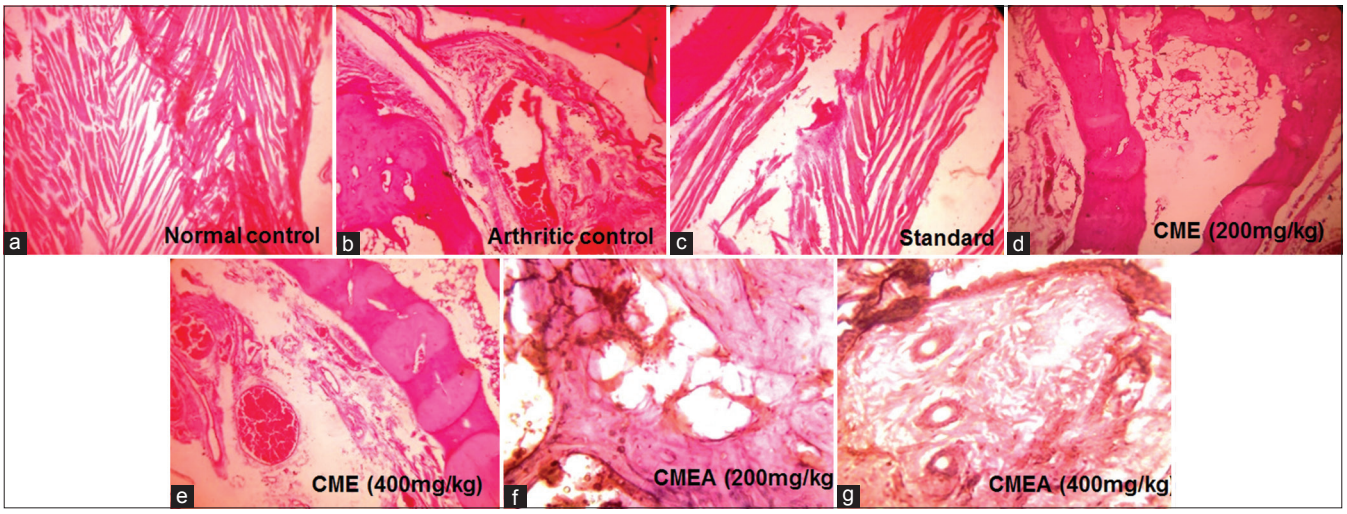

Figure 7: Effect of crithmum maritimum leaves ethyl acetate and ethanol extract (200 and 400 mg/kg) on the histo-architecture of joints tissues in Complete Freund's adjuvant-induced arthritis. Images of hematoxylin and eosin stained sections (magnification: ×10). (a) Group 1: Normal control represents the normal soft tissue and cartilage. (b) Group 2: Arthritic control represents infiltration of inflammatory cells, and synovial hyperplasia, complete destruction of articular tissue and fully inflamed synovial tissues and thinning of articular cartilage. (c) Group 3: Standard represents the decreased hyperplasia and inflammatory cell infiltration was scanty, smooth and monolayer of synovial cell lining. (d) Group 4: *Callicarpa macrophylla* ethyl acetate extract (CMEA) (200 mg/Kg) represents the moderate inflammation, synovitis, and moderate thinning of articular cartilage. (e) Group 5: CMEA (400 mg/Kg) represents mild inflammation in the internal lining of the joints and small permeation of cells. (f) Group 6: *C. macrophylla* ethanolic extract (CME) (200 mg/Kg) represents moderate synovitis, moderate inflammation of cells and bone erosion. (g) Group 7: CME (400 mg/Kg) represents intact synovial cells, prominent plasma cells and decreased proliferation of synovial cells and inflammatory cells

C. macrophylla ethanolic extracts were having better anti-rheumatic activity than ethyl acetate extract. The CRP was an inflammatory marker derived from hepatocytes. The level of CRP usually increased in most of the acute inflammatory conditions. Further, during the inflammatory process, the CRP level increases due to increased concentration of IL-6 in plasma, which was produced by increased circulating macrophages as well as adipocytes.[33-36] The significant increase in CRP level, which was attenuated with CM ethanol extract treated rats. The most conclusive evidence for the therapeutic efficacy of *C. macrophylla* leaves extract in CFA-induced arthritic animals resulted from the histopathological status of joints revealed by extract treated groups. The reduction in joint injury, evidenced from reduced synovial joint hyperplasia, inflammatory cell accumulation reduction, and cartilage destruction. Further, histopathology of the ethanolic *C. macrophylla* extract showed better prevention and healing of histoarchitecture than ethyl acetate *C. macrophylla* extract. The results of this study concluded that the antioxidants such as flavonoids, phenolic, and steroidal compounds would be the possible factor responsible for its antiarthritic activity that might results intracellular signaling through reduction of oxidative stress, inflammation, and immune-modulation. Thereby, this plant extracts could be studied as a potent natural remedy by isolation, characterization and identification of lead compounds responsible for the management of RA in the future.

CONCLUSION

On the basis of our results, we conclude that the arthritic rats when provided with the ethanolic and ethyl acetate extract of *C. macrophylla,* their inflammatory mediators such as IL-6 and CRP were lowered. The serum bilirubin, creatinine, blood urea nitrogen, and uric acid also decreased upon ingestion of *C. macrophylla* leaves extracts for 14 days. Besides this, the dose was also effective in protecting the liver, kidney, and synovial membrane from abnormalities. Thus, the polar extracts of *C. macrophylla* possess a great anti-rheumatic potential which could be used in future to cure arthritis associated symptoms and might prove as one of the potent herbal medicine.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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