



Antioxidant and Anti-arthritic Potential of *Casuarina equisetifolia* Fruit Methanolic Extract

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Authors' contributions

This work was carried out in collaboration among all authors. Authors DKG and SS carried out the sample collection, authenticate and investigate phytochemical analysis of plant material. Authors MVS and DSP helps in statistical data analysis. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2020/v31i1130204

Editor(s):

- (1) Dr. Paola Angelini, Department of Chemistry, Biology and Biotechnology, University of Perugia, Italy.
(2) Marcello Iriti, Professor of Plant Biology and Pathology, Department of Agricultural and Environmental Sciences, Milan State University, Italy.

Reviewers:

- (1) Fatma Gonca Kocanci, Turkey.
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(3) Md Afjalus Siraj, University of Hawai'i at Hilo, USA.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/54842>

Original Research Article

Received 10 December 2019

Accepted 15 February 2020

Published 18 February 2020

ABSTRACT

Aims: Antioxidant and anti-arthritic potential of *Casuarina equisetifolia* fruit methanolic extract.
Place: C.U. Shah College of Pharmacy and Research, Wadhwan, Surendranagar, Gujarat, India.
Methodology: Extract was subjected to qualitative and quantitative investigation then antioxidant properties of extract was determine by two method namely Nitric Oxide free radical scavenging activity and Reducing power by FeCl₃ method. Based on Phytochemical and antioxidant result anti-arthritic activity was performed on wistar rats using Complete Freund's Adjuvant (CFA) and evaluated different parameter like paw volume, arthritic index, biochemical parameter and hematological parameter.
Results: In this study found that the methanolic extract of *Casuarina equisetifolia* fruit (MLCEF) contain significant percentage of secondary metabolite like poly phenol that properties was proved antioxidant activity. Antioxidant properties was determined by two methods the MLCEF IC₅₀ Value 30.27±2.43 and 158.45±7.15 was found IC₅₀ value reveled that MLCEF is a potent free radical

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scavenge capacity. *Casuarina equisetifolia* fruit (MLCEF) of 400 mg/kg, p.o. showed significant increase in body wt from day 12th to 21st and significant reduction in rat paw edema was observed from day 6th to 21st and it could be significantly normalized the haematological (like Hb, WBC and ESR) and biochemical abnormalities (like urea, ALT, AST and ALP) adjuvant induced arthritic rats in both developing and developed phases of CFA induced arthritis.

Conclusion: The study outcome revealed that MLCEF contains significant amount of polyphenol and that properties exhibit good antioxidant properties and significant antiarthritic properties. The actual mechanism of action of extracts on adjuvant induced arthritis is not clear with these studies. The action of extracts on proinflammatory mediators like TNF- α , Interleukins and other relevant mediators will be carried out in future to study its mechanism.

Keywords: Anti-citrullinated; auto-oxidative; premature aging; TNF- α .

1. INTRODUCTION

The free radicals may be either Oxygen derived (ROS) or Nitrogen derived (RNS). The most common reactive oxygen species include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), peroxy radicals (ROO) and reactive hydroxyl radicals (OH). The nitrogen derived free radicals are nitric oxide (NO), peroxy nitrite anion (ONOO), Nitrogen dioxide (NO₂) and Dinitrogen trioxide (N₂O₃) [1].

These reactive species are by-products of the normal cellular functional activities, presenting an important role in cell signaling, apoptosis, gene expression and ion transportation. Nevertheless, if ROS levels increase intensely, it can result in damage of many molecules, including proteins, lipids, RNA and DNA, since they are highly reactive. Furthermore, the production of free radicals is not only associated with the normal metabolic processes in the body (endogenous sources), but can also be due to environmental factors (exogenous sources) such as stress, ozone radiation, pollution, pesticides and industrial chemicals [2–7]. When higher production of ROS in relation to their removal by biological systems (antioxidant defenses) occurs, it is called oxidative stress [8]. That has long been associated with increased risk for several diseases, such as cancer [7,9], diabetes, atherosclerosis [9], arthritis [10], neurodegenerative diseases [11] and premature aging [12].

Rheumatoid arthritis (RA) is a Persistent joint synovial fluid inflammation termed as RA. In RA, body's immune system attacks body's own tissues; such as cartilage degradation and loss of joint function [13]. The higher secretion of synovial cells activated by proinflammatory factors like IL-1, TNF α , and PGE2 is thought to

be a key step in the destruction of cartilaginous and bony tissues in RA joints. IL-1, TNF α , and PGE2 overproduction play potential pathogenic roles in the establishment of rheumatoid synovitis, in the formation of pannus tissue and in the process of joint destruction [14]. Hallmark of RA is persistent symmetric polyarthritis resulting in muscle aches and pain. Anti-citrullinated protein antibodies (ACPA) produced by plasma cells during the preclinical phase of RA, stimulate osteoclast differentiation while, synovitis at the onset of disease leads to the production of cytokines, thus ensuing in bone erosion [15].

Antioxidants may protect cells by a variety of mechanisms, including the conversion of ROS to non-radical species (which are dependent on the antioxidant involved), breaking the auto-oxidative chain reaction initiated by ROS and decreasing localized oxygen concentrations [16,17]. The intake of exogenous antioxidants, such as ascorbic acid (Vitamin C),-tocopherol (Vitamin E), carotenoids and polyphenols, that can be found in commonly consumed fruits, vegetables, beverages, cereals and others food products, may support the antioxidative defense [9,18,19,20].

In a normal cell there is an appropriate pro-oxidant: antioxidant balance. However, this balance can be shifted towards the pro-oxidant when production of oxygen species is increased or when levels of antioxidants are diminished. This state is called 'oxidative stress' and can result in serious cell damage if the stress is massive or prolonged. Herbal antioxidants have been successfully employed as rejuvenators or employed as part of adjuvant therapy and treatment of deferent type of cancer and autoimmune diseases like Rheumatoid arthritis [21-24].

The plant *Casuarina equisetifolia* Lin. (Casuarinaceae), trees are monoecious. The male and female flowers are light brown and inconspicuously tiny, fruits rounded, wide, hard, warty, brown, pinecone-like. The seeds are winged samaras. The leaf twigs are jointed. The dead, brown, fallen leaf twigs litter the ground under the trees like pine needles. The bark on older trees is rough, gray brown and flaking on the exterior and beefy red brown on the interior [25-28].

It is commonly found, along the coastal area and open forests in both wet and dry zones. It is native to South-East Asia, Australia and Polynesia. It is also cultivated as an ornamental, for wind-breaks or as a medicinal plant in some tropical countries in the South Pacific [29,30].

The plant is a source of biologically active compounds such as catechin, ellagic acid, gallic acid, quercetin, and lupeol [31], coumaroyl triterpenes [32], d-galocatechin [29], tannin [33] and proline [34], *Casuarina equisetifolia* has been reported to be used as an astringents [35], antidiarrhea, dysentery, headache, fever, cough, ulcers, toothache [36], anticancer antibacterial, antifungal, anthelmintic, antispasmodic, antidiabetic [37,38], inflammation, stomachache, diarrhea, dysentery and nervous disorders [39]. In view of the fact that the plant parts rich in secondary metabolite (tannin Triterpenes, gallic acid, ellagic acid, catechin) have significant capacity to scavenge free radical and free radical are the biggest cause of generation tissue damage and generation of inflammatory mediators. Based on chemical constituents exploited for its medicinal values, this study focused on investigating its proximate constituents, Thus the purpose of the present study has been carried out to explore the Antioxidant and anti-arthritis potential of *Casuarina equisetifolia* fruit methanolic Extract.

2. MATERIALS AND METHODS

2.1 Collection of Plant

Plants were collected from Wadhwan, Surendranagar District, Gujarat. The plants were identified and authenticated by Dr. HB Singh, Scientist, National Institute Scientific Communication and Research (NISCAIR), New Delhi (India).

2.2 Preparation of Plant Extract

The shade dried *Casuarina equisetifolia* fruits were powdered to get a course power. About 300

gm of dried powder were extracted with 90% methanol by continuous hot percolation, using soxhlet apparatus. The extract was concentrated up to 100 ml on Rota vapour under reduced pressure. The concentrated crude extracts were lyophilized in to powder and used for the study [40,41].

2.3 Experimental Design

2.3.1 Phytochemical investigation

2.3.1.1 Preliminary phytochemical investigation

The preliminary Phytochemical studies were subjected to perform for testing different chemical groups and secondary metabolites present in methanolic extract of *Casuarina equisetifolia* fruit (MLCEF) [41,42].

2.3.1.2 Total polyphenols determination

The total polyphenol content (TPC) was determined by spectrophotometry method, using tannic acid as standard, according to the method described by the International Organization for Standardization (ISO) 14502-1. Briefly, 1.0 ml of the diluted sample extract was transferred in duplicate to separate tubes containing 5.0 ml of a 1/10 dilution of Folin - Ciocalteu's reagent in water. Then, 4.0 ml of a sodium carbonate solution (7.5% w/v) was added. The tubes were then allowed to stand at room temperature for 60 min before absorbance at 765 nm was measured against water. The concentration of polyphenols in samples was derived from a standard curve of tannic acid ranging from 10 to 50 µg/ml and expressed in terms percentage [42].

2.4 Antioxidant Activity

2.4.1 Reducing power by FeCl₃ method

Various concentrations of the extracts (MLCEF) in 1.0 ml of deionized water were mixed with phosphate buffer (2.5 ml) and potassium ferricyanide (2.5 ml) and incubated at 50°C for 20 min. Aliquots of trichloroacetic acid (2.5 ml) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min whenever necessary. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml). The absorbance was measured at 700 nm. A blank was prepared without adding extract. Ascorbic acid at various concentrations was used as standard. Increased absorbance of the

reaction mixture indicates increase in reducing power [43].

2.4.2 Nitric oxide free radical scavenging activity

Various concentrations of the extracts (MLCEF) in 1.0 ml of methanol were mixed with Sodium nitro prusside (10 mM) in phosphate buffer phosphate buffer and made upto 200 µl with methanol mixture of solution Incubate at room temperature for 150 minutes. After the incubation period 5ml of Griess reagent was added then absorbance was taken at 546 nm. A blank was prepared without adding extract. Curcumin at various concentrations was used as standard the % reduction and IC50 were calculated [44].

2.5 Complete Freund's Adjuvant (CFA) Induced Arthritis in Rats

2.5.1 Experimental animals

Adult wistar rat of either sex weighing (230-250 gm) obtain from Zydus Research centre, Ahmedabad, for experimental purpose were all acclimatized for 7 days under standard husbandry conditions i.e.; room temperature of (25±1) °C; relative humidity of 45%-55% and a 12:12 h light/ dark cycle.

2.5.2 Acute oral toxicity studies

The acute oral toxicity study of MALCEF was carried out in wistar rat, weighing (230-250 gm), using the Organization for Economic Co-operation and Development (OECD) guidelines (OECD 425). The animals received a single dose of 2000 mg/kg orally by gavages and were observed for toxic symptoms and mortality, continuously for first 4 h after dosing. Finally, the number of survivors was noted after 24 hrs and these animals were then maintained for further 14 days with observations made daily.

2.5.3 Inducedction arthritis in rats

Either sex of Wister rats with an initial body weight of 230-250 gm were taken, and divided into Five groups each group containing six animals (n=06). On day zero, all rats (except normal control animals) were injected into the sub plantar region of the left hind paw with 0.1 ml of Complete Freund's Adjuvant (FCA) (Sigma Aldrich). This consist of 6 mg *Mycobacterium butyricum* 1% suspension in sterile paraffin oil by

thorough grinding with motor and pestle to give a concentration of 10 mg/ml. Dosing with the test and standard compounds were started on the same day and continued for 21 days according to the following schedule [45].

- Group 01 :** Received 2% acacia (10 ml/kg/p.o.) (Normal Control)
- Group 02 :** Complete Freund's Adjuvant Control (0.1 ml FCA, sub plantar) (CFA Control)
- Group 03 :** Standard group received Dexamethasone (10 mg/kg/p.o.)
- Group 04 :** CFA + MLCEF 400 mg/kg in 2% acacia (10 ml/kg/p.o.)
- Group 05 :** CFA + MLCEF 200 mg/kg in 2% acacia (10 ml/kg/p.o.)

Purposely from day 13th to 21st, the animals were not dosed with the test or the standard compounds. On the day 21st, the severities of the secondary lesions were evaluated by the following parameters.

2.5.3.1 Body weight

Body weight of each animal was measured on the day of CFA administration, and later, on each 3rd day upto 21 day. The mean percentage reduction in body weights with respect to that on day of CFA administration was calculated for each drug treated group and compared with that of disease control group [45].

2.5.3.2 PAW Edema

Paw volumes of hind limb was recorded on the day of CFA injection, and again measured on every 3rd day up to 21 day using mercury column Plethysmometer. The sixth day measurement is indicative of primary lesions and eighteenth day measurement will aid in estimating secondary lesions. On the day twenty-first, the secondary phase of rheumatoid arthritis becomes more evident and inflammatory changes spreads systemically and becomes observable in the limb not injected with Freund's adjuvant [45].

2.5.3.3 Arthritic index

An arthritic index for each animal was calculated as the sum of these scores. The average scores for each group of drug treated animals were compared with that of disease control animals [46].

Table 1. Arthritic index

Organs	Indications	Scoring
Ears	Absence of nodules/redness	0
	Presence of nodules/ redness	1
Nose	No swelling of connective tissue	0
	Intensive swelling of connective tissue/redness	1
Tail	Absence of nodules/ redness	0
	Presence of nodules/ redness	1
Fore paws	Absence of inflammation	0
	Inflammation of at least one joint	1
Hind paws	Absence of inflammation	0
	Slight inflammation	1
	Moderate inflammation	2
	Marked inflammation	3
	Redness, severe edema and stiffness in movement	4

2.5.3.4 Hematological parameters

Prior to termination of the experiment on day 21, blood samples were collected by retro orbital route under ether anesthesia, using glass capillary into Ethylene Diamine Tetra-acetic Acid (EDTA)-coated sample bottles for full blood count, which included RBC,WBC count and Hb, ESR determination [45].

2.5.3.5 Biochemical profile

Prior to termination of the experiment on day 21 serum samples were analyzed for Total Protein, Albumin, Urea, Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and Alkaline Phosphatase (ALP), by commercial kits as per manufacturer's instruction (Span Diagnostic Ltd) [45,47].

2.6 Statistical Analysis

Values were expressed as mean \pm SEM from 6 animals. Statistical difference in mean will be analyzed using one way ANOVA followed by Turkey's multiple comparison tests $P < 0.05$ were considered statically significant.

3. RESULTS AND DISCUSSION

3.1 Phytochemical

The Phytochemical studies of methanolic extracts of *Casuarina equisetifolia* fruit (MLCEF)

revealed presence of carbohydrates, glycosides, saponins, tannins, phenolic compounds, proteins & free amino acids and flavanoids.

3.2 Determination of Total Polyphenols

The estimation of polyphenolic content was performed by based on primary phytochemical investigation by Folin-Ciocalteu's reagent, the extracts exhibit that, the MLCEF found to contain 91.38% of polyphenolic compounds, Result shown in Table 2.

3.3 Antioxidant Activity

The extracts containing varying quantities of total polyphenols were comparatively studied for their antioxidant potentialities. Two different in vitro methods namely Reducing Power by FeCl_3 and Nitric Oxide Free Radical Scavenging Activity were employed, ascorbic acid was used as a standard in Reducing Power by FeCl_3 , the MLCEF significantly decreased the absorbance and the IC_{50} value $30.27 \pm 2.43 \mu\text{g/ml}$ was found, to possess more significant antioxidant activity, however the IC_{50} value is lesser then ascorbic acid. In Nitric Oxide Free Radical Scavenging activity, the Curcumin, was used as a standard, MLCEF has offered good free radical scavenging activity by decreasing the absorbance and the IC_{50} value $158.45 \pm 7.15 \mu\text{g/ml}$ the IC_{50} value is lesser then Curcumin. IC_{50} value of different antioxidant activity of *Casuarina equisetifolia* fruit (MLCEF) was shown in Table 3.

Table 2. Quantitative determination polyphenols at 760 nm

Sr. No.	Extract	Polyphenols (%)
1	Methanolic extracts of <i>Casuarina equisetifolia</i> fruit (MLCEF)	91.38

Table 3. IC50 value of different antioxidant activity

Sr. No.	Antioxidant determining method	IC50 value of ascorbic acid ($\mu\text{g/ml}$) \pm S.D	IC50 value of curcumin ($\mu\text{g/ml}$) \pm S.D	IC50 value of extract MLCEF ($\mu\text{g/ml}$) \pm S.D
01	Reducing power by FeCl_3	13.54 \pm 1.28	-----	30.27 \pm 2.43
02	Nitric oxide scavenging	-----	41.37 \pm 5.05	158.45 \pm 7.15

Value are mean \pm S.D.; n=3

3.4 Acute Oral Toxicity Studies

The Adult wistar rat treated with 2000 mg/kg, p.o dose of MLCEF (methanolic extracts *Casuarina equisetifolia* fruit) exhibited normal behavior, without any signs of passivity, stereotypy and vocalization. The motor activity and secretory signs were also normal and no mortality observed up to 14 days.

3.5 Complete Freund's Adjuvant (CFA) Induced Arthritis in Rats

3.5.1 Body weight CFA induced

Arthritis was induced by single sub plantar injection of 0.1 ml of Complete Freund's adjuvant (CFA) (Sigma Chemical) containing 1.0 mg. After Administration of CFA in the sub plantar region of left hind paw. Lose of body weight even before the external signs of the illness were manifested, on day 21st after adjuvant injection, lose of body wt reached their severest level; the decrease in body weight in arthritic rats is associated with a marked decrease in skeletal muscle and white adipose tissue (WAT) mass [48]. In RA, the hyper metabolism that causes loss of weight has been directly associated with the production of TNF- α and IL.TNF- α , a pivotal cytokine in rheumatic disease, can reduce the appetite, increase the protein catabolism and probably decrease the anabolic hormones such as insulin-like growth factor-I (IGF-I) this leads to a loss of weight. TNF- α is a powerful regulator of adipose tissue, may increases lipolysis and altering fat body mass [49]. Whereas methanolic extracts *Casuarina equisetifolia* fruit (MLCEF) (200 and 400 mg/kg b.w.) treated compare with CFA control, body weight significantly increasing 15th, 18th and 21st days. CFA control body weights were significantly decreased as compare to normal control, normal control weight increase in time dependent manner. The detailed results are summarized in Table 4.

3.5.2 CFA induced paw odema

After FCA injection on the rat hind paw, a pronounced swelling and hyperalgesia appeared

with no involvement of contra-lateral paw. This response is usually considered as a primary reaction. There is also a delayed hypersensitive response which is considered as latent secondary systemic response known to induce arthritis occurs after few days on the contra-lateral paw and characterized by tibiotarsal joint swelling and nodule formation in the tail. The secondary response could be due to the liberation and over-production of bradykinin, prostaglandins and kinins in paw tissue, which accompanies leukocyte migration [50]. According to result and investigation more pronounced and reliable anti-inflammatory activity was observed the extracts *Casuarina equisetifolia* fruit (MLCEF) (200 and 400 mg/kg b.w.) on chronic arthritis by CFA induced paw edema in rats. Before 72 hr of MLCEF (200 and 400 mg/kg b.w.) and Dexamethasone (DEXA) (10 mg/kg b.w.) treatment. CFA (0.1 ml) were injected into the sub plantar region of the left hind paw. The volume was gradually increasing and Paw volume was measured in CFA control, MLCEF treated, DEXA treated and untreated group, up to 21 days. MLCEF (200 and 400 mg/kg b.w.) treated animal significantly reduced the paw volume 6th day to 21st day. The detailed results are summarized in Table 5.

3.5.3 CFA induced rats arthritis index

Symmetric involvement of small hand joints (especially proximal interphalangeal and metacarpophalangeal), foot joints (metatarsophalangeal), wrists, elbows, and ankles is typical, but initial manifestations which may occur in any joint. Inflammation and/or nodules are observed on ears, nose, tail, fore paws and hind paws. Arthritic index is the average of the score given to severity of the lesions [51]. Arthritic index were evaluated on 3rd 9th 15th and 19th day, CFA control Arthritic index was significantly increased, where as Dexamethasone (10 mg/kg/p.o.) and MLCEF 400 mg/kg b.w. treated animals decrease arthritic index as compared to disease control animals. The detailed results are summarized in Table 6.

Table 4. Effect of MLCEF on body weights of CFA induced rats

Group	01 Day	03 Days	06 Days	09 Days	12 Days	15 Days	18 Days	21 Days
Normal control (Acacia 2% in water)	236.12±0.613	236.46±1.504	237.82±1.154	238.79±1.215	239.25±1.318	239.67±1.563	241.85±1.313	242.38±1.310
CFA (0.1mlCFA subplantar)	235.21±0.143 ^{ns}	225.15±1.102 [*]	224.23±1.032 ^{**}	223.37±1.279 ^{***}	217.64±1.421 ^{***}	212.13±1.105 ^{***}	208.72±1.138 ^{***}	205.24±1.314 ^{***}
CFA + DEXA (10mg/kg/p.o.)	236.83±0.713 ^{ns}	235.31±1.602 ^{ns}	234.45±1.294 ^{ns}	233.13±1.023 ^{##}	232.87±1.096 ^{###}	231.76±1.245 ^{###}	230.84±1.312 ^{###}	230.19±1.572 ^{###}
CFA+ MLCEF (400mg/kg /p.o.)	235.38±0.503 ^{ns}	234.38±1.195 ^{ns}	231.80±1.103 ^{ns}	229.34±1.261 ^{ns}	228.59±1.112 ^{##}	227.17±1.314 ^{###}	225.76±1.127 ^{###}	224.54±2.056 ^{###}
CFA+ MLCEF (200mg/kg/p.o.)	235.92±0.721 ^{ns}	232.55±1.501 ^{ns}	229.13±1.787 ^{ns}	227.58±1.434 ^{ns}	225.73±1.482 ^{ns}	223.42±1.401 ^{##}	221.25±1.136 ^{###}	218.87±1.357 ^{###}

All the values are expressed as Mean± SEM, n= 6. ***P< 0.001 vs. normal control group, **P< 0.01 vs. normal control group and *P< 0.05 vs. normal control group
^{###}P< 0.001 vs. CFA induced group, ^{##}P< 0.01 vs. CFA induced group and [#]P< 0.05 vs. CFA induced group, ns – Non-significant

Table 5. Effect of MLCEF on CFA induced rats paw odema

Group	01 Day	03 Days	06 Days	09 Days	12 Days	15 Days	18 Days	21 Days
Normal control (Acacia 2% in water)	0.217±0.017	0.225±0.028	0.231±0.093	0.226±0.010	0.240±0.058	0.235±0.0107	0.228±0.021	0.234±0.015
CFA(0.1ml FCA sub plantar)	0.332±0.043 ^{***}	0.464±0.026 ^{***}	0.512±0.0221 ^{***}	0.627±0.0247 ^{***}	0.768±0.0132 ^{***}	0.837±0.0134 ^{***}	0.925±0.0141 ^{***}	0.985±0.0143 ^{***}
CFA +DEXA (10 mg/kg/p.o.)	0.236±0.013 ^{###}	0.268±0.014 ^{###}	0.287±0.016 ^{###}	0.325±0.015 ^{###}	0.318±0.012 ^{###}	0.349±0.018 ^{###}	0.376±0.014 ^{###}	0.418±0.010 ^{###}
CFA+ MLCEF (400 mg/kg ,/p.o.)	0.287±0.022 ^{ns}	0.329±0.015 ^{ns}	0.367±0.012 ^{###}	0.405±0.021 ^{###}	0.453±0.025 ^{###}	0.476±0.023 ^{###}	0.491±0.116 ^{###}	0.517±0.025 ^{###}
CFA+ MLCEF (200 mg/kg, /p.o.)	0.325±0.014 ^{ns}	0.357±0.056 ^{ns}	0.378±0.024 ^{##}	0.415±0.023 ^{###}	0.461±0.016 ^{###}	0.519±0.014 ^{###}	0.532±0.023 ^{###}	0.557±0.032 ^{###}

All the values are expressed as Mean± SEM, n= 6. ***P< 0.001 vs. normal control group, **P< 0.01 vs. normal control group and *P< 0.05 vs. normal control group. ^{###}P< 0.001 vs. CFA induced group, ^{##}P< 0.01 vs. CFA induced group and [#]P< 0.05 vs. CFA induced group, ns – Non-significant

Table 6. Effect of MLCEF on CFA induced rats arthritis index

Group↓	3 Days	9 Days	15 Days	21 Days
Normal control (Acacia 2% in water)	00	00	00	00
CFA(0.1 ml FCA, sub plantar)	2.8±0.314 ^{***}	4.6±0.345 ^{***}	5.9±0.231 ^{***}	7.3±0.401 ^{***}
CFA + DEXA (10 mg/kg/p.o.)	0.4±0.241 ^{###}	0.8±0.201 ^{###}	1.5±0.203 ^{###}	1.9±0.024 ^{###}
CFA+ MLCEF (400 mg/kg, p.o.)	0.8±0.210 [#]	2.2±0.487 ^{##}	2.6±0.364 ^{###}	3.1±0.245 ^{###}
CFA+ MLCEF (200 mg/kg, po)	2.1±0.447 ^{ns}	3.5±0.232 ^{ns}	4.6±0.245 ^{ns}	5.8±0.373 ^{ns}

All the values are expressed as Mean± SEM, n= 6. ***P< 0.001 vs. normal control group, **P< 0.01 vs. normal control group and *P< 0.05 vs. normal control group. ^{###}P< 0.001 vs. CFA induced group, ^{##}P< 0.01 vs. CFA induced group and [#]P< 0.05 vs. CFA induced group, ns – Non-significant

Table 7. Effect of MLCEF on CFA induced rats hematological parameters

Group↓	Hb (gm %)	RBC (10 ⁶ Cell/Cu mm)	WBC (10 ³ Cell/Cu mm)	ESR
Normal control (Acacia 2% in water)	13.23±0.421	5.27±0.417	10.42±0.535	5.25±0.312
CFA (0.1 ml FCA, sub plantar)	5.34±0.328 ^{***}	3.57±0.451 ^{ns}	17.35±0.236 ^{***}	11.53±0.263 ^{***}
CFA + DEXA (10 mg/kg/po)	12.83±0.203 ^{###}	5.16±0.219 ^{ns}	10.86±0.431 ^{###}	5.79±0.350 ^{###}
CFA+ MLCEF (400 mg/kg, p.o.)	10.54±0.334 ^{###}	4.26±0.422 ^{ns}	11.82±0.313 [#]	6.13±0.467 ^{###}
CFA+ MLCEF (200 mg/kg, p.o.)	6.35±0.592 ^{ns}	3.78±0.435 ^{ns}	13.91±0.235 [#]	7.41±0.514 [#]

All the values are expressed as Mean± SEM, n= 6. ^{***}P< 0.001 vs. normal control group, ^{**}P< 0.01 vs. normal control group and ^{*}P< 0.05 vs. normal control group. ^{###}P< 0.001 vs. CFA induced group, ^{##}P< 0.01 vs. CFA induced group and [#]P< 0.05 vs. CFA induced group, ns – Non-significant

Table 8. Effect of MLCEF on CFA induced rats biochemical parameter

Group	Total protein g/dl	Albumin g/dl	UREA Mg/dl	AST IU/L	ALT IU/L	ALP IU/L
Normal control (Acacia 2% in water)	6.82±0.242	3.54±0.384	34.34±1.253	33.51±1.243	28.67±1.208	87.18±1.250
CFA(0.1 ml FCA, sub plantar)	2.23±0.354 ^{***}	1.37±0.125 ^{**}	59.25±1.321 ^{***}	65.35±0.488 ^{***}	59.62±1.421 ^{***}	145.4±1.372 ^{***}
CFA+DEXA (10 mg/kg/p.o.)	5.38±0.327 ^{###}	3.15±0.402 [*]	36.43±1.213 ^{###}	35.24±1.160 ^{###}	30.20±1.104 ^{###}	95.71±1.154 ^{###}
CFA+ MLCEF (400 mg/kg, p.o.)	3.17±0.428 ^{ns}	2.16±0.172 ^{ns}	40.37±1.460 ^{###}	40.16±0.524 ^{###}	33.43±0.426 ^{###}	112.24±1.213 ^{###}
CFA+ MLCEF (200 mg/kg, p.o.)	2.95±0.312 ^{ns}	1.54±0.501 ^{ns}	43.65±1.415 ^{###}	42.37±0.632 ^{###}	36.31±0.703 ^{###}	115.36±1.234 ^{###}

All the values are expressed as Mean± SEM, n= 6. ^{***}P< 0.001 vs. normal control group, ^{**}P< 0.01 vs. normal control group and ^{*}P< 0.05 vs. normal control group. ^{###}P< 0.001 vs. CFA induced group, ^{##}P< 0.01 vs. CFA induced group and [#]P< 0.05 vs. CFA induced group, ns – Non-significant

3.5.4 CFA induced rats hematological parameter

Arthritic rats exhibited a reduced RBC count and an increased ESR. These indicate the anemic condition, which is a common diagnostic feature in chronic arthritis. Erythrocyte sedimentation rate (ESR) is an estimate of suspension stability of RBC's in plasma. It is related to the number and size of the red blood cells and to the relative concentration of plasma proteins, especially fibrinogen and β globulins. Increase in the rate is an indication of active but obscure disease processes. When an inflammatory process is present, the high proportion of fibrinogen in the blood causes red blood cells to stick to each other [52]. The treatment with the MLCEF (dose 200 and 400 mg/kg b.w.) improved the RBC count and the ESR to a near normal level indicating the significant recovery from the anemic condition and arthritis progression thus justifying its significant role in arthritic conditions.

In arthritis condition there is a rise in WBC count due to release of IL- β inflammatory response, IL- β increase the production of both granulocyte and macrophages colony stimulating factor. In differential count, rise in neutrophil count, the migration of leukocytes into the inflamed area [50]. WBC was increased in all the groups as compared to control group. However in MLCEF (dose 200 and 400 mg/kg b.w.) treatment the increase in WBC was comparatively less than in CFA groups. is suppressed. The detailed results are summarized in Table 7.

3.5.5 CFA induced rats biochemical parameter

Increased blood urea, AST, ALT and ALP level also found in the arthritic control group which indicates the liver and kidney dysfunction in arthritic rats [53]. MLCEF (dose 200 and 400 mg/kg b.w.) treatment has significantly reduced the altered urea, AST, ALT and ALP levels. Increased blood urea level was reported in arthritic rats and it was hypothesized that substantial fraction of blood urea in arthritic rats comes from arginine synthesized in the kidneys.

The adjuvant induced arthritis causes changes in plasma protein concentrations that are manifested as a decrease in the albumin fraction [54]. It was also postulated that during inflammation, inflammatory mediator likes histamine, bradykinin and prostaglandins

increase the permeability of vascular tissues to albumin leading to reduction in its serum levels [41]. Thus MLCEF (dose 200 and 400 mg/kg b.w.) treatment could increase the albumin level in arthritic rats which indicates that extracts might have a suppressive action on inflammatory mediator. The detailed results are summarized in Table 8.

4. CONCLUSION

In this study found that the methanolic extract of *Casuarina equisetifolia* fruit (MLCEF) contain significant percentage of secondary metabolite like poly phenol that properties was proved antioxidant activity. Antioxidant proterpties was determined by two method both reveled that the MLCEF have significant capacity to scavenge free radical.

Casuarina equisetifolia fruit (MLCEF) of 400 mg/kg, p.o. showed reduction in rat paw edema volume and it could normalize the haematological and biochemical abnormalities in adjuvant induced arthritic rats in both developing and developed phases of CFA induced arthritis. The actual mechanism of action of extracts on adjuvant induced arthritis is not clear with these studies. The action of extracts on proinflammatory mediators like TNF- α , Interleukins and other relevant mediators will be carried out in future to study its mechanism.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The experimental protocols were approved by Institutional Animal Ethical Committee (IAEC) of C.U. Shah College of Pharmacy and Research, Surendranagar (Gujarat) and were conducted in strict compliance according to ethical principles and guidelines provided by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

ACKNOWLEDGEMENTS

The authors are thankful to Dr. J.G. Sanghvi, Chairman and management members of C.U. Shah College of Pharmacy and research for providing all necessary facilities to carry out the research work.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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