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IN VIVO ANTI-ARTHRITIC ACTIVITY OF *CALOTROPIS GIGANTEA* ROOT BARK EXTRACT

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ABSTRACT

In the present paper anti-arthritic activity of *C. gigantea* root bark extract in experimental rats was explored. The root bark of plant was powdered and defatted with petroleum ether then extracted with a mixture of ethanol and water having ratio 9:1 to yield hydroalcoholic extract. The extract underwent various phytochemical analyses to estimate the secondary phytoconstituent present in plant. The antiarthritic activity was estimated by adjuvant induced arthritis in experiment rat and various parameters have been determined. Percentage yield (% w/w) of hydroalcholic extract was found to be 7.69 % w/w. The secondary metabolites were found to be Alkaloids, Steroid, Tannins, Saponin and Triterponoids. The arthritic score was found to be significantly decreased on treatment with the drug. Oral administration of test extract produced a dose dependent decrease in paw volume it was 0.94± 0.26 mm and 0.76±0.17 mm in test extract CGE 200/400 mg/kg respectively as compared with day of peak inflammation. Radiographic analysis revealed that clear and minimal narrowing of joint space. Arthritic rats treated with *C. gigantea* root bark extract showed a significant ($p < 0.05$) decrease in the activity of AST and ALT enzymes. *C. gigantea* root bark extract has shown significant anti-arthritic activity.

Keywords: Hydroalcoholic, Antiarthritic, *C. gigantia, AST, ALT*

1. INTRODUCTION

Rheumatoid arthritis (RA) is a common chronic and systemic autoimmune disorder characterized by inflammation of the synovial joints and concomitant destruction of cartilage and bone [1]. The inflammation of rheumatoid disease can also occur in tissues around the joints, such as the tendons, ligaments, and muscles [2].

The precise mechanisms of action of drugs are unclear. All the DMARDs inhibit the release or reduce the activity of inflammatory cytokines. Activated T-lymphocytes appeared to be particularly important in this process and it is known that methotrexate, leflunomide, and cyclosporine all inhibits T-cells, cytokines which appears to be important in the inflammatory cascade, include TNF, interlukin IL-1, IL-2, and IL-6. These are good evidence that DMARDs inhibits these cytokines in-vitro and in-vivo [3].

Calotropis gigantea Linn. (*C. gigantea*) is a well-known medicinal herb commonly known as milk weed and has been used in Unani, Ayurveda and Siddha system of medicine for years. It is a native of India, China and Malaysia and it is distributed in almost all over world. *C. gigantea* Linn belongs to the family *Asclepiadaceae* which includes more than 280 genera and approximately 2,000 species. *C. gigantea* (Linn) R.Br. and *Calotropis procera* (Ait) R.Br. are the two common and closely related species [4]. The present study has envisaged to explore the anti-arthritic activity of *C. gigantea* root bark extract in experimental rats

2. MATERIAL AND METHODS

2.1. Plant Material

The Plant *C. gigantea* was collected from the various places of and local area of Mandsaur city during the month of Aug-Sept, 2007. The plant was identified and authenticated by Dr. Gyanendra Tiwari, (Sr. Scientist, medicinal and aromatic plant project, K.N.K. College of Horticulture, Mandsaur, M.P.). The Hebarium was deposited in the Department of Pharmacognocy, B.R. Nahata college of Pharmacy, Mandsaur (M.P.) which has a voucher specimen number: BRNCP/C/008/2008/*C. gigantea*/Dileep Bharati. The root bark was separated from the fresh root of the plant growing in the wild. It was cleaned, and dried under shade at ambient temperature and crushed to get coarse Powder.

2.2. Solvent Extraction

The root bark of plant was powdered and defatted with petroleum ether (40-60^0) to remove chlorophyll, cell debris and fatty materials. Then defatted plant material extracted with a mixture of ethanol and water having ratio 9:1 to yield Hydroalcoholic extract. The Hydroalcoholic extract was the filtered and concentrated for further studies.

2.3. Qualitative Chemical Evaluation

The extract obtained from above procedure was subjected to qualitative test for the identification of various constituents such as alkaloids, flavonoids, glycosides, steroids, terpenoids, carbohydrates etc. [5, 6].

2.4. Pharmacological Investigation

2.4.1. Animals

Male albino rats of wister strain weighing around 100-180g were procured from Animal house, Department of Pharmacology, B. R. Nahata College of Pharmacy, Mandsaur, M.P., Allanimals were housed in polypropylene cages in a temperature - controlled animal house room at 24 ±1°C temperature, 60 ± 5 % relative humidity and 12 hour light and 12 hour dark cycle. The animals were fed with pelleted feed with standard rat diet and tap water throughout the experiment. These animals were used for anti-inflammatory and locomotor activity. The experiment were designed and conducted in accordance with the ethical norms approved by Institutional Animal Ethical Committee Guidelines (Reg. No.98/MPH/09/IAEC/BRNCP/08-09/Mandsaur).

The animal experimentation was carried out in accordance to the guidelines mentioned in the CPCSEA,

2.4.2. Acute Toxicity studies

The preliminary pharmacological studies were conducted to assess the acute pharmacological effects and LD_{50} of the hydroalcholic drug extract.The acute toxicity study was carried out in adult female albino rats (100-180) by "fixed dose" method of OECD (Organization for Economic Co-operation and Development) guideline number-420. The animals were fasted overnight and next day extract (root bark, CGE) of plant *C. gigantea* were administered orally at dose level 5, 50, 200, 400, mg/kg (1% v/v tween 80) for sighting study then the animals were observed continuously for three hour for change in general Behavioral, Neurological, Autonomic, profile and then every 30 minutes for next three hour and finally for mortality (death) after 24 hour. The observation was tabulated according to 'Irwin's table'.

2.4.3. Induction of Arthritis in rats

Arthritis was induced by a single intra-dermal injection of 0.1 ml of Freund's complete adjuvant (FCA) containing 1.0 mg dry heat-killed Mycobacterium tuberculosis per milliliter sterile paraffin oil. The rats were inoculated intradermally into planter surface of right hind paw. A glass syringe (1 ml) with the locking hubs and a 26G needle was used for injection. The rats were anesthetized with ether inhalation prior to and during adjuvant injection, as the very viscous nature of the adjuvant exerts difficulty while injecting. The swelling in hind paws were periodically examined in each paw from the ankle using plethysmometer [7].

2.5. Experimental setup

Before any treatment rats were divided into five groups, each group contains six animals as followed for analysis of histological and biochemical parameters [8].

Table 1: Experimental design for anti-arthritic activity

Groups	Nomenclature	Dose Details
Group I	Normal control,	rats were injected intradermal saline 0.1 ml.
Group II	Arthritic control	Injected 0.1 ml Freund complete adjuvant (FCA, containing 10 mg of heat-killed *Mycobacterium tuberculosis* into right hind paw of the rats on day 0
Group III	Treatment group I (200mg/kg CGE)	Adjuvant induced arthritic rats were administered with hydroalcholic extract of *C. gigantea* root bark extract of dose 200mg/kg body weight/rat/day from day 15-24 after adjuvant injection by oral administration
Group IV	Treatment group II (400mg/kg CGE)	Adjuvant induced arthritic rats were administered with hydroalcholic extract of *C. gigantea* root bark extract of dose 400mg/kg body weight/rat/day from day 15-24 after adjuvant injection by oral route administration
Group V	Standard group,	treated with 0.7 mg/kg body weight/rat/day Dexamethasone from day 15-24 after adjuvant injection by i.p. administration

In the normal control group no treatment was given. In the every treated group, drugs were administered orally as suspension in normal saline. Adjuvant was given to each animals of each group other than normal control and treatments starts from day 14 which is immunization period called as onset of arthritis treatment was continued till day 24 [9]. The standard drug, Dexamethasone i. p. used in the experiment, was purchased from the market having the dose of 2mg/ml in the form of liquid injection. At the end of the experimental period, rats were fasted overnight and anesthetized with xylazine/ ketamine (10/50 mg/kg, i. p.) the anesthetized rats were sacrificed by cervical decapitation and the blood was collected into tubes by cardiac puncture prior to sacrifice. Blood samples were immediately centrifuged at 3000 rpm for 10 min. and plasma or serum samples were stored under freezer until assayed [7].

2.5.1. Clinical Assessment of Adjuvant Arthritis by Arthritic score (polyarthritic index):

For clinical evaluation of AA, the polyarthritis severity was graded on a 0-4 scale, each paw was graded, and 4 grades were summed to a maximum possible score of 16. Rats of each group were evaluated daily for arthritis using a macroscopic scoring system describe as follows: 0 = Normal or no sign of arthritis or no swelling; 1 = Swelling and/or redness in one joint; 2 = Swelling and/or redness in more than one joint; 3 = Swelling and/ or redness in the entire paw; 4 = Deformity and/ or ankylosis [10].

2.5.2. Inflammation Parameter

Measurement of paw volume indicates the effect of drugs on inflammation. Anti-inflammatory effect of the drugs was evaluated by measurement of physical changes in right hind paw of rats. It was evaluated by two different ways as follows:

2.5.2.1. Paw volume (Plethysmometer)

Paw volume were examined after every 3-4 days. The right hind paw volume was measured with plethysmometer (basic value, day 0) and repeated on days 5, 10, 14, 20 and 24. Intensity of oedema in paw (in joint and soft tissue) was determined by measuring the paw volume of entire inflamed paw (right hind paw) with the help of mercury plethysmometer, equipped for accurate measurement of the rats paw swelling through dislocation of fluid volume. The change in volume of the affected paw was evaluated on before the induction of inflammation or arthritis ($V_{b.i.}$), 14 days after induction (V_{14}) and 24 days after induction (V_{24}) and paw volume index was calculated [11-13].

2.5.2.2. Assessment of Pain Behavior by Hot Plate Paw Withdrawal Latency method

In the arthritic rats hind paw has inflamed so the heat sensitivity of the right hind paw has been changed. Heat sensitivity of the paw has been measured in arthritic rats by using the hot-plate test and paw withdrawal latency. As the inflammation progresses in the paw the sensitivity to heat of the rat paw decreases. Thus the paw withdrawal latency decreases. The rat was kept on the hot plate having temperature maintained at 55^0C, and the maximum exposure time to hot plate was 15 sec. For the evaluation of heat sensitivity reaction time (paw licking or paw withdrawal) were recorded for each animals of each group [14].

2.5.3. Radiographic Analysis (Evaluation of bone Destruction by X- rays)

At the end of experiment on the day 25 after adjuvant injection, rats were anesthetized by inhalation of anaesthetic ether and imaged on Fuji HR-Fast film (Fuji photo film), using a siemens X-rays tube assembly (Siemens AG, Munich, Germany). Whole bodies were X-rayed using a 90^0 projection from the dorsal ventral aspect. Radiographs of each rat were evaluated for soft tissue swelling, Bone matrix resorption, Periosteal new bone formation and bone erosion, and were scored in a blind fashion by two independent observers graded as follows: 0 = Normal, No change; 1 = Slight change; 2 = moderate change; 3 = severe change. The total radiological scores were calculated from the sum of both hind paws, with a maximum possible score of 6 for each radiological parameter per rat [9,10].

2.5.4. Biochemical Assays

2.5.4.1. Aspartate transaminase (AST)

One milliliter of buffered substrate was incubated for 10 min at 37^0C. Then 0.2 ml of the enzyme was added and incubation was continued for an hour. To the control tubes, the enzyme was added after arresting the reaction with 1.0 ml of DNPH and the tubes were kept at room temperature for 20 min. Then 5.0 ml of 0.4 N NaOH was added. A set of standard pyruvate were also treated in a similar manner. The colour developed was read at 540 nm. The enzyme activity was expressed as micromoles of pyruvate liberated per milligram of protein per minute [7].

2.5.4.2. Alanine transaminase (ALT)

One milliliter of the buffered substrate was incubated for 10 min at 37^0C. Then 0.2 ml of enzyme was added. The tubes were incubated at 37^0C for 30 min. To the control tubes, enzymes was added after arresting the reaction with 1.0 ml of DNPH and the tubes were kept at room temperature for 20 min. Then 5.0 ml of 0.4 N NaOH was added. A set of standard pyruvate were also treated in similar manner. The colour developed was read at 540 nm. The enzyme activity was expressed as moles of pyruvate liberated per milligram of protein per minute [7].

2.6. Measurement of Inflammatory Mediators

2.6.1. Lipid Peroxidation in Erythrocytes (TBARS)

Lipid peroxidation in erythrocytes was estimated by measuring thiobarbituric acid reacting substances (TBARS). The method is based on spectrometric measurement of purple colour generated by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA). 2.5 ml of TCA solution (10% w/v) was added to 0.5 % supernatant of the tissue preparation in each centrifuge tube and tubes were placed in a boiling water bath for 15 min. After cooling to room temperature, the tubes were centrifuged at 1000X g for 10 min. and 2 ml of each sample supernatant was transferred to a test tube containing 1 ml of TBA solution (0.67 % w/v). Each tube was placed in a boiling water bath for 15 min. after cooling to room temperature; the absorbance was measured at 532 nm. The concentration of MDA was calculated based on the absorbance coefficient of TBA-MDA complex ($\varepsilon = 1.56 \times 10^5$/cm/M) and it was expressed as nmol/mg protein [15].

2.6.2. Determination of Reduced Glutathione (GSH)

The liver was quickly removed from sacrificed rat, and was homogenized in 5 ml of distilled water. The homogenate centrifuged at 6000 rpm for 10 min. 0.5 ml of above solution was mixed with 0.5 ml of 10 % TCA and a protein free supernatant was obtained by centrifugation. 0.5 ml of TCA was mixed with 1 ml of 0.6 M Na_2HSO_4 and 0.5 ml of DTNB reagent. The absorbance of this solution was measured at 410 nm. The absorbance was reading from Concentration Vs Absorbance standard graph of pure glutathione [16].

2.7. Body Weight Measurement

Every rats which was used in experiment weighed at the starting (Baseline) and at the end of experiment which is final day after injection of adjuvant (day 25). The body weight based on pre-study values are 0 = < 5 % decrease; 1 = 6 -10 % decrease; 2 = 11-20 % decrease; 3 = 21-25 % decrease; 4 = > 25 % decrease [17].

2.8. Index of Immune Organ (Spleen)

In the course of experiment, the body weight of rats was measured. At day 28 after immunization, the animals were killed, and the spleen was promptly removed and weighed. The index of spleen was expressed as the percentage (%) of spleen wet weight Vs body weight, respectively [18].

2.9. Balance Beam Activity

The unit feature of balance beam designed for the adult rat, constructed of solid oak, and mounted on each height pole stand. It contains 45 cm long open field and dark black colored box are present at the opposite side of the beam. For determining the balance beam activity of rats, trained animals (training for three days) of each group one by one placed at the one side of the beam and length of time for which animal remained on the beam was recorded with a maximum time limit being 3 min.

2.10. Histopathological Analysis

At the end of experiment Rats paw were collected on day 25, after the injection of adjuvant, by first sacrificing animals and the hind paws were excised from the femur bone then the following process was done:

1. Fixation: The tissue was fixed in 10 % buffered formalin for 5 days.

2. Decalcification: The decalcification was done by 5 % nitric acid for 3- 14 days. Fresh solution of nitric acid were prepared daily and changed. The end point was determined by either chemical mean or direct mechanical method. The bone was pierced with sharp pin if it was properly decalcified it would easily penetrate the bone.

3. Processing: the fragments were then dehydrated by successive solvent treatment (Acetone 50 %, 70 %, 90 %, Absolute, Benzene)cleared and embedded in paraffin. Serial sagittal sections of the whole paw were cut (5 μm thik), stained with hematoxylin and eosin (H&E) and examined for the degree of synovitis and bone destruction in the blinded manner by the pathologist.

4. Evaluation: The following parameters were assessed: Grading of cellular infiltration (polymorphonuclear cells, Macrophages or Lymphocytes), joint space narrowing, synovial hyperplasia, synovial inflammation, pannus formation and bone and cartilage erosion of the ankle joists were examined by the blind observer by using a semiquantitative scale from 0-3 as follows: 0 = Normal;

1 = Mild change; 2 = Moderate change; 3 = Severe change. Histopathological scores were expessed as to give a maximum histological score of 3 for each histological parameter per rat [11].

2.11. Statistical analysis

The experimental results were expressed as Mean ± S.E.M. of n = 6 rats per group. Their 95% Confidence intervals (95% CI) were calculated by linear regression analysis. Software Graph Pad Prism 5.01.336 was used for data analysis. Statistical analysis was evaluated by independent Student's t-test or one-way ANOVA followed by Dunnett's test, with the level of significance chosen at $P < 0.05$.

3. RESULTS

3.1. Percentage yield of extract (w/w)

Percentage yield (% w/w) of hydroalcholic extract (CGE) obtained by solvent extraction was determined was found to be **7.69** % w/w.

3.2. Qualitative chemical evaluation

The qualitative chemical examination for the determination of major photochemical by different methods was found to be Alkaloids, Steroid, Tannins, Saponin and Triterponoids.

3.3. Pharmacological Investigation

3.3.1. Acute toxicity study

The hydroalcholic extract of root bark (CGE) of *C. gigantea* was found to be toxic at 2000 mg/kg and non-toxic safe up to 400 mg/kg body weight by oral route. After 24 h animal were found well tolerated. We have fixed 1000 mg/kg as cutoff LD_{50}. So dose level i.e. 200 mg/kg and 400 mg/kg were selected for present study**.**

3.3.2. Evaluation of adjuvant arthritis: Arthritic score (Polyarthritic index)

The arthritic score was found to be significantly decreased on treatment with the drug as shown in table **(Table 2).**

Table 2: Effect of *C. gigantea* root bark extract (CGE) on Arthritic score (Polyarthritic score) in adjuvant induced arthritis in rats

Groups	Arthritic score (Poly Arthritic score)				
	Day 5	Day 10	Day 14	Day 20	Day 24
Normal Control	0	0	0	0	0
Arthritic control	1.40 ± 0.24	1.80 ± 0.36	2.40 ± 0.45	2.00 ± 0.27	2.20 ± 0.22
CGE-I	1.80 ± 0.45	2.00 ± 0.28	1.80 ± 0.23	2.20 ± 0.42	1.60 ± 0.48*
CGE-II	2.20 ± 0.35	2.20 ± 0.49	1.60 ± 0.29	2.20 ± 0.40	1.80 ± 0.28*
Standard	2.20 ± 0.29	2.40 ± 0.28	2.20 ± 0.35	1.60 ± 0.42	1.40 ± 0.35*

*Values are expressed as mean ± SEM, n = 6 rats in each group. ns = not significant *p < 0.05 when compared with arthritic control. One way ANOVA followed by Dunnet's test*

Table 3: Effect of *C. gigantea* root bark extract (CGE) on change in paw volume (ml)

Groups	Paw volume of rats on different days in ml					
	Day 0	Day 5	Day 10	Day 14	Day 20	Day 24
Normal Control	0.53 ± 0.03	0.59 ± 0.08	0.60 ± 0.02	0.58±0.17	0.52±0.13	0.51 ± 0.16
Arthritic control	0.62 ± 0.02	0.86 ± 0.04	0.98 ± 0.16	1.13±0.27	1.15±0.18	1.16 ± 0.27
CGE-I	0.61 ± 0.04	0.95 ± 0.17	0.85 ± 0.23	0.87±0.27	0.89±0.23	0.94 ± 0.26
CGE-II	0.65 ± 0.06	0.92 ± 0.28	0.73 ± 0.31	0.82±0.24	0.79±0.14	0.76 ± 0.17
Standard	0.60 ± 0.03	0.96 ± 0.32	0.72 ± 0.7	0.79±0.18	0.72±0.24	0.70 ± 0.11

*Values are expressed as mean ± SEM, n = 6 rats in each group. ns = not significant. *p < 0.05 when compared with arthritic control. One way ANOVA followed by Dunnet's test*

3.3.3. Effect on inflammation parameter

3.3.3.1. Paw Volume

Injection of FCA in right hind paw of rat produced an increase in paw volume that was maximum at day 14 (1.13 ± 0.27 mm), and there after it gradually declined. The inhibitory effect of CGE was evaluated on the day of peak inflammation that is day 14. Oral administration of test extract produced a dose dependent decrease in paw volume it was 0.94 ± 0.26 mm and 0.76 ± 0.17 mm in test extract CGE 200/400 mg/kg respectively as compared with day of peak inflammation. The

immunosuppressant, Dexamethasone was more effective in inhibiting joint inflammation as compared to test extracts. The decrease in paw volume in standard drug Dexamethasone treated rats was 0.70 ± 0.11 mm as compared with day of peak inflammation as shown in table 3.

3.3.4. Assessment of Pain Parameter

3.3.4.1. Hot Plate Paw Withdrawal Latency method

As the pain progress the latency of bearing heat by arthritic rats reduced as shown in table 4 when compared with the normal rats. Increment of latency by treatment as dose dependent manner in extract treated rats as comparable with Dexamethasone treated rats. Better result was shown by Dexamethasone as increase in heat latency.

3.3.5. Radiographic Analysis (Evaluation of Bone Destruction by X- Rays)

The radiographic pictures of the joints of arthritic animals, which shows the narrowing of a number of joint space, severe soft tissue swelling, pronounced decrease in bone density, marked destruction of bones, and abnormal ossification in the tarsal, metatarsal, and interphalangeal regions, marginal erosion of joint can be seen in drug treated group (CGE and dexamethasone), in which these changes were normalized **(Fig. 1).**

Table 4: Effect of *C. gigantea* root bark extract (CGE) on Hot Plate Paw Withdrawal Latency method (in seconds)

Groups	Paw withdrawal time in second					
	Day 0	Day 5	Day 10	Day 14	Day 20	Day 24
Normal Control	8.61 ± 0.37	9.14 ± 0.24	9.53 ± 0.27	8.73 ± 0.38	9.94 ± 0.74	10.78 ± 0.42
Arthritic control	5.43 ± 0.26	3.52 ± 0.42	3.28 ± 0.59	3.65 ± 0.63	4.34 ± 0.26	4.24 ± 0.48
CGE-I	5.56 ± 0.28	2.47 ± 0.68	2.16 ± 0.45	3.44 ± 0.65	4.15 ± 0.29	5.64 ± 0.63*
CGE-II	4.91 ± 0.36	2.42 ± 0.59	2.15 ± 0.38	3.46 ± 0.31	5.46 ± 0.42*	5.94 ± 0.52*
Standard	4.86 ± 0.34	1.17 ± 0.73	1.18 ± 0.26	3.35 ± 0.27	5.63 ± 0.52**	6.47 ± 0.76**

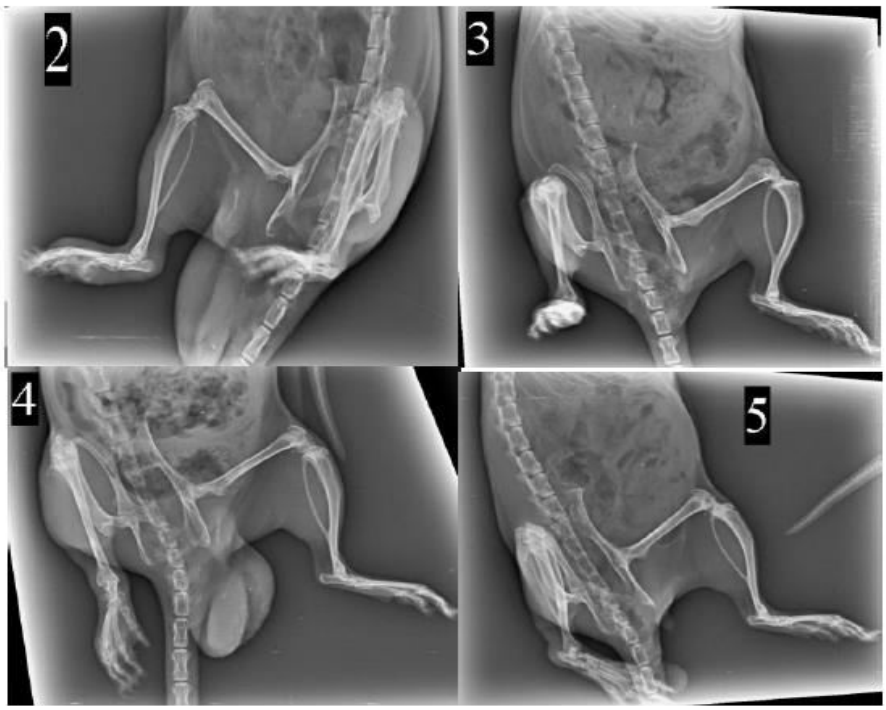

Fig. 1: X-rays pictures of the joints of Control and Experimental animals. 2) Arthritic rat: Showing the diffused joint and narrowing of joint space. **3) Treatment group [CGE-I] (200 mg/kg):** Showing clear and minimal narrowing of joint space. **4) Treatment group [CGE-II] (400 mg/kg):** Showing clear and minimal narrowing of joint space. **5) Treatment group Standard [Dexamethasone] (0.7mg/kg):** Showing clear and minimal narrowing of joint space

3.4. Biochemical Assays

3.4.1. Aspartate transaminase (AST)

A marked increase in the activity of AST (membrane marker enzymes) were observed in the blood homogenate**,** liver tissues homogenate, and spleen tissue homogenate of arthritic rats (group II) when compared to normal control rats (Group I). Arthritic rats treated with *C. gigantea* root bark extract showed a significant ($p < 0.05$) decrease in the activity of AST enzymes **(Table 5)**.

3.4.2. Alanine transaminase (ALT)

A marked increase in the activity of ALT (membrane marker enzymes) were observed in the blood homogenate, liver tissues homogenate, and spleen tissue homogenate of arthritic rats (group II) when compared to normal control rats (Group I). Arthritic rats treated with *C. gigantea* root bark extract showed a significant ($p < 0.05$) decrease in the activity of ALT enzymes **(Table 5).**

Table 5: Effect of *C. gigantea* root bark extract (CGE) on blood homogenate for membrane markers (Aspartate transaminase, AST)

Groups	Treatment	Dose (mg/kg)	Aspartate transaminase (AST)			Alanine transaminase (ALT)		
			Blood homo-genate	Liver homo-genate	Spleen tissue homogenate	Blood homo-genate	Liver homo-genate	Spleen tissue homogenate
Normal Control	Vehicle	-	2.86 ±0.24***	2.25 ± 0.17***	3.18 ± 0.29***	2.26 ± 0.15***	3.29 ± 0.16***	2.88 ± 0.29***
Arthritic control	Vehicle	-	6.44 ± 0.07	6.72 ± 0.06	7.25 ± 0.06	5.54 ± 0.02	6.82 ± 0.03	8.25 ± 0.06
CGE-I	Extract	200 mg/kg	3.63 ± 0.24*	3.53 ± 0.07*	3.37 ± 0.03*	3.53 ± 0.06*	3.83 ± 0.05*	4.37 ± 0.05*
CGE-II	Extract	400 mg/kg	3.43 ± 0.04*	3.41 ± 0.16*	3.62 ± 0.26*	3.71 ± 0.18*	3.91 ± 0.18*	4.62 ± 0.16*
Standard	Dexa-methasone	0.7 mg/kg	3.32 ± 0.14**	3.12 ± 0.14**	3.21 ± 0.15**	3.62 ± 0.1**	3.62 ± 0.1**	4.21 ± 0.19**

*Values are expressed as mean ± SEM, One way ANOVA followed by Dunnet's test, (n=6) *p < 0.05, **p<0.01 when compared with arthritic control, ***p<0.001 when compared with normal control.*

Table 6: Effect of *C. gigantea* root bark extract (CGE) on lipid peroxides (TBARS) of liver tissue homogenate (in nmol/ml)

Groups	Treatment	Dose (mg/kg)	TBARS (nmol/ml tissue supernatant)	GSH (mg/ml)
Normal Control	Vehicle	-	0.23 ± 0.01*	11.38 ± 1.16***
Arthritic control	Vehicle	-	0.34 ± 0.03	3.26 ± 2.32
CGE-I	Extract	200 mg/kg	0.31 ± 0.02^{ns}	4.82 ± 0.72*
CGE-II	Extract	400 mg/kg	0.20 ± 0.01***	3.98 ± 0.37*
Standard	Dexamethasone	0.7 mg/kg	0.14 ± 0.01***	5.28 ± 0.69**

*Values are expressed as mean ± SEM, The statistical difference was evaluated by One way ANOVA followed by Dunnet's test, *p < 0.05 when compared with normal control, ***p<0.001 when compared with arthritic control, p<ns = not significant*

3.5. Measurement of Inflammatory Mediators

3.5.1. Lipid Peroxidation in Erythrocytes (TBARS) on Liver tissue

FCA injection produced an increased in level of tissue TBARS (Thiobarbituric acid reacting substances) expressed as nmol of MDA normal in control rats to increase in arthritic rats tissue supernatant.Table 6 shows the level of lipid peroxides in liver. Lipid peroxide MDA level was found to be significantly increased (p<0/001). After drug treatment for 14 days, the level was found to be significantly reduced in CGE-I (200 mg/kg) and CGE –II (400mg/kg) shows dose dependent reduction in MDA level. Standard drug dexamethasone shows significantly better reduction of tissue peroxidase than CGE group.

3.5.2. Determination of Reduced Glutathione (GSH) on Liver tissue

Oxidative stress associated with FCA-induced polyarthritis was evaluated by measuring level of GSH in the inflamed liver tissues; FCA injection into right hind

paws decreased the tissue GSH, which is naturally occurring antioxidant in body. In normal control rats tissue supernatant level changes from 11.38 ± 1.16 mg/ml to 3.26 ± 2.32 mg/ml in arthritic rats. Both the extract produces an increase in the level of GSH Table 6.

3.6. Body Weight Measurement

During experimental period significant changes in body weight are shown in table 7**.** It was observed that the normal rats gain body weight during experiment, whereas arthritic rats reduce their body weight. Extract treated rats significantly improved the body weight and in standard group rats, dexamethasone shows more weight increment than extract treated rats.

Table 7. Effect of *C. gigantea* root bark extract (CGE) on changes in body weight in grams

Groups	Treatment	Dose (mg/kg)	Body Weight			
			Initial	Final	% wt. change	Score
Control	Vehicle	-	108.00±8.68	130.50±3.75	20.83 (I)	0
Arthritic control	Vehicle	-	136.7±14.06	80.8±4.64	40.8 (D)	4
CGE-I	Extract	200 mg/kg	136.7±10.85	108.7±5.75*	20.48 (D)	3
CGE-II	Extract	400 mg/kg	136.7±9.45	101.8±4.27*	25.53 (D)	4
Standard	Dexamethasone	0.7 mg/kg	126.7±8.43	105.00±6.73*	17.12 (D)	3

*Values are expressed as mean ± SEM, The statistical difference was evaluated by One way ANOVA followed by Dunnet's test, *p<0.05, when compared with adjuvant arthritic group, n=6 animals. I: Increase, D: Decrease.*

Table 8: Effect of *C. gigantea* root bark extract (CGE) on weight measurement index of immune organ (spleen)

Groups	Treatment	Dose (mg/kg)	Index (100%)
Normal Control	Vehicle	-	0.53 ± 0.14
Arthritic control	Vehicle	-	0.29 ± 0.02
CGE-I	Extract	200 mg/kg	0.37 ± 0.17*
CGE-II	Extract	400 mg/kg	0.31 ± 0.15
Standard	Dexamethasone	0.7 mg/kg	0.42 ± 0.09**

3.7. Index of Immune Organ (Spleen)

The index of spleen of adjuvant arthritic rats was determined at day 24 after immunization (Table 8). It was found that there was a decrease of spleen in arthritic rats. The administration of CGE (200 and 400 mg/kg) evidently increased the weight of spleen of adjuvant arthritic rats, but increased better in higher dose (400 mg/kg) of extract. Significantly increases the body weight of dexamethasone treated group.

3.8. Balance Beam Activity

Balance beam performances of each animals of each group were observed which was showed that the arthritic control rats were spending more time in the balance beam as compared to the other animals. Normal control rats were taken very less time in balance beam. Drug treated animals also shows more length of time in balance beam than normal control rats **(Table 9).**

Table 9: Effect of *C. gigantea* root bark extract (CGE) on balance beam activity:

Groups	Treatment	Dose (mg/kg)	Time (sec.)
Normal Control	Vehicle	-	35.66 ± 4.27
Arthritic control	Vehicle	-	91.67 ± 2.261
CGE-I	Extract	200 mg/kg	80.83 ± 3.146*
CGE-II	Extract	400 mg/kg	76.50 ± 3.16**
Standard	Dexamethasone	0.7 mg/kg	71.00 ± 3.19***

*Values are expressed as mean ± SEM, The statistical difference was evaluated by One way ANOVA followed by Dunnet's test, *p<0.05, **p<0.01. ***p<0.001 when compared with adjuvant arthritic group, n=6 animals*

3.9. Histopathological Analysis

Histopathological study was carried out after completion of 24 days of the activity. It shows some changes like leukocytes infiltration cartilage and bone destruction in treated animals. CGE treated animals had a lower degree of sub-synovial infiltration that was cure, found in non-arthritic animals.

A histopathological study produced marked infiltration of leukocytes, edema, fibrosis, bone and cartilaginous erosion, cellular inflammatory changes in the nearer tissues, and also shows joint space narrowing, vascular proliferation, and congetion. Figure 2 showed the different types of changes occurs in hind paw of treated and non-treated animals.

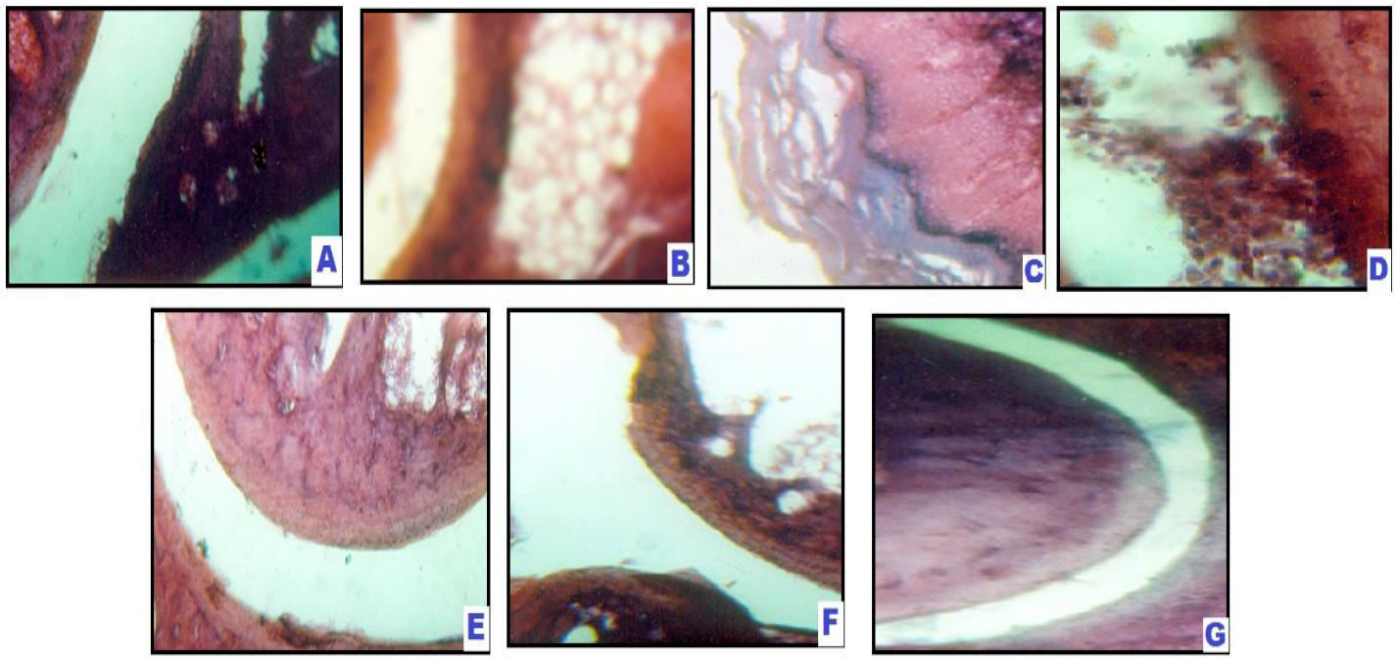

Fig. 2: Histopathological Analysis. A: Normal control: Histopathology of normal control rat showing normal structure of joint and normal synovial cavity. **B:**Histopathology of normal control rat showing normal structure of bones and cartilages**. C: Arthritic control:** Histopathology or microphotograph of arthritic rats showing cartilage destruction.**D:** Microphotographs of model control or arthritic control rat shows cellular infiltration in synovial cavity. **E:** Microphotograph of CGE-I (100mg/kg) treated rat showing normalization of joints and bones.**F**: Microphotographs of CGE-2 (200 mg/kg) treated rats showing normal structure of joints and bones, less inflammation and normalization of joint space, synovial space. **G:** Microphotographs of Dexamethasone (0.7 mg/kg) treated rats showing normal structure of joints and bones

4. DISCUSSION

The present study demonstrates that freund's complete adjuvant (FCA) containing killed *M. tuberculosis* induced AA (adjuvant arthritis) in rats. Treatment of AIA (adjuvant induced arthritis) in rats with *C. gigantea* root bark extract shown decline in inflammation which was comparable to Dexamethasone treated groups (standard drug).

Adjuvant induced arthritis (AIA) is thought to occur through cell mediated autoimmunity by structural mimicry between mycobacteria and cartilage proteoglycans in rats. Thus activated macrophages and lymphocytes by adjuvant inoculation or their product monokines, cytokines, chemokines, may be involved in abnormal lipid and protein metabolism. Lipid peroxide formed by auto oxidation of polyunsaturated fatty acids of cell membranes. In the damaged cells concentration is increased. In the present study lipid peroxide were significantly increased in blood, spleen and liver cells of arthritic rats. This may be due to less activity of antioxidants and inactivation of them. Inhibition of inflammation and accumulation of lipid peroxides may be due to presence of flavonoids in the *C. gigantea*. Flavonoids have antioxidation property by scavenging singlet O_2[17].

Adjuvant induced arthritis (AIA) is a rather aggressive and monophasic form of arthritis, usually the disease is quite severe and finally leads to complete ankylosis and

permanent joint mal-formations. Therefore AIA is most frequently used as a model for screening and testing anti-arthritic agents, especially NSAIDs, as the inflammation associated with AIA is very dependent on prostaglandin E_2 (PGE_2) generated by cyclooxygenase (COX) [18,19].

The FCA administered rats showed soft tissue swelling around the ankle joints during the development of arthritis, which was considered as edema of the particular tissues. As the disease progressed, a more diffused demineralization developed in the extremities [20]. Secondary lesions of adjuvant arthritis occurred after a delay of approximately 10 days and were characterized by inflammation of non-injected rats (normal control rats) and further increase in the volume of the injected hind leg. Reduction of paw swelling in the *C. gigantea* root bark extract (CGE) treated rats from the third week onwards may be due to immunological protection rendered by the plant extract. Cellular enzymes, such as aspartate transaminase (AST), alanine transaminase (ALT), membrane bound indicator of type II cell secretary activity or the lysosomal enzyme b-glucuronidase, an indicator of phagocytic activity, can also be used as sensitive markers of cellular integrity and cellular toxicity induced by pathological conditions. Increased activities of these enzymes were observed in arthritic rats. This may be attributed towards persistent inflammation.

The pathogenesis of RA is perpetuated by the activity of a complex network of cytokines. As a consequence of the inflammatory processes, a large number of cytokines and growth factors with overlapping biological effects are found in the synovium [21]. Several cytokines such as TNF-α, IL-1 and IL-6 have been implicated in the pathological mechanism of synovial tissue proliferation, joint destruction and programmed cell death in rheumatoid joint. It was reported that the expression of inflammatory cytokines such as TNF- α and IL-1 β, and the tissue enzymes such as cathepsin and matrix metalloproteinases were observed to be increased in the subchondral bone region of the knee joint samples from human osteoarthritis or rheumatoid arthritic patients. Biological agents that specifically inhibit the effects of TNF-α or IL-1 represent a major advancement in the treatment of RA [22].

In the histological studies of joints showed the destruction of inflamed joints are the continued migration into the synovium and joint fluid of polymorphonuclear leukocytes, lymphocytes and monocytes/macrophages, all of which produce inflammatory cytokines. Thus, pharmacological inhibition of this leukocyte migration and accumulation in arthritis may have beneficial effects for joint preservation. In our study, our histological observations supported that the administration of *C. gigantea* root bark extract may be protective by decreasing the leukocytic migration. It has also been reported that the flavonoids significantly inhibit the leukocyte migration in a dose dependant manner [7].

The bones become thin and fragile of the arthritic rats on inspection and were easily crushed during cutting and homogenizing. Change in body weight is in response to the incidence and severity of arthritis and used to assess the onset of the disease. It was reported that Arthritis characterized by reduced body weight and loss of body weight is associated with increased production of pro-inflammatory cytokines such as TNF-α and IL-1 [23]. Treatment with *C. gigantea* and dexamethasone recovered the body weights, which support and confirms the above observations.

5. CONCLUSION

C. gigantea root bark extract has shown significant anti-arthritic activity which may be due to some short of Phytochemicals such as Flavonoids, Triterpenes and Steroids and compared with dexamethasone as standard drug.

Conflict of Interest: NIL

6. REFERENCES

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