EFFECT OF ETHANOLIC EXTRACT OF COUROUPITA GUIANENSIS LEAF ON FRUEND'SCOMPLETE ADJUVANT INDUCED RHEUMATOID ARTHRITIS IN RATS

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Abstract:

In traditional system of medicine, *couroupita guianensis* Linn leaves were used for various ailments including diabetes, inflammation, depression, hypertension, malaria, antibiotic, skin diseases etc. To investigate the anti-arthritic activity of the above leaves, the present study was carried out on Fruend's adjuvant induced arthritis in rats.The present study states that the effect of the ethanolic extract of Fruend's complete adjuvant induced rheumatoid arthritis in rat paw edema, body weight changes and alterations haematological and biochemical parameters in both developing and developed phases of arthritis. Histopathology of proximal interphalangeal joints and radiology of hind legs were studied. In FCA induced arthritic rats, there was significant increase in rat paw volume and decrease in body weight increment, there as *couroupita guianensis* treated groups showed significant reduction in paw volume and normal gain in body weight. The altered haematological parameters and serum parameters in the arthritic rats were significantly brought back to near normal by the *couroupita guianensis* leaf treatment at the dose of 200mg/kg in both developing phases of arthritis. Further the histopathological and radiological studies revealed the anti-arthritic activity of *couroupita guianensis* leaf by indicating fewer abnormalities in these groups when compared to the arthritic control group.In conclusion, *couroupita guianensis* leaf extract at the specified dose level of 200mg/kg, showed reduction in rat paw edema volume and it could significantly normalize the haematological and biochemical abnormalities in adjuvant induced arthritic rats in both developing and developed phases of FCA induced arthritis. Further the histopathological and radiological studies confirmed the antiarthritic activity of *couroupita guianensis.*

Keywords: *Couroupita guianensis*, Histopathological studies, Radiological studies.

Introduction:

Rheumatoid arthritis (RA) is a chronic multi system disease characterized by persistent inflammatory synovitis involving peripheral joints leading to progressive functional impairment.[1] About 1 % of the world's population and 0.7 % (88 lakhs) of Indian population are afflicted with RA. It is more common in women and generally occurs between 40 and 60 years of age.[2, 3]

Arthritis causes disability, compromised quality of life, and premature mortality.[4] It affects approximately 0.5%–1.0% of the global adult population, with an estimated annual incidence of 12.0–24.5 males and 23.9–54.0 females per 100,000.[3,5,6] The incidence is largely consistent racially and geographically, and the peak age of onset lies between the ages of 45 and 65 years. The economic cost of arthritic-related diseases is estimated to consume about 3% of gross domestic product in developed countries, and is somewhat lower in developing countries due to lower life expectancy.[7,8] The present decade has therefore been declared the "Bone and Joint Decade" by the World Health Organization,[9] in order to further our understanding of the impact of musculoskeletal diseases on society and individuals and to assist advancement on this front. It is against this background that we attempted to assess the antiarthritic effect of aqueous and ethanolic leaf extracts of *Pistia stratiotes* in a rodent adjuvant-induced arthritis model to ascertain its importance in the traditional management of inflammatory disorders.[10,11]

couroupita guianensis, known by several common names, including **Cannonball tree,** is a deciduous tree in the family Lecythidaceae, which also contains the Brazil nut (Bertholletia excelsa). It is native to the rainforest of central and southamerica[12].It is cultivated in many other places[13].

Materials and methods:

Collection of leaves:

Fresh plant leaves of ***couroupita guianensis*** were collected and authentified by M.R.Paul satyakeerthi.

Preparation of powder:

Fresh leaves of the plant were collected and dried under shade. These dried materials were mechanically powdered sieved using 80 meshes and stored in an airtight container. The powdered material was used for phytochemical analysi[14].

Plant extraction:

The extract of the study plant was prepared according to the methodology of Indian pharmacopoeia. The leaves were dried in shade and the dried leaves were subjected to the pulverization to get coarse powder. The coarse powder material was subjected to soxhlet extraction. The powdered plant material was packed into soxhlet column, defattying with petroleum ether and then extracted with ethanol by using soxhlet apparatus. The extract was filtered through a whatmann filterpaper no.1 and concentrated to dryness in flash evaporator under reduced pressure and controlled temperature to get a constant weight. Just prior to use, the substance was dissolved in physiological saline solution. This ethanol extract was used for the further study [15].

Drugs and chemicals: Freund's Complete Adjuvant (FCA) was procured from Sigma chemicals Co. ELISA kits of TNF-α and IL-6 were purchased from Ray Biotech, diclofenac and methotrexate from M/S Alkem laboratories ltd. All other chemicals were of the highest purity and analytical grade.

Test extract:

Ethanolic extract of couroupita guianensis defattying with petroleum ether was employed for assessing anti arthritic activity.

Chemicals: 10%formalin, petroleum ether, Ethanol (95%).

Standard drug: Diclofenac

Inducing agents: Freund's complete Adjuvant.

Vehicle: 1%tween 80

Instruments: Mercury plethysmometer, ESR stand, Research microscope sahil's Haemoglobin meter.

Preparation of dose formulations:

The required quantity of compound and extract were taken and dissolved in particular vehicle to prepare dose formulations which can be given to the animals.

Justification for Test system:

Wistar rats is the species recommended for the assessment of antiarthritic activity as they are easily prone to arthritis and they are well reported in the literature.

Personnel safety precautions:

Gloves, head cap, facemask, and goggles will be used in addition to protective body garments and shoes to ensure adequate personal health and safety. Appropriate measures will be taken to avoid inhalation and skin contact with test item. In case of eye contact, the eye will be washed thoroughly with copious amount of water and medical treatment will be sought. In case of skin contact, the compound residue will be washed with the soap and water.

Institutional animal ethics committee (IAEC) approval:

The following study protocol has been reviewed and approved by the Institutional animal ethics committee (IAEC) of sicra lab. The recommendations as per the animal welfare guideline regarding animal care and handling will be strictly adhered to. The approval has been documented in the committee for the purpose of control and supervision of experiments on animals specified from B protocol. The procedure used in the protocol will be designated to conform to the accepted practices and to minimized /avoid risk of selected for use in this study will be the considered to be the minimum requirements to meet rationale scientific end points.

Acclimatization of animals:

Animals were kept for one week, before enrollment into the study. Food, housing and water to the animals are also taken care.

Randomization and grouping:

A group of animals will be examined for health and healthy wistar rats will be selected for the study and will be randomly assigned to different groups based on the body weight.

Study design:

The selected rats were divided into normal control, disease control, standard, test group 1 and 2. Disease induction is done. Administration of the disease, extract and standard drugs were done at time. Route and frequency of administration of formulations must be planned prior to the experiment.

Preparation of dose formulation:

Fruend's complete Adjuvant:

The required quantity of Fruend's complete Adjuvant will be weighed using analytical balance and transferred into mortar and pestle. Desired quantity of normal water added and triturated well to get final concentration of 0.1ml/kg body weight [16].

Diclofenac:

The required quantity of Diclofenac will be using analytical balance and transferred into mortar and pestle. Desired quantity of normal water added and triturated well to get final concentration of 25mg/ml solution [17].

Ethanolic Extract couroupita Guianensis (EECG):

The required quantity of **EECG** will be the weighed using an analytical balance and transferred to a mortar and pestle. Desired volume of 0.5%w/v carboxymethyl cellulose sodium medium viscosity in water will be added and triturated to get the final concentration of 100 and 200 mg/kg body weight suspensions. The suspension formulation will be transferred to a centrifuge tube and subjected to vortexing for 2 minutes to obtain homogenous suspensions.

Animal selection:

Albino rats of either sex weighing between 150-200 gm were selected for present study. The food and water were supplied ad libitum. All the animals were kept under standard laboratory conditions in a 12k:12h light and dark cycles and maintained under controlled temperature 27±2˚C for acclimatization the experiment was conducted in accordance with the direction ofInstitutional Animal Ethical Committee. Before performing the experiment, the Ethical clearance was obtained from Institutional Animal Ethical Committee [18].

Acute toxicity study:

Albino mice of either sex weighing between 20-30mg were used during investigation. The animals were fasted overnight. The OECD guidelines no.420 fixed dose method was adopted and accordingly doses of the extract were calculated. As per following the OECD guidelines no.420 fixed dose method procedure, the safest dose of extract is 1000mg/kg body weight. The safe dose was found to be the 1000mg/kg body weight, hence 1/10*of the dose was taken as effective dose which is found to be 100mg/kg body weight for the extract [19].

Procedure:

In order to evaluate possible toxic effects on animals, 1000mg/kg dose was administered to animals. After 5 days of a single administration, treated mice did not present behavioral alterations and no lesions or bleedings in stomach were observed. No sign of in toxification such as convulsions, death were present during the period of observation. This indicates that the dose was nearly nontoxic in mice up to an oral dose of 1000mg/kg of body weight.

Experimental design:

Male wistar rats weighing between 150-200gm were selected for the experiment. They are grouped in a group of 6 animals each into 6 groups. The treatment schedules of rats belonging to the different groups are shown below:

Group1: normal (10%tween 80)

Group2: control (0.1ml freund's complete adjuvant)

Group3: Diclofenac (10mg/kg)

Group4: Ethanolic extract of couroupita guianensis(1000mg/kg)

Group5: Ethanolic extract of couroupita guianensis(2000mg/kg)

On the 0^{th} day, the basal paw volume of left hind paw of each animal was measured using mercury plethysmometer, on the 1^{st} day, all the animals of all groups were once anasthetized, they were injected into the ankle joint of left hind paw with 0.1ml of complete freund's adjuvant containing o.1mg of heat killed mycobacterium tuberculosis cells in liquid paraffin and were allowed to recover to serve as control. Doing with standard drug Diclofenac and extract 100mg/kg body weight was started on the same day i.e 1^{st} day and continued for 21 days. Merest experimental groups animals receives respective treatment once daily by oral route. The 1% tween 80 was used as vehicle for suspended the extract. Paw volume change from day 19-21. The animal will receive the test compound orally. On day 21, the paw volume will be recorded again. The body weight of the animals was measured by digital balance to access the course of the disease at the initial day before induction and at the end of 21^{st} day.

At the end of day 21,the animals were anesthetized with anesthetic ether and blood was isolated from retro orbital route to all the groups of animals and various haematolagical parameters such as haemoglobin content, total WBC, RBC and Erythrocyte sedimentation rate were estimated using routine laboratory methods. At the end of study, all the animals were sacrificed and ankle joint portion of the rats were separated and joint bone was separated. The bone portion was kept in10%formalin solution in glass container for histopathological studies.

Evaluation:

Arthritis was induced by complete freund's adjuvant injection, the inflammation was induced with a maximum at 4 to 5 days of injection. The paw edema was measured on first week, second week, third week and forth week respectively after the induction. The mean change in injected paw edema with respect to initial paw volume, were calculated on respective weeks and %inhibition of paw edema with respect to untreated group was calculated using the following formula.

Calculation of paw edema:

Paw edema=paw volume after injection-paw volume before injection

For initial week=0week

Initial paw edema= paw volume after injection-paw volume before injection

For first week:

Paw edema=paw volume- paw volume before injection

For second week:

Paw edema=paw volume- paw volume before injection

For third week:

Paw edema=paw volume- paw volume before injection

For fourth week:

Paw edema=paw volume- paw volume before injection

Calculation for percentage inhibition:

%inhibition for paw edema(ml)I=[cn-ct/cn]^{x}100

Where

I=% inhibition for paw edema

Cn=mean paw edema (ml) control group

Ct= mean paw edema (ml) treated group

The body weight of the animals was measured by digital balance to access the course of the disease at the initial day before induction and at the end of 21st day.

Statistical analysis:

All the data obtained from the various parameters were statistically evaluate by one way analysis of variance test followed by Dunnet's post-test value less than 0.05 were used as the significant level. The results are presented as mean ±S.D [20].

Results:

Paw edema volume :

Adjuvant induction model:

Mono arthritis will be induced according to the method of newbould et al[76]. Once animal was anaesthetized i.e. one ankle joint will be injected with 0.1 ml of fraud's complete adjuvant (FCA) containing 0.1mg mycobacterium tuberculosis in mineral oil was injected intradermally into the planar aspect of the hyind paw of each animal. The contra lateral knee will be injected with 0.1ml (0.9%) physiological saline. The animals will be allowed to recover. To serve as controls. Another group of anaesthetized rats will be injected with 0.1ml saline in one knee and the contra lateral knee will be left untreated.

The degree of inflammation was measured plethyswmographically accordingly,l edema formation and the percentage of inhibition was calculated as described on the days 1,4,8,14,21 and the primary and the seconadary lesions were measured.rats

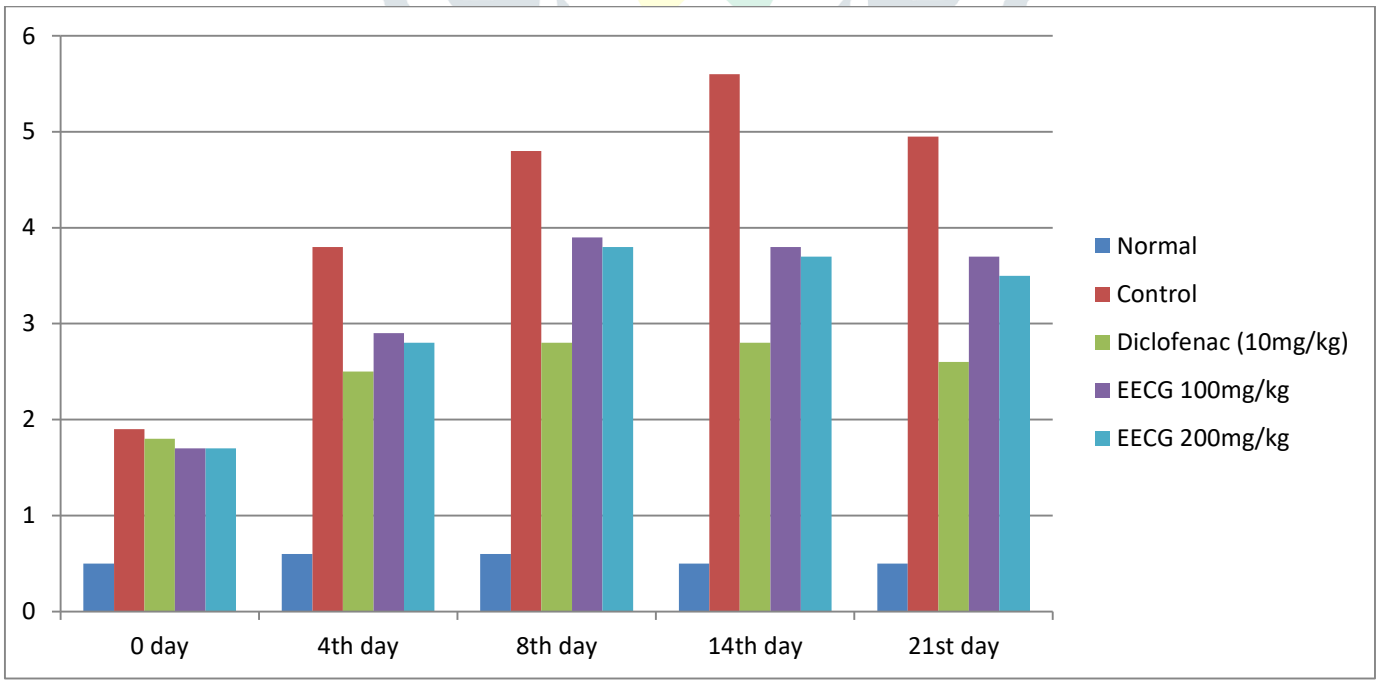
will be examined form visual appearance of arthrities in peripheral joints and scores for severity of arthritis. Rats will be considered arthritic when significant changes in redness and or swelling will be noticed in digits or in thr part of paw. The clinical severity of the arthritis in each paw will be quantified weakely by the clinical score [21].

Anti-arthritic effect of MECG was significantly studied by using in-vitro inhibition of protein denaturation model. MECG at different concentrations (50, 100, 150, 300 and 500µg/ml) provide protection against denaturation of proteins. Most of the investigators have reported that denaturation of protein is cause of rheumatoid arthritis. Production of auto-antigens in most of the rheumatic diseases may be due to in-vivo denaturation of proteins. Mechanism of denaturation may involves changes in electrostatic, hydrogen, hydrophobic and disulphide bonding. The maximal protein denaturation inhibition of MECG was found to be 87.41 % .

Platelets activation may not play a key role in homeostasis, moreover their hypersensitivity is reported that to be related with progression of atherosclerosis. The MECG showed dose dependent inhibition of ADP-induced human platelet aggregation and more over 20% inhibition were observed at the initial dose of 25 µg/ml. The MECG shows dose dependent inhibition of ADP-induced human platelet aggregation.

Table-1: Effect of Ethanolic Extract of Couroupita guianenesis leaf on rat paw edema in FCA induced rats.

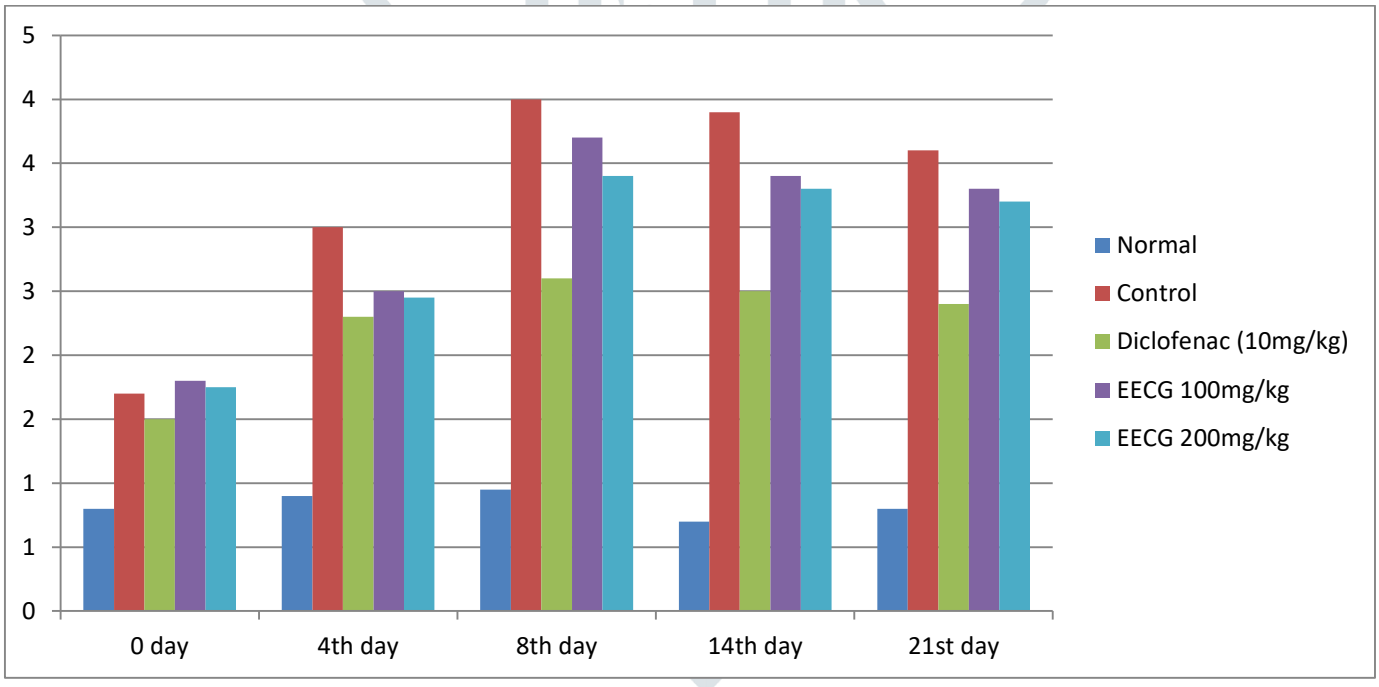
Groups	Paw edema volume (m1) ± SEM				
	Day 0	Day 4	Day 8	Day 14	Day 21
Normal	0.22 ± 0.05	0.025 ± 0.09	0.26 ± 0.09	0.21 ± 0.05	0.22 ± 0.09
Control (FCA 0.1 ml)	0.94 ± 0.004	1.85 ± 0.004	2.41 ± 0.004	2.76 ± 0.004	2.47 ± 0.09
Diclofenac (10 mg/kg)	0.93 ± 0.005	1.25 ± 0.005	1.41 ± 0.004	1.31 ± 0.004	1.31 ± 0.06
EECG 100 mg/kg	0.92 ± 0.005	1.41 ± 0.003	1.92 ± 0.006	1.87 ± 0.004	1.78 ± 0.12



H-1: Histogram showing the mean changes in paw edema in FCA induced arthritic rats.

Table-2: Effect of Ethanolic Extract of Couroupita guianenesis leaf on rat paw diameter in FCA induced arthritic rats.

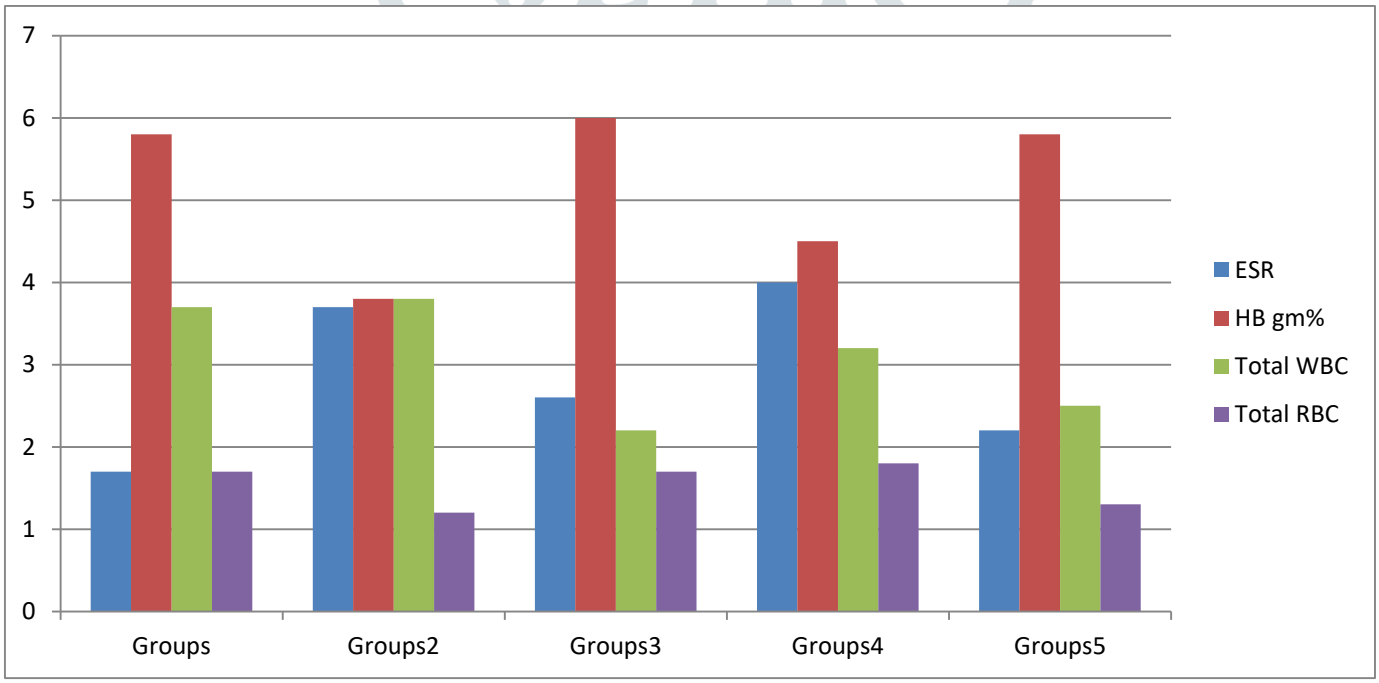
Groups	Rat Paw Diameter (mm)				
	Day 0	Day 4	Day 8	Day 14	Day 21
Normal	4.02 ± 0.02	4.87 ± 0.02	4.98 ± 0.07	3.72 ± 0.65	3.68 ± 0.604
Control 0.1 ml FCA	8.04 ± 0.03	15.02 ± 0.004	20.03 ± 0.004	19.03 ± 0.63	18.01 ± 0.062
Diclofenac (10 mg/kg)	7.32 ± 0.005	12.02 ± 0.005	13.44 ± 0.006	13.04 ± 0.008	12.31 ± 0.006
EECG 100 mg/kg	9.01 ± 0.004	13.45 ± 0.004	18.67 ± 0.006	17.15 ± 0.007	16.04 ± 0.004
EECG 200 mg/kg	8.44 ± 0.004	13.07 ± 0.004	17.04 ± 0.006	16.04 ± 0.004	15.02 ± 0.005



H-2: Histogram showing the rat paw diameter in FCA induced arthritic rats.

Table-3: Effect of Ethanolic Extract of Couroupita guianenesis leaf on Haematological parameters in FCA induced arthritic rats.

Groups	ESR and Haematological Parameters			
	ESR (mm/hr)	**HB (gm %)**	**Total WBC**	**Total RBC**
Normal	3.30 ± 0.05	11.56 ± 0.71	7.15 ± 0.67	3.49 ± 0.56
Control (FCA 0.1 ml)	7.06 ± 0.36	7.35 ± 0.72	7.31 ± 0.57	2.48 ± 0.67
Diclofenac (10 mg/kg)	5.07 ± 0.03	12.04 ± 0.71	4.73 ± 0.79	3.44 ± 0.70
EECG 100 mg/kg	8.02 ± 0.03	8.81 ± 0.69	6.28 ± 0.77	3.14 ± 0.42
EECG 200 mg/kg	4.58 ± 0.3	11.56 ± 0.60	5.46 ± 0.81	3.47 ± 0.67

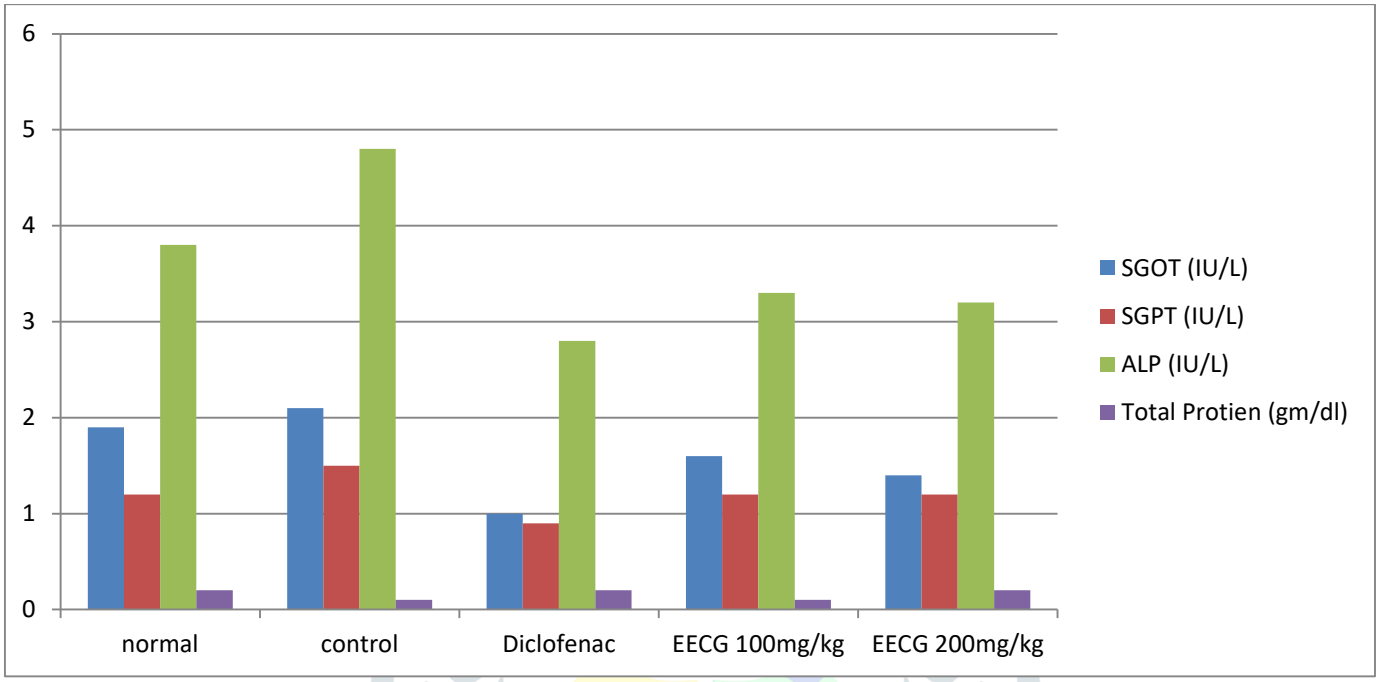


H-3: Histogram showing the Haematological parameters in FCA induced arthritic rats.

Table-4: Ethanolic Extract of Couroupita guianenesis leaf on serum parameters in FCA induced arthritic rats.

Groups	ESR and Haematological Parameters			
	SGOT (IU/L)	**SGPT (IU/L)**	**ALP (IU/L)**	**Total Protien (gm/dl)**
Normal	95.4 ± 0.26	55.56 ± 0.89	187.5 ± 5.70	7.91 ± 0.21
Control (FCA 0.1 ml)	103.5 ± 3.69	70.82 ± 1.57	245.0 ± 9.99	2.91 ± 0.23

Diclofenac (10 mg/kg)	49.60 ± 2.08	46.18 ± 1.20	133.0 ± 4.54	6.76 ± 0.59
EECG 100 mg/kg	73.81 ± 1.89	56.37 ± 2.42	169.9 ± 7.20	4.78 ± 0.36
EECG 200 mg/kg	69.06 ± 2.14	54.86 ± 1.71	154.8 ± 3.28	5.23 ± 0.68



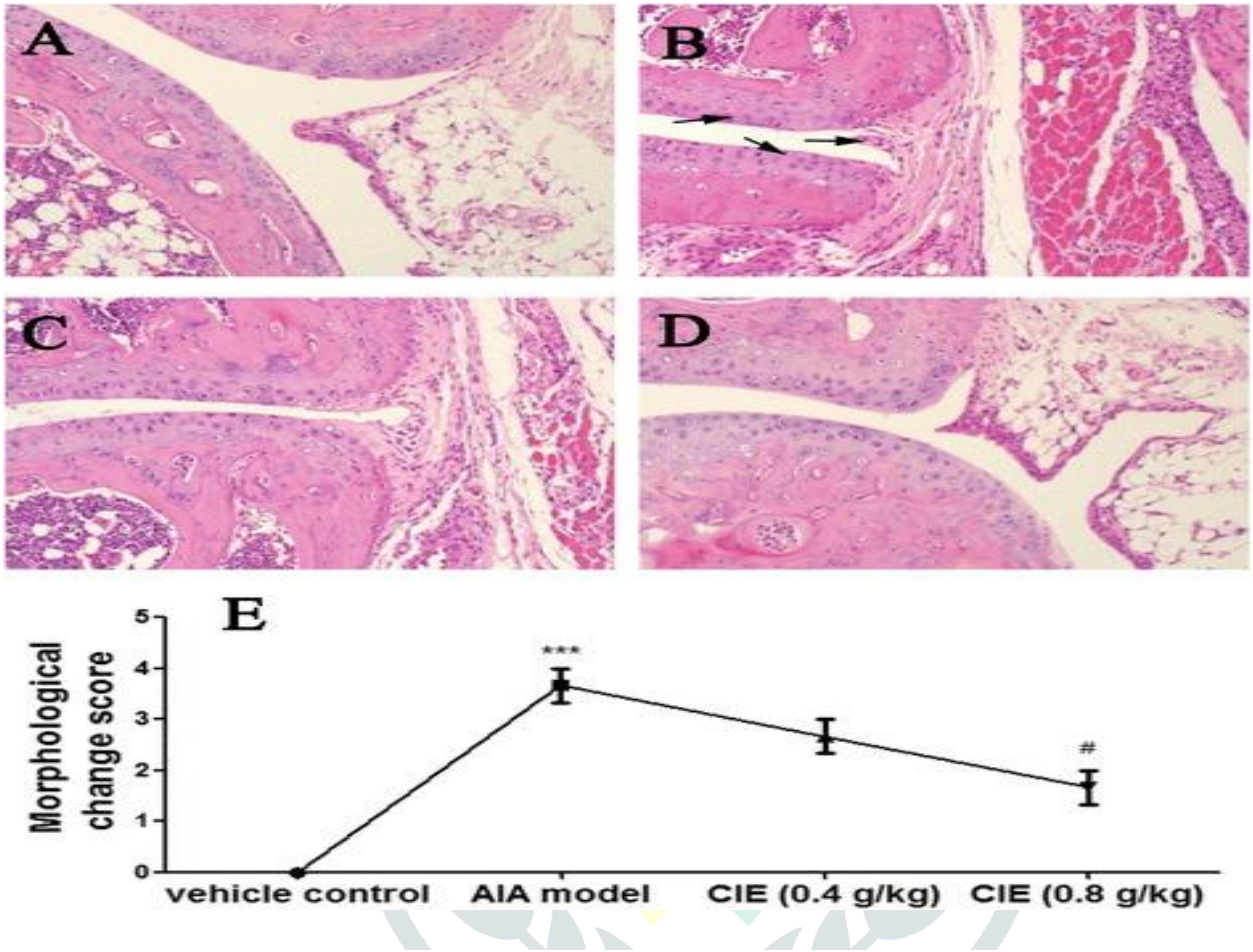
H-4: Histogram showing the rat serum parameters in FCA induced arthritic rats.

Table-5: Changes in Body weight in FCA induced rats.

Groups	**Mean Body Weight (gms)**				**Mean changes ± SEM**
	Intial Week	**1st Week**	**2nd Week**	**3rd Week**	
Control (FCA 0.1 ml)	172.8 ± 6.7898	171.2 ± 6.580	169.0 ± 5.882	154.3 ± 6.086	-22 ± 0.660
Diclofenac (10 mg/kg)	169.3 ± 4.161	169.3 ± 4.161	17.20 ± 5.37	175.7 ± 4.828	6.9 ± 1.089
EECG 100 mg/kg	165.2 ± 4.872	167.2 ± 4.674	171.0 ± 4.147	172.3 ± 4.74	9.5 ± 0.399
EECG 200 mg/kg	164.2 ± 2.929	165.3 ± 2.929	170.08 ± 3.509	171.4 ± 3.509	10.0 ± 0.973

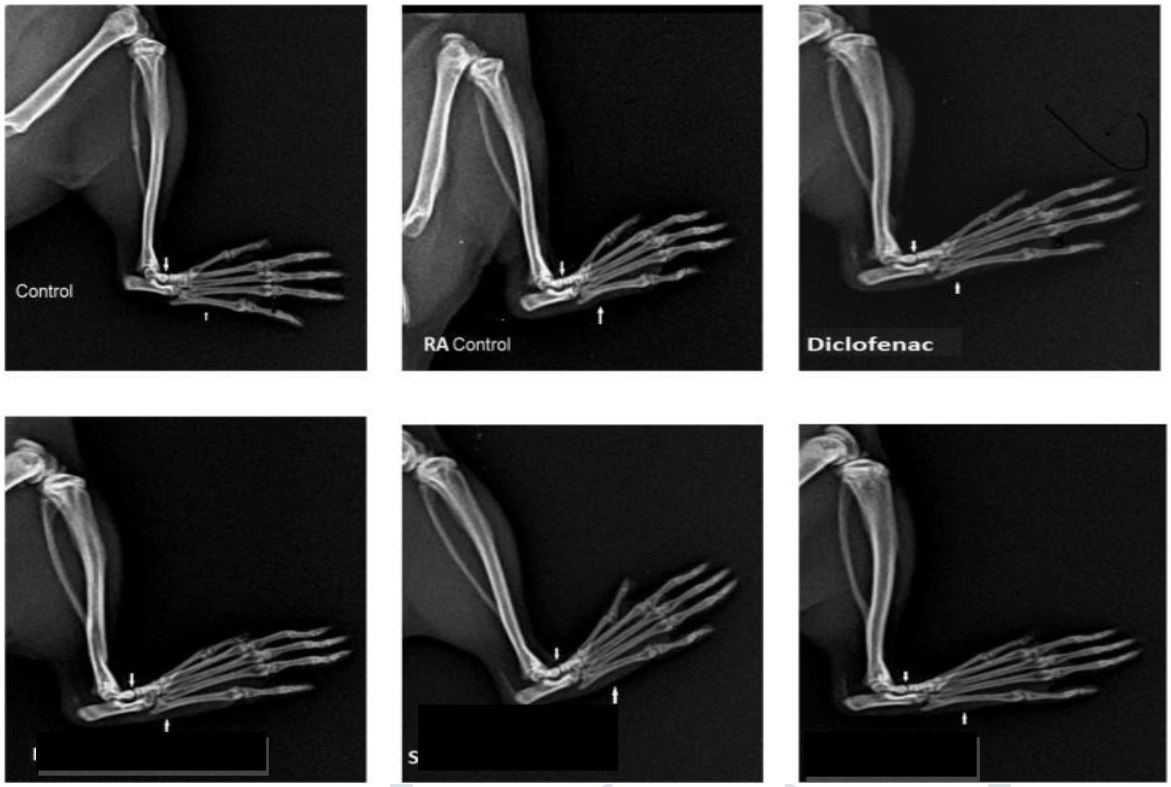
Histopathological changes :

The joints of hind paw in NC rats had shown intact articular cartilage and normal joint space without inflammation. Inflammatory changes, distorted articular cartilage and narrowing of joint space were seen in RAC rats. There was varying degrees of reduction in inflammation and improvement in joint space in groups treated with leaves .



Radiological changes :

RA-induced joint showed soft tissue swelling and narrowing of joint space. The joint space was intact and soft tissue swelling was reduced in rats treated with D, M and EESI. But there was no difference in joint space between standards and tests.



DISCUSSION :

The latex of Calotropis procera is well known for its anti-inflammatory properties in many majore experimental models. It has also been shown to afford protection towards functional impairment produced by FCA in rat model of monoarthritis.

Intra-articular injection of FCA produced maximal inflammatory response in the joint on 4th day that is associated with fluid exudation, neutrophil infiltration, and mast cell activation.

Thus, present study visualises that the latex of C. proceramarkedly reduces cell influx, release of mediators, and oxidative stress associated with arthritic condition, and therefore has the potential to be used as an antiarthritic agent.

CONCLUSION :

Based on the results of the present study it can be concluded that ethanolic extract of couroupita guianensis leaf extract has anti-rheumatoid activity. The anti-inflammatory, antioxidant, analgesic activities of couroupita guianensis leaf have contributed to its anti-rheumatoid activity. The standard drug and ethanolic extract reduced the paw edema by 52.34 and 49.64% om 21st day. Hence couroupita guianensis leaf extract can be further evaluated for its use as an effective future alternative in the treatment of rheumatoid arthritis.

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