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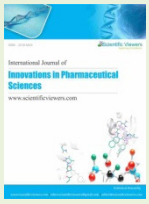

EFFECT OF ETHANOLIC EXTRACT OF COUROUPITA GUIANENSIS LEAF ON FRUEND'S COMPLETE ADJUVANT INDUCED RHEUMATOID ARTHRITIS IN RATS

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ABSTRACT

Couroupita guianensis Linn (Lecythidaceae) is a large deciduous tropical tree 90' tall and indigenous to the Amazon rainforest. It is native to tropical northern South America and the Southern Caribbean. The trees are grown extensively in Shiva temples in India. It is known as "Cannonball tree."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 antiarthritic activity of the above leaves, the present study was carried out on Fruend's adjuvant induced arthritic rats. The present study states the effect of the ethanolic extract of *Couroupita guianensis* leaf on the Fruend's complete adjuvant (FCA) induced arthritic rat paw edema, body weight changes and alterations in 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, where as *Couroupita guianensis* treated groups showed significant reduction in paw volume and normal gain in body weight. The altered haematological parameters (Hb, RBC, WBC and ESR) and serum parameters (SGPT, SGOT, total proteins and ALP) in the arthritic rats were significantly brought back to near normal by the *Couroupita guianensis* leaf treatment at the dose of 200 mg/kg in both developing and developed 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.
KEY WORDS: Rheumatoid Arthritis, *Couroupita guianensis*, FCA, SGPT, SGOT.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune systemic disease with chronic inflammation of the synovial joint and progressive destruction of cartilage and bone [1]. Rheumatoid arthritis is a chronic systemic inflammatory disorder that may affect many tissue and organs- skin, blood vessels, heart, limes and muscles- but principally attacks the joints, producing a nonsuperlative proliferative and inflammatory synovitis that s to destruction of the articular cartilage and ankylosis of the joints[2].

Rheumatoid arthritis is characterized by the infiltration of a variety of inflammatory cells in to the joint. The synovial membrane becomes highly vascularized, synovial fibroblasts proliferate and inflammatory cells release numerous cytokines and growth factors into the joint. These agents subsequently cause synovial cells to release photolytic enzymes resulting in destruction of bone and cartilage [3].

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 rainforests of Central and South America [4]. It is cultivated in many other places [5]. *Couroupita guianensis* grows up to 35 meters in height. The clustered leaves vary in length, generally from 8 to 31 centimeters, but reaching

up to 57[6]. These are alternate, oblong up to 20 cm long, entire to slightly serrate and hairy on the veins beneath. Inflorescence is racemose, arising from the trunk and other large branches. The clustered leaves vary in length, generally from 8 to 31 centimeters, but reaching up to 57. These are green in colour, and the extract is brown in colour. A stout, straight tree, it possesses a dense, often narrow crown, with leaves clustered at the tip of branches. In Florida, leaves tend to lack a strong green color. The tree have been reported for various pharmacological activities such as antibiotic, antifungal, antiseptic, analgesic, antioxidant, antidiabetic, antiulcer, hepato protective, anti-rheumatic and anti-inflammatory. To the best of our knowledge, this is the first study to evaluate the ethanolic extract of *Couroupita guianensis* leaf on FCA induced arthritis in rats.

MATERIALS AND METHODS

Chemicals: sodium corboxy methyl cellulose, Diclofenac, FCA, 1% tween 80. All the drugs were suspended in 0.1 percentage Na-CMC [vehicle control] just before use. All other reagents used were of analytical grade.

Plant Material: *Couroupita guianensis* [CG] (Aubl. Lecythidaceae) leaves were collected during rainy season in the month of July – August from local tropical area. The plant material was taxonomically identified and authenticated by M. R. Paul Satya Keerthi, H.O.D, Professor in Botany at AC College, Guntur.

Preparation of extract: The leaves after collection were shade dried and powdered using a mechanical grinder and passed through 40 mesh sieve. Powder (100 g) was defatted using 1.5L of petroleum ether (bp: 60- 80 ºC) and subjected to extraction in a Soxhlet apparatus using methanol for 16h. The EECG fraction was concentrated under reduced pressure and controlled temperature (40-50 ºC). The yielding ratio of ethanolic extract of CG was 15.3% w/w respectively. The extract was stored in tightly closed container in refrigerator and was screened for phytochemicals [7, 8].

Animals: Male Wistar rats (weighing around100-150g) obtained from Mahaveer enterprises, Hyderabad were used in the study. They were maintained at 22 ± 5 ºC, relative humidity 55 ± 5 ºC with free access to food and water *ad libitum*, under a 12:12 light /dark cycle (light on at 8:00 h). All manipulations were carried out between 9:00 and 15:00 h. with each animal used only once. The experimental protocol was approved by the Institutional Animal Ethics committee of institute as per the direction of the Committee for the Purpose of Control and Supervision of Experiments on Animals (769/2010/CPCSEA). The animals were acclimatized for a period of 7 days before the study. All efforts were made to reduce the number of animals used and treated humanely to minimize their pain and discomfort [9].

Acute toxicity study [10]: The acute toxicity study in mice was performed as per the OECD guidelines (No. 423) to evaluate the undesirable effects or toxicity of EECG extract. Wistar rats were divided into the groups of 4 animals per group and were administered once orally with dose of 1000 mg/kg of EECG extract. The mice were then critically observed for clinical signs, gross behavioural changes and mortality after 30min, 1hr, 2hr, 3hr and then after 24hr. These observations were continued for a period of 7 days.

Induction of rheumatoid arthritis: All the animals of all groups were once anaesthetized, they were injected into the ankle joint of left hind paw with 0.1 ml of Complete Freund's adjuvant (Sigma Aldrich. USA) containing 0.1 mg of heat killed mycobacterium tuberculosis cells in liquid paraffin. Paw edema was observed on 19-21 days [11].

Dose preparation and administration of standard Diclofenac and EECG: Standard diclofenac at a dose of 10mg/kg was prepared by suspending bulk diclofenac in aqueous 0.5% Methyl Cellulose. The Ethanolic extract of CG was dissolved in carboxy methylcellulose and at doses of 100, 200 mg/kg were given to the rats once in a day along with the 0.1ml of FCA was given once throughout the study. Treatment was given daily for 21 days.

Protocol for anti-arthritic activity:
The experimental animals were divided into four groups, 3 animals each group.

- Group 1 normal
- Group 2 FCA (0.1ml)
- Group 3 Diclofenac(10mg/kg)
- Group 4 Ethanolic extract of *Couroupita guianensis* (100mg/kg)
- Group 5 Ethanolic extract of *Couroupita guianensis* (200mg/ kg)

Blood sample collection and analysis: At the end of day 21st, the rats were anaesthetized with anaesthetic ether and blood was isolated from the retro orbital route to animal of each group and estimate various Haematological parameters such as Haemoglobin content, Total WBC, RBC, and Erythrocyte Sedimentation Rate (ESR) by using routine laboratory methods. Liver markers such as SGOT, SGPT were analysed by using an auto-analyser.
At the end of experiment, animal of each group were sacrificed by cervical decapitation and blood was collected in EDTA containing tubes – for plasma/serum separation. The homogenized samples were subjected to biochemical examination for the estimation of total protein. Parameters are estimated by Sicra Lab, Hyderabad

Statistical analysis: All the data obtained from the various parameters were statistically evaluated by one way analysis of variance test (ANNOVA) followed by Dunnet's post-test. "P" value less than 0.01 ($p < 0.01$) were used as the significant level. The results are presented as mean ± S.D [12].

RESULTS

Rat Paw Edema: Challenge with FCA (0.1ml) shows development of paw edema which reached peak edema on 21st day of injection in control group ($p<0.001$). Diclofenac treated group shows significant inhibition of paw edema on day 4th ($P<0.001$), 8th($P<0.001$), 14th ($P<0.001$) and day 21st ($P<0.001$). EECG (100mg/kg) shows significant inhibition of paw edema on day 4th ($p<0.001$), 8th ($p<0.001$), 14th day ($p<0.001$), and day 21st with $P<0.001$. Also rats treated with EECG (200mg/kg) shows significant inhibition of paw edema on day 4th($P<0.001$), 8th ($P<0.001$), 14th ($P<0.001$) and day 21st($P<0.001$).

Paw diameter was increased upto 8th day of adjuvant induction and after that it slightly decreased. Diclofenac treated group shows significant inhibition of paw diameter on day 4th ($p<0.001$), 8th ($p<0.001$), 14th ($p<0.001$), 21st ($p<0.001$). EECG (100mg/kg) shows significant inhibition of paw diameter on day 4th to 21st with $P<0.001$. Also rats treated with EECG (200mg/kg) shows significant inhibition of paw diameter on day4th, 8th, 14th and day 21st with $P<0.001$.

Increased in level of ESR (Erythrocyte sedimentation rate) and decreased in level of HB (Haemoglobin) in Diclofenac (10mg/kg) shows significant change in ESR and HB with $P<0.001$. EECG (100mg/kg) shows significant change in HB with $P<0.01$. EECG (200mg/kg) shows significant change in ESR and HB ($P<0.001$).
There was decrease in RBCs and increase in WBCs in control rats. Animals treated with Standard drug Diclofenac sodium (10 mg/kg) showed significant decrease ($P < 0.01$) in WBCs and increased in ($p<0.05$) RBCs level as compared to control group animals. There was no change in WBC in animals treated with EECG (100 mg/kg), ($P < 0.05$) as compared to control group animals. Where as EECG (200 mg/kg), significantly increases ($P<0.05$) in the RBCs level and significant decrease in WBC as compared to control group animals.

Haematological parameters: Increased in level of ESR (Erythrocyte sedimentation rate) and decreased in level of HB (Haemoglobin) in Diclofenac (10mg/kg) shows significant change in ESR and HB with $P<0.001$. EECG (100mg/kg) shows significant change in HB with $P<0.01$. EECG (200mg/kg) shows significant change in ESR and HB ($P<0.001$).
There was decrease in RBCs and increase in WBCs in control rats. Animals treated with Standard drug Diclofenac sodium (10 mg/kg) showed significant decrease ($P < 0.01$) in WBCs and increased in ($p<0.05$) RBCs level as compared to control group animals. There was no change in WBC in animals treated with EECG (100 mg/kg), ($P < 0.05$) as compared to control group animals. Where as EECG (200 mg/kg), significantly increases ($P<0.05$) in the RBCs level and significant decrease in WBC as compared to control group animals.

Serum parameters: Increased in level of SGOT, SGPT, ALP and decreased in level of total protein in control group ($p<0.001$). Diclofenac treated group shows decreased in level of SGOT($P<0.001$),

Table1: Phytochemical evaluation of EECG extract: [3]

Phytoconstituents	Test/Reagent	EECG
Alkaloids	Dragendroff's test	-
	Hager's test	-
	Wagner's test	+
Carbohydrates	Molisch's test	+
	Fehling's test	+
	Barfoed's test	+
Phenolic and Tannins	Ferric chloride test	+
	Lead acetate test	+
Flavonoids	Shinodas test	+

Table 2: Effect of Ethanolic Extract of *Couroupita guianensis* leaf on rat paw edema in FCA induced rats.

Groups	Paw edema volume (ml)±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 0.1ml)	0.94± 0.004	1.85± 0.004	2.41± 0.004	2.76± 0.004	2.47±0.09
Diclofenac (10mg/kg)	0.93± 0.005	1.25± 0.005	1.41± 0.004	1.33± 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

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

Table 3: 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 0.1mlFCA	8.04± 0.03	15.02± 0.004	20.03± 0.004	19.03± 0.63	18.02± 0.006
Diclofenac (10mg/kg)	7.32± 0.005	12.02± 0.005	13.44± 0.006	13.04± 0.008	12.04± 0.006
EECG 100mg/kg	9.01± 0.004	13.45± 0.004	18.67± 0.006	17.15± 0.007	16.04± 0.004
EECG 200mg/kg	8.44± 0.004	13.07± 0.004	17.04± 0.006	16.04± 0.004	15.02± 0.005

Values are expressed as mean ± SEM (n=6). P<0.001 as compared with control (One-way ANOVA followed by Dunnet's test).

Table 4: Effect of Ethanolic Extract of *Couroupita guianenesis* leaf on Haematological parameters in FCA induced arthritic rats:

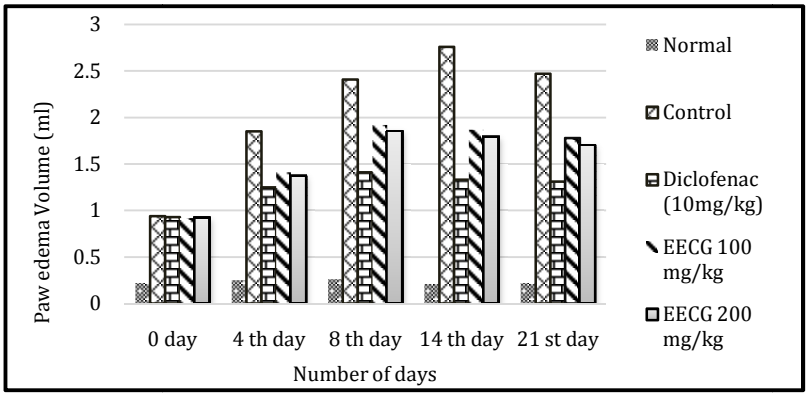
Groups	ESR and Haematological Parameters			
	ESR (mm/hr)	ESR (mm/hr)	ESR (mm/hr)	ESR (mm/hr)
Normal	3.30±0.05	3.30±0.05	3.30±0.05	3.30±0.05
Control (FCA 0.1ml)	7.06±0.36	7.06±0.36	7.06±0.36	7.06±0.36
Diclofenac (10mg/kg)	5.07±0.03	5.07±0.03	5.07±0.03	5.07±0.03
EECG 100mg/kg	8.02±0.03	8.02±0.03	8.02±0.03	8.02±0.03
EECG 200mg/kg	4.58±0.3	4.58±0.3	4.58±0.3	4.58±0.3

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

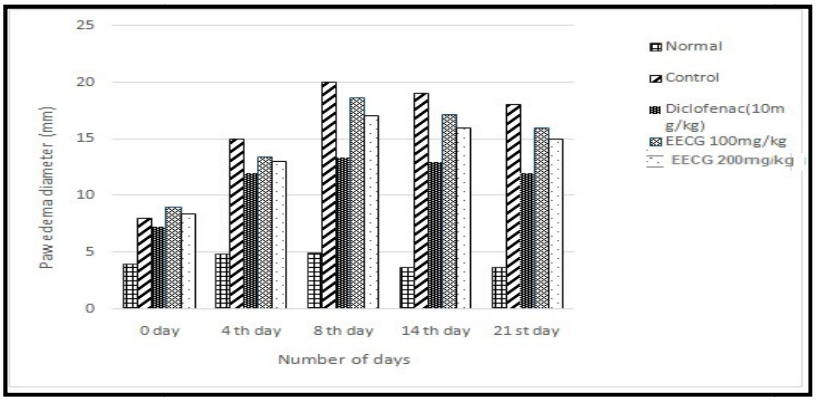
Table 5: Ethanolic Extract of Couroupita guianenesis leaf on serum parameters in FCA induced arthritic rats.

Groups	Serum parameters			
	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	Total Protein (gm/dl)
Normal	95.4±0.26	55.56±0.89	187.5±5.70	7.91±0.21
Control (FCA 0.1ml)	103.5±3.69	70.82±1.57	245.0±9.99	2.91±0.23
Diclofenac (10mg/kg)	49.60±2.08	46.18±1.20	133.0±4.54	6.76±0.59
EECG 100mg/kg	73.81±1.89	56.37±2.42	169.9±7.20	4.78±0.36
EECG 200mg/kg	69.06±2.14	54.86±1.71	154.8±3.28	5.23±0.68

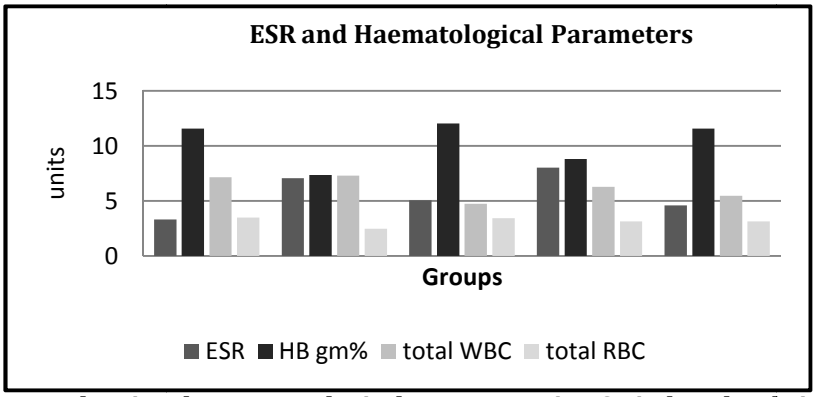
Values are expressed as mean ± SEM (n=6). P<0.05, P<0.001 as compared with control (One-way ANOVA followed by Dunnet's test).



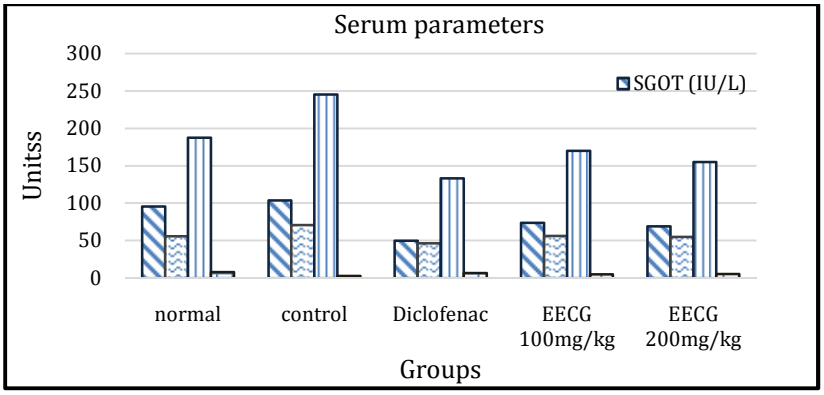
H-1: Histogram showing the mean changes in paw edema in FCA induced arthritic rats.



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

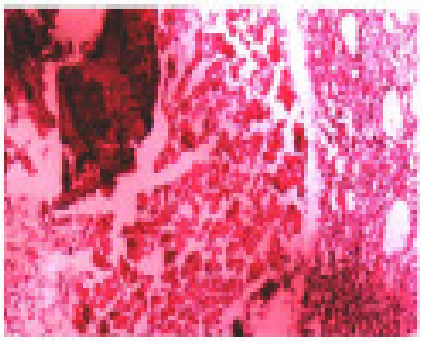


H-3: Histogram showing the Haematological parameters in FCA induced arthritic rats.

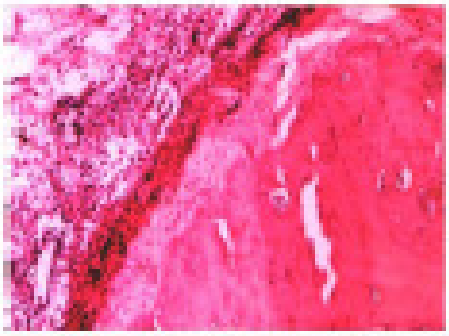


H-4: Histogram showing the rat serum parameters in FCA induced arthritis in rats.

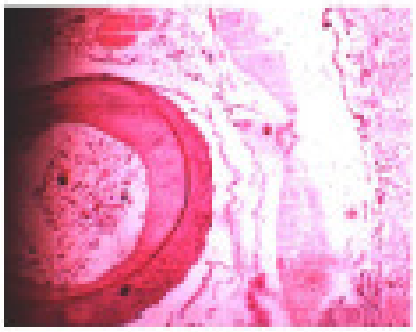
Group-I



Group-II



Group-III



Group-IV

Group-V

Figure 1: Histopathological changes of proximal interphalangeal joints.

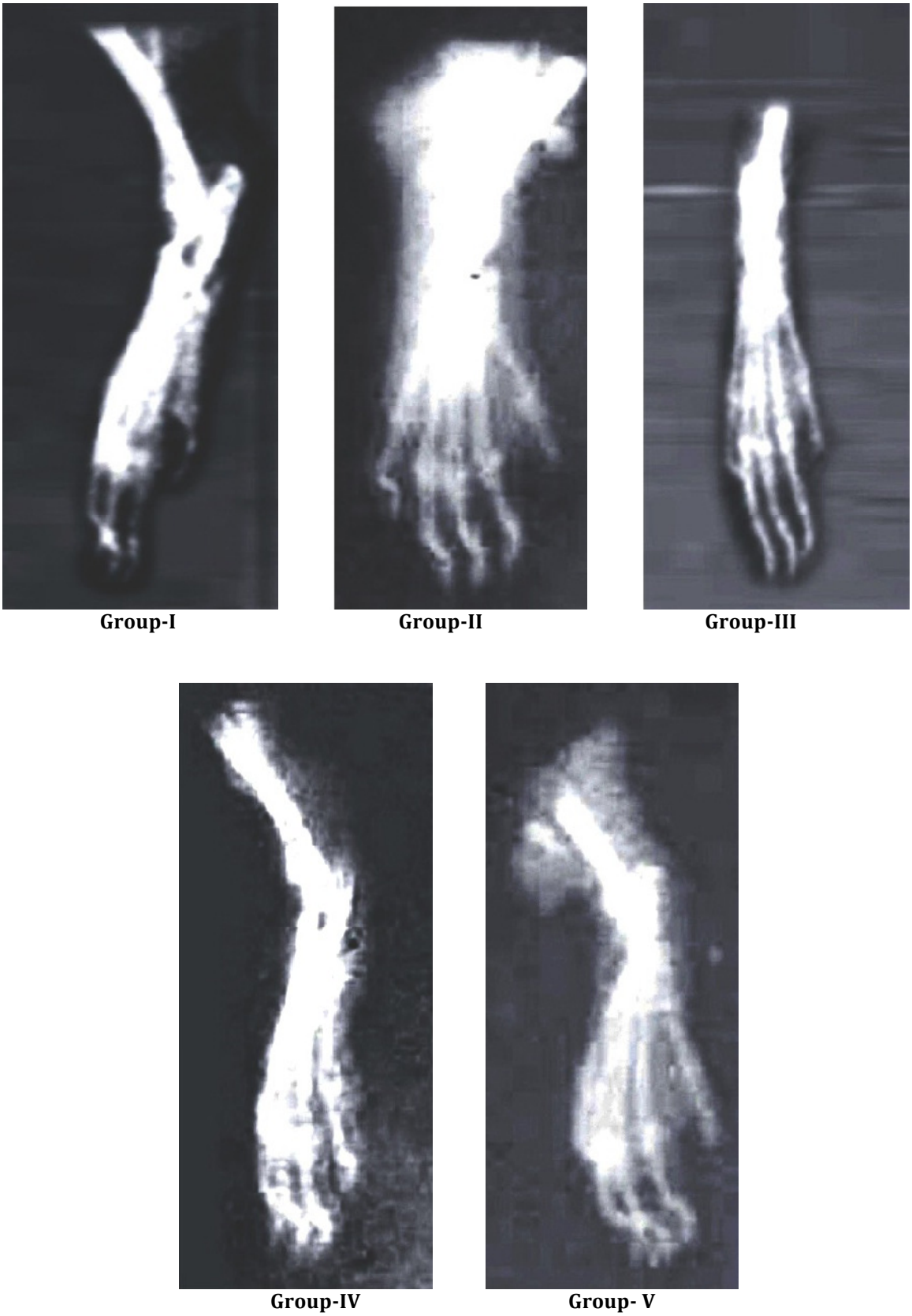


Figure 2: X rays of Knee joints

SGPT (P<0.001), ALP (P<0.001) and increased in level of Total protein (P<0.001). EECG(100mg/kg) shows significant decreased in level of SGPT(P<0.001),SGOT (P<0.001), ALP (P<0.001) and increased in level of Total protein (P<0.05). EECG (200mg/kg) treated group shows decreased in level of SGOT(P<0.001), SGPT(P<0.001), ALP(P<0.001) and arthritis on the increased in level of Total protein (P<0.05).

Histopathology: The proximal interphalangeal joints were removed, washed with saline and stored in 10% formalin. The interphalangeal joint sections were obtained, stained with eosin – haemoglobin stain and viewed under 100 X magnifications. The hind arthritis induced legs of the experimental rats were taken X-ray, and examined for the soft tissue swelling, bony erosions and narrowing of the spaces between joints[13].

Radiographic analysis: At the end of the study, the animals were anaesthetized using diethyl ether, and digital x-rays were taken for radiographic analysis of the knee joints. X-rays were taken of the knee joints for confirmation and evaluation of the severity of arthritis in FCA-induced rats.

Soft tissue swelling is the earlier radiographic sign, where as prominent radiographic changes like bony erosions and narrowing of joint spaces can be observed only in the final stages of arthritis [14].

DISCUSSION

In Freund's adjuvant arthritic rat model, treatment with c.guianensis plant extract showed significant inhibitory effect on injected hind paw edema and maximum inhibition was observed on the 21st day. In the present study, the increased lymphocyte count and migration of leucocytes into the inflammed area of arthritic rats were significantly prevented with the treatment of the herbal product and the standard drug as reflected from the significant decrease in total WBC count. The erythrocyte sedimentation rate (ESR) level which was markedly elevated in arthritic control group of rats was decreased significantly with herbal product at different doses (100 mg/kg and 200mg/kg) and the effect was comparable to standard drug.

The chronic inflammation involves the release of various inflammatory mediators like cytokines (IL-1α and TNF-α), granulocyte monocytes colony stimulating factor (GM-CSF), platelet derived growth factor (PGDF) and others. These mediators are responsible for the pain, destruction of cartilage and leads to severe disability. Paw swelling is one of the major factors in assessing the degree of inflammation and efficacy of the drugs. Adjuvant induced arthritis is non-specific immune response within the joint can also result in inflammatory and erosion disease.

Paw swelling is an index of measuring the anti-arthritic activity of various drugs and it is employed here to determine the activity of EECG. Reference standard Diclofenac sodium, EECG administered groups showed marked reduction in paw volume when compared with the arthritic control group by inhibiting the release of inflammatory mediators. As inflammation is progressed, a more diffused demineralization developed in the extremities. In the present study, the significant role of the EECG is due to decreased demineralization in the joint extremities.

The cytoplasmic enzymes like AST and ALT serves as indicators and suggestive for disturbances of the cellular integrity induced by pathological conditions. These enzymes are used as sensitive markers for evaluation of protective activity, these markers attribute towards persistent inflammation. The increased enzyme activity may result from one of the several mechanisms which include the release of various enzymes from leukocytes, from necrotic or inflamed synovial tissue and production and release of an increased amount of enzymes due to altered synovial tissue. A positive correlation observed between the leukocytes in the field and the enzyme levels is considered as evidence for the release of enzymes from the leukocytes. A loss of semi-permeability of the synovial membrane has also been correlated with the significant elevation of enzyme levels. In present study, the decreased level of cytoplasmic enzyme ALT supports the protective role of the EECG.

Decrease in RBC count and haemoglobin level represents the anaemic condition in arthritic rats. The more important causes are the abnormal storage of iron in the reticulo endothelial system and synovial tissue and failure of bone marrow to respond to anaemia. It has been reported that a moderate rise in the WBC count occurs in arthritic conditions due to an IL-1β-mediated rise in the respective colony stimulating factor. In the present study, EECG exhibited significant increase in the ESR is attributed to the accelerated formation of endogenous protein such as fibrinogen and α/β globulin, and such a rise in the ESR indicates an active but obscure disease process. In the present study, ethanolic extract of

Couoropita guianensis leaf treatment restored the altered haematological profile by decreasing the ESR provides support for its anti-arthritic effect.

CONCLUSION

The present study was carried out to evaluate the *Couroupita guianensis* leaf for antiarthritic activity. Diclofenac (10mg/kg) used as a standard drug.

In conclusion, *Couroupita guianensis* at the specified dose level of 200mg/kg b.w produced significant reduction in inflammation and redness of rat paw edema on the 21 day treatment when compared with control group rats. It could normalize the hematological 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 c.guianensis in FCA induced arthritis. It exerts potent anti-arthritic activity by significantly altering the pathogenesis during arthritis without exerting any side effects in FCA induced arthritis in rats.

The results showed that ethanol extract showed high percentage inhibition in paw edema on 21 day treatment. Therefore, we can conclude the flavonoids present in the ethanolic extract may be responsible for the antiarthritic activity.

The standard drug and ethanol extract reduced the paw edema by 52.34% and 49.64% respectively on 21st day, Which may be due to the suppression of inflammatory mediator released due to induction of Fruend's adjuvant.

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