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Research Article

Evaluation of anti-arthritic activity of Spathodea Campanulata of root bark extract on freund's adjuvant induced arthritis in rats models

Sankari M[1*], Thamizharasi S[1], Mariyammal A[2], Meghana K[2], Parvatha Raja Kumaran V.S[2], Chetan A[2]

[1]Associate Professor, [2]M. Pharm Student, Department of Pharmacology, K.K. College of Pharmacy, Chennai, Tamil Nadu, India

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ABSTRACT

Rheumatoid arthritis is a chronic systemic inflammation of the synovial joint and progressive destruction of cartilage and bone that may affect many tissues and organs- skin, blood vessels, heart, lungs and muscles but principally attacks the joints, producing a non-suppurative proliferative and inflammatory synovitis that often progresses to destruction of the articular cartilage and ankylosis of the joints. Angiogenesis and microvascular lesions are common features of RA inflammation, which leads to abnormal serum protein infiltration into the synovium. The objective of the study was to determine the preliminary phytochemical constituents, to evaluate acute toxicity studies, *In-vitro* anti-oxidant studies, *In vitro* and *In-vivo* anti-arthritic activity of the root bark of *Spathodea campanulata*.. Paw edema was induced in rats by intra plantar injection of 0.1ml of Complete Freund's Adjuvant (CFA) in left hind paw. The ethanolic extract of Spathodea campanulata (150mg/Kg & 300mg/Kg) and Diclofenac Sodium were orally administered for 28 consecutive days. Percentage inhibition was determined by Protein denaturation method and Membrane Stabilizing Activity Hematological, Histopathology, Radiological Analysis of CFA-induced arthritis in rat was performed. Statistical significant was analyzed by one-way ANOVA followed by Tukey test. Our study concluded that ethanolic extract at the dose of 300 mg/kg showed significant anti-arthritic activity when compared to Diclofenac sodium.

Keywords: Angiogenesis, Phytochemical, Acute toxicity, DDPH Assay

INTRODUCTION

Rheumatoid arthritis is a chronic systemic inflammatory disorder that may affect many tissue and organs- skin, blood vessels, heart, lungs and muscles- but principally attacks the joints, producing a non-suppurative proliferative and inflammatory synovitis that often progresses to destruction of the articular cartilage and ankylosis of the joints. [1] It has affected about 1% of the population throughout the world with male and female ratio of 1: 2.5 [2]. The potential target for the

Address for Correspondence: M. Sankari, Associate Professor, K.K. College of Pharmacy, 1/161, Sankaralinganar Road, Gerugambakkam, Chennai-600128; E-mail: vs_vellaisamy@yahoo.com

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treatment of chronic inflammatory conditions can be the regulatory checks of these mediators secreted by immune cells and the inhibition of enzymes like COX and LOX for the metabolic modulation of arachidonic acid [3, 4]. Although different categories like non-steroidal anti-inflammatory drugs (NSAIDs), immunosuppressant, and steroidal anti-inflammatory drugs are in use, the limitation is their potential side effects which include irritation of gastric mucosa, belching, gastric ulceration and bleeding [5]. Long term use of NSAIDS may impair renal and hepatic functions, influencing the patient to cardiovascular diseases. [6]. Hence, there is a continuous search for alternative drugs from plants and other natural sources [7]. The similarities in the joint pathology between rat arthritis and human RA are most widely used for studying the pathogenesis of human RA and for searching new drugs for RA management. Therefore, AA is most frequently used as a model for screening and testing anti-arthritic agents [8].

Several phytochemical studies were performed with different parts of *S.campanulata*, including stem barks, flowers, leaves, and fruits. Spathodic acid, steroids, saponins, ursolic acid, tomentosolic acid and pectic substances have ever been isolated from the stem bark. Flowers and stem bark extracts have shown molluscicidal activity. These are also employed in diuretic and anti-inflammatory treatments. The leaves have been found to contain spathodol, caffeic acid, other phenolic acids and flavonoids [9]. The main aim of the study was to evaluate the anti-arthritic activity of root bark extract of *Spathodea campanula* on Freund's adjuvant induced arthritis model in rats.

MATERIALS AND METHOD

Preparation of Extracts: Fresh root bark was collected, dried in shade, coarsely powdered and extracted with Ethanol by Continuous hot percolation process using Soxhlet apparatus. After extraction each extract was concentrated by using Rotary vacuum evaporator. It was dried and the percentage yield was calculated. Appearances and consistency of the extract was noted.

Preliminary Phytochemical Screening: [10]

Root bark powder and extracts was subjected to qualitative chemical analysis for the identification of active constituents. The screening was performed for triterpenes/steroids, alkaloids, anthraquinones, coumarins, flavonoids, saponins, tannins, and phenolic acids. The color intensity or the precipitate formation was used as analytical responses.

INVITRO ANTI-ARTHRITIC ACTIVITY

Protein Denaturation Method: [11, 12]

The test and standard solutions were prepared and pH was adjusted to 6.3 to all the solution by using 1N HCL. All the sample solution was incubated at 37ºC for 20 minutes; temperature was increased to 57ºC for 3 minutes and cooled. 2.5ml of phosphate buffer was added to all the above solutions. The absorbance was measured at 416nm using UV visible spectrophotometer. The percentage inhibition of protein denaturation was calculated.

$$\text{Protein denaturation assay} = \frac{(\text{O.D test compound-O.D Control})}{(\text{O.D Control})} \text{x } 100$$

Membrane Stabilizing Activity: [12, 13, 14]

In this model percentage membrane stabilizing activity was used to assess the anti-inflammatory activity. The standard drug was Diclofenac sodium. The reaction mixtures 4.5ml consists of 2ml hypotonic saline (0.25% NaCl) + 1ml 0.15M phosphate buffer (pH 7.4) + 1ml test solution (100-500µg/ml) in normal saline + 0.5ml of 10% rat RBC in normal saline. The mixture was incubated at 56°C for 30 minutes and the tubes were cooled for 20 minutes. Mixture was centrifuged for 3000rpm for 10min and the absorbance of the supernatant was measured at 560nm. Percentage membrane stabilizing activity was calculated based on the following formula,

$$\% \text{ Membrane stabilization} = \frac{\text{Abs (Control)} - \text{Abs (Sample)}}{\text{Abs (Control)}} \text{ x } 100$$

- ❖ Microscopical observation of RBC was done.
- ❖ IC50 value of extract as well as standard for inhibition of hemolysis was calculated

DPPH Assay (2, 2-Diphenyl -1- Picrylhydrazyl) [11, 14, 15, 16]

Anti-oxidants react with DPPH and reduce it to DPPH-H and as consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the anti-oxidant compounds in terms of hydrogen donating ability. Different volumes (1.25-10µl) of plant extracts were made up to 40µl with DMSO and 2.96 ml DPPH (0.1mM) solution was added. The reaction mixture was incubated in dark condition at room temperature for 20 minutes. After 20 minutes, the absorbance of the mixture was read at 517nm. 3ml of DPPH was taken as control.

$$\% \text{ Inhibition} = \frac{\text{Abs (Control)} - \text{Abs (Sample)}}{\text{Abs (Control)}} \text{ x } 100$$

INVIVO ACTIVITY:

ACUTE TOXICITY STUDIES (OECD 423 GUIDELINES) [17, 18]

Procedure: Acute toxicity study was performed as per the OECD guidelines 423. Adult Wistar albino rats of either sex weighing between 150-200 g of 4 groups of each contain 3 animals for study. All the animals were fasted overnight provided with water ad libitum. Following period of fasting the test compound was administered at the dose of 2000 mg/kg body weight orally. Animals were observed individually after dosing periodically during the first 30 minutes to the first 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total of 14 days. The animals were observed individually for signs of acute toxicity and behavioral changes (Table 2). Attention should be directed to observation of tremors convulsions, salivation, diarrhea, lethargy, sleep and coma. The rats were observed regularly for 14 days to note the mortality or toxic symptoms. If no death was reported, the study was repeated with same dose to confirm the results. The study was approved by Institution of Animal Ethical Committee in K.K. College of Pharmacy with approval number: KKCP/2019/10 and the work was carried out as per CPCSEA guidelines, New Delhi

Adjuvant Induced Paw Edema Model [19, 20]

This is one of the most commonly used animal models for evaluating anti-inflammatory activity. Paw edema was induced in rats by intra plantar injection of 0.1ml of Complete Freund's Adjuvant (CFA) in left hind paw except for the vehicle control. The adjuvant contained heat killed *mycobacterium tuberculosis* in sterile paraffin oil. The test drug and the standard drug were administered orally for a period of 28 days and the paw volume was measured periodically using plethysmograph. Test drug – Ethanolic extract was given at the dose of (low dose) and (high dose) for 28 days.

Experimental Analysis

A total of 30 adult Wistar albino rats weighing (150-200 g) were divided into 5 groups of 6 animals in each group. Group I received the vehicle, Group II received the Complete Freund's Adjuvant (CFA) alone, Group III received the standard drug Diclofenac sodium and Complete Freund's Adjuvant (CFA), Group IV and Group V received the Test -CFA+ EESC (LOW DOSE) and Test -2 CFA+ EESC (HIGH DOSE) respectively.

Paw Volume Measurement

Paw volume of the all the animal groups was measured by using plethysmograph at 0, 7, 14 and 21, 28 days.

$$Vt = \frac{Vc - V0) - (Vt - V0)}{(Vc - V0)} \times 100$$

Where, Vc - is the paw volume after induction, V0 – is the paw volume before induction and
Vt – is the paw volume after treatment

RADIOLOGICAL ANALYSIS OF ANKLE JOINT [21, 22]

At the end of the experiments, all rats were anesthetized with 40 mg/kg sodium thiopental intraperitoneal injection. Once anesthetized, the animals were constantly kept on X-ray plate; the projections of the left ankle joint were taken at day 28. The following parameters was evaluated using the tarsometatarsal region: erosion, a destruction of bony structure resulting in irregular bone surface; periosteal reaction, a fine ossified line, paralleling normal bone producing bone thickening; increase in soft tissue and calcification. The parameters were calculated using score which follows: 0, no sign; 1, mild; 2, moderate; and 3, severe.

HISTOPATHOLOGICAL STUDY OF JOINTS [21, 22]

Rats were sacrificed by administering high dose of halothane. Ankle joints were removed and fixed in 10 % buffered formalin. The bones was decalcified in 5 % formic acid, processed for paraffin embedding, sectioned at 5 mm thickness and subsequently stained with hematoxylin-eosin for examination under a light micro-scope for the presence of changes in synovium, cartilage and joint space.

HEMATOLOGICAL PARAMETER [21, 22, 23]

At the end of day 28th day, the animals were anaesthetized. Blood was collected from the retro orbital route and various hematological parameters such as Hemoglobin content, Total WBC, RBC, and Erythrocyte Sedimentation Rate (ESR) were estimated using routine laboratory methods**.**

SPLEEN AND THYMUS WEIGHT [23]

At the end of day 28th day, the thymus and spleen of all the animals was removed and weighed.

STATISTICAL EVALUATION

The experimental results are represented as Mean ± SEM. The data will be statistical analyses by one way analysis of variance (ANOVA) Followed by Tukey HSD test.

RESULT AND DISCUSSION

Phytochemical investigations were carried and the results are as follows,

QUANTITATIVE PHYTOCHEMICAL ANALYSIS

Table No 1: PRELIMINARY PHYTOCHEMICAL ANALYSES

S.NO	CHEMICAL CONSITUENTS	POWDERED DRUG	ETHANOL EXTRACT
1.	**Steroids**	+	+
2.	**Glycosides**	-	-
3.	**Saponins**	-	-
4.	**Flavonoids**	+	+
5.	**Tannins**	-	-
6.	**Triterpenes**	+	+
7.	**Proteins**	+	+
8.	**Alkaloid**	+	+
9.	**Carbohydrates**	+	+
10	**Phenolic compounds**	+	+

Note: + and - indicates the presences and absence

ACUTE TOXICITY STUDIES

Table No 2: ACUTE TOXICITY- PARAMETER STUDIED

OBSERVATION	30 minutes	4 hours	24 hours	14th day
Body weight	-	-	-	-
Pre terminal deaths	-	-	-	-
Cage side observation	+	+	+	+
Motor activity	+	+	+	+
Convulsion	-	-	-	-
Piloerection	-	-	-	-
Righting reflex	+	+	+	+
Lacrimation	-	-	-	-
Salivation	-	-	-	-
Respiration	+	+	+	+
Skin colour	-	-	-	+
Diarrhoea	-	-	-	-
Loss of corneal reflex	-	-	-	-
Loss of pinnal reflex	-	-	-	-
Grooming	-	-	-	-
Sedation	-	-	-	-
Excitation	-	-	-	-
Aggression	-	-	+	-

NOTE: + and – indicates presence or absence

INVITRO STUDY

1. **Anti-Oxidant Activity**

Table No 3: PERCENTAGE INHIBITIONS OF EXTRACTS - DPPH ASSAY

EXTRACT	PERCENTAGE INHIBITION AT VARIOUS CONCENTRATIONS				
	10µg/ml	20 µg/ml	40 µg/ml	80 µg/ml	100µg/ml
Ethanol	41.26	54.65	60.56	70.34	81.43
Ascorbic acid (Standard)	58.09	63.53	68.15	72.56	80.13

2. **Anti-Arthritic Activity**

Table No 4: PERCENTAGE INHIBITION OF ETHANOLIC EXTRACT PROTEIN DENATURATION METHOD

EXTRACT	PERCENTAGE INHIBITION AT VARIOUS CONCENTRATIONS				
	10µg/ml	20 µg/ml	40 µg/ml	80 µg/ml	100 µg/ml
Ethanol	38.28	44.22	55.87	65.13	75.66
Ascorbic acid (Standard)	41.26	54.65	60.56	70.34	81.43

3. **Membrane Stabilization Effect**

Table No 5: MEMBRANE STABILIZING EFFECT OF ETHANOLIC EXTRACT

TREATMENT	CONCENTRATION (µG/ML)	PERCENTAGE OF MEMBRANE STABILISATION
Standard	100	27.88±0.93
	200	37.45±1.30
	300	59.61±0.516
	400	74.057±0.990
	500	84.04±0.511
Extract	100	11.29±0.628
	200	23.36±1.26
	300	34.89±1.016
	400	50.65±1.182
	500	72.64±0.765

All the values are expressed as Mean+ SEM (n=3).

***INVIVO* STUDY**

Anti-Arthritic Activity

Table No 6: MEASUREMENT OF PAW VOLUME USING PLETHYSMOGRAPH-ADJUVANT-INDUCED INFLAMMATORY RATS

TREATMENT	0 day	7th day	14th day	21st day	28th day
Group I (control)	1.04±2.68	1.12±2.68***	1.16±5.96***	1.23±5.96***	1.22±5.96***
Group II (Inflammatory control)	1.06±5.96	2.73±2.68	2.96±2.68	3.03±5.96	3.33±5.96
Group III (Diclofenac sodium)	1.10±2.98	1.08±5.96***	1.06±5.96***	1.01±5.96***	1.0±2.98***

Group IV (150mg/kg)	2.68±2.68	2.56±2.68*	2.35±2.68**	1.17±5.96***	1.12±5.96***
Group V (300mg/kg)	2.91±2.68	2.23±5.96**	1.16±5.96***	1.08±5.96***	1.05±5.96***

*p≤0.05, **p≤0.01, ***p≤0.001 when compared with negative control. The data was analyzed using one way analysis of variance (ANOVA) followed by Dunnett's Test

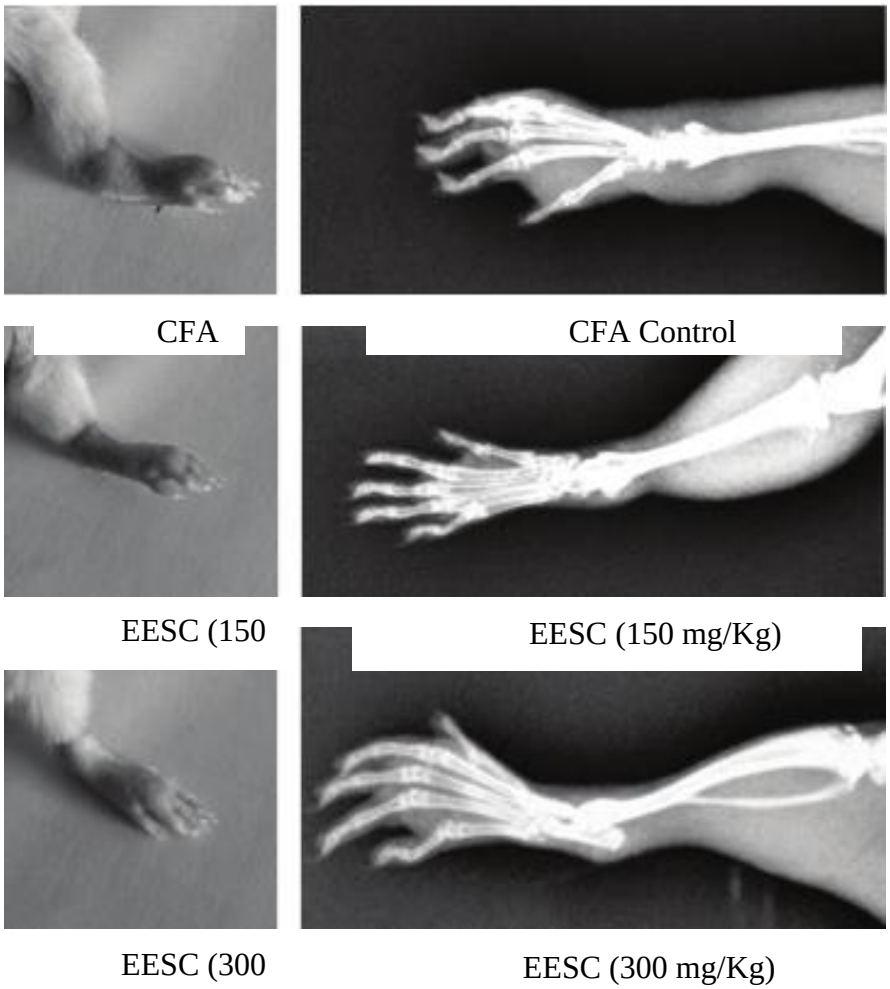
Table No 7: EFFECT OF EESC (150 AND 300 MG/KG) ON HAEMATOLOGICAL PROFILE.

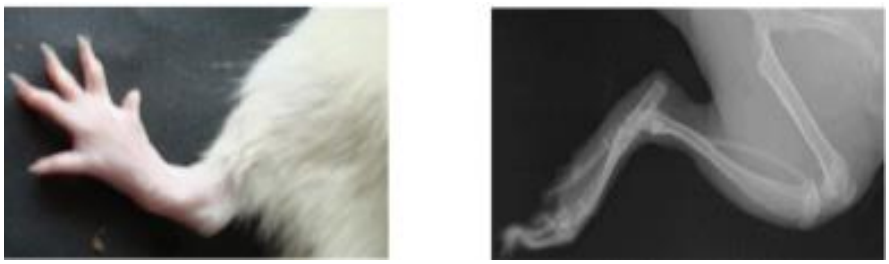
Treatment	**Dose (mg/kg)**	**RBC(×10^6/lL)**	**WBC (×10^3/lL)**	**Hb (g/dL)**	**ESR (mm)**
Vehicle control	-	8.05 ± 0.12	11.48 ± 0.16	13.21 ± 0.10	3.05 ± 0.07
FCA control	-	7.58 ± 0.11	14.15 ± 0.18	11.56 ± 0.18	9.43 ± 0.12
EESC	150	8.00 ± 0.07	12.88 ± 0.61	12.40 ± 0.37	6.31 ±0.19**
EESC	300	7.96 ± 0.08	12.85 ± 0.72	12.43 ± 0.33	4.08 ±0.12**
Diclofenac	10	8.00 ± 0.12	12.21 ± 0.43	13.08 ±0.09**	3.53 ±0.10**

Values are plotted as the mean ± SEM, n = 6 in each group; significant change was analysed by one-way analysis of variance followed by Dunnett's **P < 0.01 compared to FCA control.

RADIOLOGICAL ANALYSIS OF ANKLE JOINT

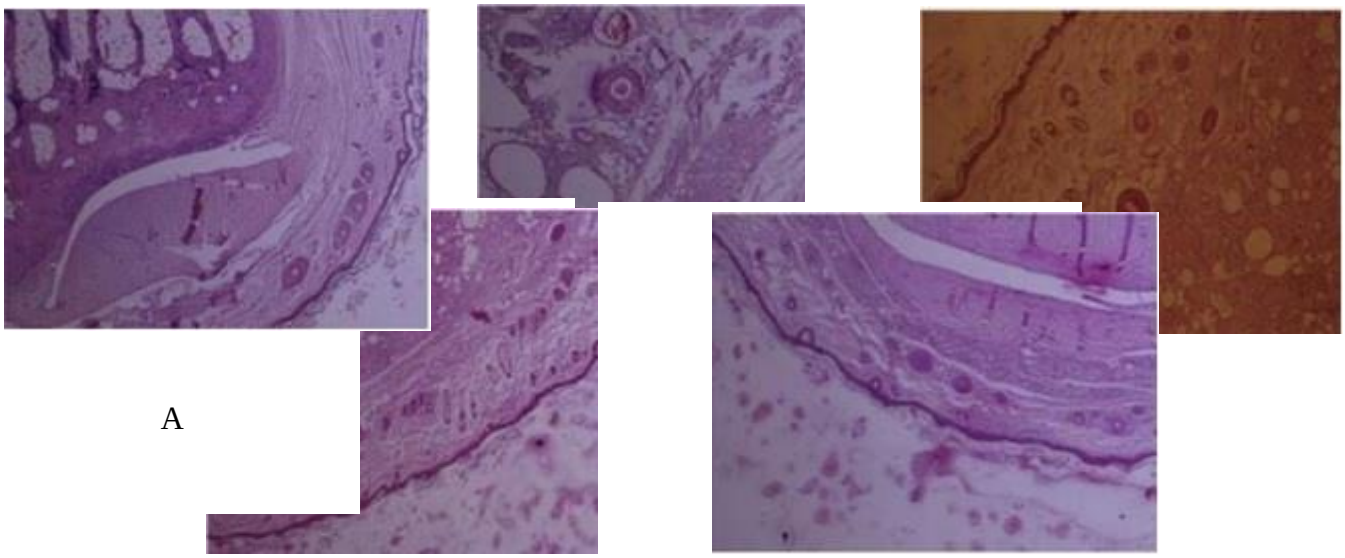
Figure 1: PHOTOGRAPHIC AND RADIOGRAPHIC ANALYSIS OF CFA-INDUCED ARTHRITIS IN RATS.





Diclofenac (10 **OF JC** Diclofenac (10

Figure 2: HISTOPATHOLOGICAL STUDY OF JOINTS



A

E

in the synovial membrane. D) EABP (300mg/kg) showed decrease in inflammation, decreased edematous spaces, reorganization of synovial membrane with perceptible decrease of injury. E) Diclofenac (10mg/kg) showed re-establishing with less edema and inflammatory cell. Magnification ×100; thickness: 5 lm

DISUSSION

The phytochemical active compounds of Root bark powder and ethanolic extract of *Spathodea Campanulata* were qualitatively analyzed and the results are presented in Table 1. Phytochemical screening of the Root bark powder and ethanolic extract of *Spathodea Campanulata* were reported the presence of phytoconstituents such as alkaloids, flavonoids, steroids, Proteins, Carbohydrates, Phenolic compounds and triterpenoid which have various medicinal values such as anti-inflammatory, anti-oxidant, anti-diabetic, anti-microbial and analgesic activities. The medicinal value of plants lies in some chemical substances that have a definite physiological action on the human body. Phytochemicals have been found to possess a wide range of activities, which may help in providing therapy against diseases. For example, alkaloids protect against chronic diseases. Steroids and triterpenoids show the analgesic properties. Fluorescence analysis shows absence of fluorescent substance in the extract.

In vitro antioxidant activity by DPPH radical scavenging assay:

DPPH is a stable organic free radical with an absorption band at 517 nm. It loses the purple colour that absorbs at this wavelength when accepting an electron or a free radical species, which results in a yellow colour [24]. The extract at varying concentration for antioxidant activity in the DPPH assay was observed. The IC50 values ranged between 7.72 µg/ml and 154.77 µg/ml. The ethanolic extract of root bark of Spathodea Campanulata had good radical scavenging activity with IC50 values of 60.56, 70.34 and 81.43 at 40, 80, and 100 µg/ml respectively (Table 5). The IC50 value of a compound is inversely related to its antioxidant capacity. A lower IC50 value indicates a stronger antioxidant activity of the extract or compound [25]. Ascorbic acid, a known potent

antioxidant, had the highest DPPH scavenging activity (80.13 at 100µg/ml).

In vitro anti-arthritic activity by inhibition of protein denaturation method:

The effects of ethanolic extract of root bark of *Spathodea Campanulata* on inhibition of protein denaturation are shown in Table 4. Extract at different concentrations (dose levels) provided significant protection against denaturation of proteins. The maximum percentage inhibition was observed in ethanolic extract of leaves (75.66% at 100µg/ml) as compared to standard. It possesses significant activity comparable with that of the standard Diclofenac sodium. Most of the investigators have reported that denaturation of protein is one of the causes of rheumatoid arthritis. From the results of present study, it can be stated that ethanolic extract of root bark of *Spathodea Campanulata* is capable of controlling the production of auto antigen and inhibits denaturation of protein in rheumatic disease. Production of auto-antigen in certain arthritic diseases may be due to denaturation of protein. [12]

In vitro anti-inflammatory activity by membrane stabilization method:

Now a day, there is a need for newer agents from herbal source having highly potent agents with lesser side effects for therapy. The effect of the ethanolic extract of *Spathodea Campanulata* on stabilization of RBC membrane is shown in Table 5. The maximum percentage stabilization observed in the ethanolic extract of *Spathodea Campanulata* (72.64% at 500µg/ml). It possesses significant activity when compared with standard Diclofenac sodium.

Acute Oral Toxicity Study:

A study on the acute oral toxicity of the ethanolic extract of *Spathodea Campanulata* was reported and was found to be non-toxic up to the dose of 2000 mg/kg. Since there were no death and behavioural changes observed. The extract was considered as safe to administer. From this 1/10th and 1/5th of the dose (150mg/kg and 300 mg/kg) was selected for anti-arthritis study.

Paw Volume:

FCA-induced arthritis is the most widely used model in which the clinical and pathological changes are comparable with those seen in human rheumatoid arthritis [26]. The ethanolic extract of *Spathodea Campanulata* were administered orally for a period of 28 days and the paw volume was measured periodically using plethysmograph and changes in rat paw volume were recorded on 0th, 7th, 14th, 21th and 28th days Table No.6 The Negative control groups showed signs of arthritis development as seen by the increase in paw volume. Significant ($p<0.001$) reduction in rat paw volume was observed in standard Diclofenac sodium, and ethanolic extract of *Spathodea Campanulata* treated groups on 28st day after FCA induction. When compared with standard Diclofenac sodium, the ethanolic extract of *Spathodea Campanulata* with Low dose (150 mg/kg) and High dose (300 mg/kg) showed the significant decreased in paw volume results.

From the Table – 13, in the CFA induced arthritic animals the haematological perturbations such as an increase in the WBC count, a decreased RBC count and an increased erythrocyte sedimentation rate (ESR) were also favorably altered by Diclofenac sodium and extracts treatment.

Figure 4 shows X-ray radiographs of the paws taken on the 28st day. It is clearly observed in the X-rays that the soft tissue swelling around the joints, periarticular bone resorption, periarticular bony erosions and joint space narrowing in the rats treated with BA have been protected from the CFA-induced arthritis-related joint changes.

CONCLUSION

From the above studies it can be concluded that the proper identification of the plant that could be made use of, those who deal with the species and also in the quality assurance of the plant species with the support of phytochemical, *in vitro* and *in-vivo* pharmacological studies, ethanolic extract was selected and subjected to *in vitro* and *in-vivo* anti-arthritic activity. The ethanolic extract at the dose of 300mg/kg showed significant anti-arthritic activity which was comparable with that of the standard.

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