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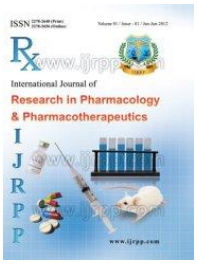

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Evaluation of anti-arthritic activity of aqueous extract of leaves of *chloroxylon swetenia* in adjuvant induced arthritis in rats

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ABSTRACT

OBJECTIVE

The aim of this study was to evaluate anti-arthritic activity of ethanolic extract of *Chloroxylon swietenia* leaves in Male Wister Albino rats using in-vitro and in-vivo methods.

METHODS

Animals were divided into five groups of six animals each as follows:

GROUP-I - Normal rats given vehicle alone 2% Tween 80, (p.o).

GROUP-II -Arthritic untreated rats.

GROUP-III -Arthritic rats treated with 10mg/kg (p.o) standard Diclofenac sodium.

GROUP-IV -Arthritic rats treated with 100mg/kg (p.o) of ethanolic extract.

GROUP-V –Arthritic rats treated with 200mg/kg (p.o) of ethanolic extract.

The inducing agent used was Complete Freund's adjuvant (CFA). The biochemical parameters like erythrocyte sedimentation rate (ESR), red blood cell (RBC), Hemoglobin (Hb), and total WBC count was observed which were the major markers of arthritis and uric acid analysis (plasma). A significant inhibition of paw edema volume and body weight was observed from day 0th, 7th, 14th, 21st and 28th day in the treated groups.

RESULTS

A significant increase in body weight, reduction in paw volume of both hind legs were observed in FCA induced arthritis rats. A decrease in levels of RBC and hemoglobin were observed in arthritic rats. There was a significant improvement in the levels of hemoglobin and RBC in *Chloroxylon swietenia* treated rats. The increased levels of WBC, ESR were significantly suppressed in the extract administered arthritic group.

CONCLUSION

It may be concluded that the ethanolic extract of *Chloroxylon swietenia* at two different concentrations (100mg/kg and 200mg/kg) possessed significant anti-arthritic activity as compared to standard drug.

KEY WORDS: Chloroxylon swietenia, Freund's adjuvant, Diclofenac sodium.

INTRODUCTION

Rheumatoid arthritis (RA), a chronic inflammatory autoimmune disease of the joints, can be effectively suppressed with glucocorticoids (GC), with or without other disease modifying anti-rheumatic drugs (DMARD's) [1-7]. GC is rapidly cleared from the circulation. Thus they are used with high and frequent dosage, thereby adversely affecting other tissues. Since the disease is often polyarthritic, involving large as well as small joints, local treatment of each inflamed joint is not a practical therapeutic option. Chloroxylon swietenia and it has been investigated for hepato protective and anti-oxidant activity, larvicidal activity, anti-inflmmatory activity, anti-bacterial and anthelminthic activity, larvicidal and ovicidal activity. *Chloroxylon swietenia* did not reported as an anti-rheumatoid activity, so in this study we investigate the anti-rheumatoid activity experimentally by using in-vitro and in-vivo methods.

MATERIALS AND METHODS

COLLECTION OF PLANT MATERIAL

PLANT

Chloroxylon swietenia collected from Near Chennuru, Adilabad district, Andhra Pradesh, India and the plant was authentified by Dr. E. Narasimha Murthy, Department of Plant Sciences, School of Life Sciences, University of Hyderabad, P.O. Central University, Andhra Pradesh, India. Plant was dried under shade.

PLANT PREPARATION AND EXTRACTION

The leaves were dried in sunlight and reduced to a coarse powder. Then the powder was subjected to macerate with ethanol for 72 hours at a temperature of 50-60 ºC. The extract was concentrated and the solvent was completely removed. They were freeze dried and stored in the vacuum desiccators until further use.

ANIMALS

Male Wister Albino rats weighing 200-220 grams were used for this experiment, procured from Sanzyme scientific, Hyderabad, India. The animals were housed in poly acrylic cages (38cmx23cmx10cm) with not more than six animals per cage, at an ambient temperature of 18±2ºC with 12-h-light/12-h-dark cycle. Rats have free access to standard chow diet and water. The maintenance and the handling of animals were performed according to CPCSEA guidelines and the institutional ethics committee approved all the experimental procedures Vaagdevi college of Pharmacy, Warangal, A.P., India.

INDUCTION OF ARTHRITIS

Arthritis was induced by single intra-dermal injection of 0.1 ml of Complete Freund's Adjuvant (CFA) containing 1 mg/ml mycobacterium tuberculosis H37Ra suspension in sterile paraffin oil into a foot pad of the left hind paw of male rats with help of glass syringe and 26 G needles. 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.

EXPERIMENTAL

Animals were divided into five groups of six animals each as follows:

GROUP-I - Normal rats given vehicle alone 2% Tween 80, (p.o).

GROUP-II -Arthritic untreated rats.

GROUP-III -Arthritic rats treated with 10mg/kg (p.o) standard diclofenac sodium.

GROUP-IV -Arthritic rats treated with 100mg/kg (p.o) of ethanolic extract.

GROUP-V –Arthritic rats treated with 200mg/kg (p.o) of ethanolic extract.

NOTE: Standard and test extracts were dissolved in 2% tween 80.

MEASUREMENT OF BODY WEIGHT AND PAW VOLUME

Body weight was measured at every four days up to 28thday after immunization. Both the injected and contra lateral hind paw volume was measured by means of a plethysmometer immediately before arthritis induction and 1, 7, 14, 21, 28 days thereafter. The following formula was used to calculate this increase:

(Volume on the test day − Volume before adjuvant injection) ×100/ Volume before adjuvant Injection

The value thus obtained was corrected for 100 g body weight [13]

MEASUREMENT OF HEMATOLOGICAL PARAMETER

On the 28thday after arthritis induction, rats were anaesthetized with ether and blood samples were

collected into Ethylenediamine tetra acetic acid (EDTA)-coated tubes from retro-orbital junction. The number of leukocytes from each rat was determined using a counting chamber (HORIBA, ABX MICRO ESP 60) and differential analysis of every sample was performed on staining blood smears using Jenner's stain. A total of 100 white cells were counted to determine the percentage of neutrophils. Erythrocyte sedimentation rate (ESR) was determined using the Wintrobe method [14] and Hemoglobin was also determined.

MEASUREMENT OF SPLEEN WEIGHT

The rats were sacrificed with ether on the 28thday, the spleen removed and the wet weight of the spleen was recorded and corrected for 100g body weight.

RADIOLOGICAL ANALYSIS OF BONE DESTRUCTION

After scarification on 28thday, knee joints were removed and experienced radiologist, who is unaware of the different drug treatments was scored the condition of tibiotarsal joints and graded as follows: periosteal reaction, 0−3 (none, slight, moderate, marked); erosions, 0-3 (none, few, many small, many large); joint space narrowing, 0−3 (none, minimal, moderate, marked); joint space destruction, 0−3 (none, minimal, extensive, ankylosis) [15].

HISTOLOGICAL PROCESSING AND ASSESSMENT OF ARTHRITIS DAMAGE

After sacrifice on 28th day, knee joints were removed and fixed in 10% formalin. After decalcification in 10% formalin, processed for paraffin embedding tissue sections (7µm thick) were stained with haematoxilin and eosin or safranin O. An experienced pathologist was unaware of the different drug treatments, scored the condition of tibiotarsal joints. Histopathological changes were scored as follows: inflammatory cells in the synovial tissues scored, 0−3; destruction of articular cartilage, 0−3 (ranging from the appearance of dead chondrocytes to complete loss of the articular cartilage); bone erosion, 0−3 (ranging from no abnormalities to complete loss of cortical and trabecular bone of the femoral head); Cartilage and bone destruction by pannus formation, 0−3 (none, mild, moderate, 3, severe); and vascularity , 0−3 (almost no, few, some, many)[16-18].

IN- VITRO METHODS

PROTEIN DENATURATION METHOD

Test solution of 0.5ml consisted of 0.45ml of bovine serum albumin (5% w/v aqueous solution) and 0.05ml of test solution (50,100,250,500, 1000µg/ml).

Control solution of 0.5ml consisted of 0.45ml of bovine serum albumin (5% w/v aqueous solution) and 0.05ml of distilled water. Standard Solution of 0.5ml consisted of 0.45 ml of bovine serum albumin (5% w/v aqueous solution) and 0.05ml of Diclofenac sodium (50,100,250,500, 1000 µg/ml).

Product Control of 0.5ml consisted of 0.45ml of distilled water and 0.05ml of test solution (50, 100, 250, 500, 1000µg/ml). All of above solutions were adjusted to pH6.3 using small amount of 1N Hcl. The samples were incubated at 37ºC for 20min and heated at 57ºC for 3min. After cooling, add 2.5ml of phosphate buffer to the above solution. The absorbance of the above solutions was measured using UV-Visible spectrophotometer at 416nm. The percentage inhibition of protein denaturation was calculated using the formula:

$$\text{Percentage inhibiton} = 100 - \frac{[(\text{OD of test solution} - \text{OD of product control})]}{(\text{OD of test control})} \times 100$$

The control represents 100% protein denaturation. The results were compared with Diclofenac sodium (250µg/ml) treated samples [3].

MEMBRANE STABILIZATION METHOD

The principle concerned in the following method is stabilization of human red blood cell membrane by hypotonicity induced membrane lysis. Blood was collected from (2ml) from healthy volunteers and was mixed with equal volume of sterilized Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid, 0.42% sodium chloride, in distilled water) and centrifuged at 3000rpm for 5min. The packed cells were washed with isosaline solution and 10%v/v suspension was prepared with normal saline and kept at 4^0C undisturbed until use Procedure is as follows: Test Solution of 4.5ml contained 2 ml hypotonic saline (0.25% w/v), 1ml

of phosphate buffer (pH-7.4), 1ml of test extract (50, 100, 200, 250, 500 1000 µg/ml) and 0.5ml of 10% HRBC suspension. Product Control of 4.5ml contained 2ml of hypotonic saline(0.25% w/v), 1ml of phosphate buffer (pH-7.4) and 1ml of test extract (50, 100, 200, 250, 500, 1000 µg/ml) in normal saline and 0.5ml of isotonic saline. Test Control of 4.5ml contained 2ml of hypotonic saline (0.25% w/v), 1ml of phosphate buffer (pH-7.4) and 1ml of isotonic saline and 0.5% of 10% HRBC suspension. Standard Solution of 4.5ml contained 2ml of hypotonic saline(0.25% w/v), 1ml of phosphate buffer (p^H-7.4) and 1ml of Diclofenac sodium (50, 100, 200, 250, 500, 1000µg/ml) in normal saline 0.5ml of 10% of HRBC suspension. The above solution was incubated at 56ºC for 30 min. The tubes were then cooled in running tap water for 30min. After that they were centrifuged and the supernatant liquid was separated and the absorbance of supernatant solution was measured at 560nm by UV-spectrophotometer. The percentage of membrane stability was calculated as follows:

$$\text{Percentage stabilization} = 100 - \frac{[(\text{OD of test solution} - \text{OD of product control})]}{(\text{OD of test control})} \times 100$$

STATISTICAL ANALYSIS

The result was expressed as mean ± S.D statistical difference between two means was determined by one-way ANOVA followed by Dunnett multiple comparisons test by using Graph pad prism V.5 software. Only those mean values showing statistical difference *P* <0.001, *P<0.01* and *P* <0.05 were considered as statistically significant.

RESULTS

BODY WEIGHT

Animals in which arthritis had been induced gained less weight after induction, which was significantly lower than negative controls on days 14 to 28 (*P* <0.01). In *Chloroxylon swietenia* treated arthritic rat's weight was not decrease as such to disease control animals in dose-dependent manner.

Table-1: Effect of C. Swietenia on Body Weight in Control and Experimental Animals.

Treatment groups	DAY 1	DAY 7	DAY 14	DAY 21	DAY 28
Control	203.33±5.16	205.83±3.76	210±4.47	215±4.47	215.83±3.76
Disease control	204.16±5.84	188.33±6.05	182.5±2.73	176.66±4.08*	171.66±2.58
Standard	200.83±5.84	189.16±7.35	197.5±4.18*	206.66±2.58**	210.83±3.76***
Test-I	202.5±5.24	190±6.32	196.66±5.16*	201.66±5.16**	209.166±7.35***
Test-II	205±5.47	195.83±3.76	198.33±4.08	208.33±4.08**	212.5±4.18***

All values expressed as mean ± SD, n=6, *p<0.05, **p<0.01, ***p<0.001 as compared to disease control group comparisons are done by one way ANOVA using Dennett's test.

PAW VOLUME

The volume of ipsilateral paw as well as contralateral paw in the adjuvant induced arthritis (AIA) rats increased progressively. The differences in the volume of ipsilateral paw and contralateral paw between the AIA and drug-treated rats were statistically significant in dose-dependent manner. Especially significant effects of *Chloroxylon swietenia* were observed at days 7 to 21 after immunization.

Table-2: Effect of *Chloroxylon Swietenia* on Paw Volume in Control and Experimental animals

Treatment groups	Day 1	Day7	Day14	Day21	Day28
Normal control	0.55±0.05	0.58±0.03	0.60±0.05	0.60±0.046	0.58±0.04
Disease control	0.56±0.051	0.98±0.01	1.21±0.07	1.3±0.08*	1.51±0.09
Diclofenac sodium 10mg/kg	0.55±0.054	0.961±0.033	1.16±0.051*	0.94±0.053**	0.73±0.10***
EECS 100mg/kg	0.54±0.069	0.96±0.030	1.2±0.12*	1.08±0.075**	0.91±0.05***
EECS 200mg/kg	0.53±0.05	0.971±0.24	1.28±0.07	0.98±0.070**	0.74±0.13***

All values expressed as mean ± SD, n=6, *p<0.05, **p<0.01, ***p<0.001 as compared to disease control group comparisons are done by one way ANOVA using Dennett's test.

HEMATOLOGICAL PARAMETERS

In relation to leukocyte analysis, total white blood cell (WBC) count increase with significantly increased neutrophil percentage in arthritic animals. ESR and RF were also significantly increased, while hemoglobin was decreased in AIA animals. Results shown in below table 3 suggest that total WBC count, neutrophil, ESR and RF are significantly decreased, while hemoglobin was slightly increased in treated animals in dose-dependent manner as compared to disease control (P <0.01).

Table 3: Effect of *Chloroxylon swietenia* on Hematological Parameters in Control and Experimental Animals

Parameters	Group-I (Control)	Group-II (Disease)	Group-III (Standard)	Group-IV (Test 1)	Group-V (Test2)
Hb (gm/dl)	12.78±0.415	9.648 ±0.11	12.69±0.142	10.10±0.08	10.29±0.137
RBC (10^6/mm^3)	5.66±0.05	4.40±0.219	5.49±0.06	4.67±0.10*	4.80±0.171
WBC (10^6/mm^3)	6.215±0.24	10.33±0.360	6.05±0.145*	5.26±0.175**	5.53±0.175***
ESR 30 min	2.28±0.047	6.59±0.09	3.10±0.048*	2.58±0.109**	2.75±0.110***
ESR 60 min	3.85±0.042	8.87±0.137	3.74±0.088	3.47±0.106**	3.54±0.029***

All values expressed as mean ± SD, n=6, *p<0.05, **p<0.01, ***p<0.001 as compared to disease control group comparisons are done by one way ANOVA using Dennett's test.

SPLEEN WEIGHT

In an experiment, the mean spleen weight of the adjuvant control rats was increased it was suggested spleenomegaly was apparent. Both extracts significantly reduced spleen weight of the adjuvant treated rats.

RADIOLOGICAL ANALYSIS OF BONE DESTRUCTION

Bone destruction, which is a common feature of adjuvant arthritis, was examined by radiological analysis. Adjuvant-treated rats had developed definite joint space narrowing of the in tertarsal joints, diffuse soft tissue swelling that included

diffuse demineralization of bone, marked periosteal thickening, and cystic enlargement of bone and extensive erosions produced narrowing or pseudo widening of all joint spaces. In contrast, in rats given ethanolic extract of *Chloroxylon swietenia* attenuate these abnormalities predominantly localized to the proximal areas of the paws ethanolic extract at 100 mg/kg dose alone failed to produce any significant improvement. Ethanolic extract at 200 mg/kg dose alone should produce slightly good significant improvement than before dose.

HISTOLOGICAL ANALYSIS OF BONE DESTRUCTION

Histological changes like infiltration of a few neutrophils and lymphocytes into mildly edematous synovium, destructive lesions in articular cartilage, destruction of synovial membrane and bony trabeculae, vascularity formation into the joint space, more extensive shown in adjuvant-treated animals. Ethanolic extracts (200 mg/kg) of *Chloroxylon swietenia* produced knee joints protective effect compared to control in dose-dependent manner (Table 4). Ethanolic extract at 100 mg/kg dose alone failed to produce any significant improvement.

Table 4: *In-Vitro* Anti-Arthritic Activity: Membrane Stabilization Method

Conc. (µg/ml)	% Membrane stabilization of *C.swietenia*	% Membrane Stabilization of Standard
50	67.75	68.86
100	79.15	81.71
200	80.1	83.19
250	83.27	84.32
500	87.98	89.9
1000	90.15	92.82

Table 5: Inhibition of protein denaturation

Conc.(µg/ml)	% Protein denaturation inhibition of *C.swietenia*	% Protein denaturation Inhibition of Standard.
50	65.81	67.78
100	70.28	72.53
200	73.2	75.8
250	78.23	81.62
500	86.8	87.88
1000	88.75	91.54

DISCUSSION

Most of the investigators have reported that inhibition of adjuvant-induced arthritis in rats is one of the most suitable test procedures to screen anti-arthritic agents since it closely resembles human arthritis. Freund's adjuvant induced arthritis is thought to occur through cell-mediated autoimmunity structural mimicry between mycobacteria and cartilage Proteoglycan in rats. It activates macrophages and lymphocytes by adjuvant inoculation or their products like monokines, cytokines, and chemokines may be

involved in abnormal lipid and protein metabolism. The CFA 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 [4]. The body weight of control AIA rats was significantly decreased compared with that of no immunized normal rats. The data suggest that oral *Chloroxylon swietenia* prevents inflammatory body weight loss in AIA rats. Thus, give protective action. Spleen is a vital organ involved in immune responses. In adjuvant arthritis, spleen serves as the reservoir for the cells and antibody formation which involved in the immune response. Increased in the weight of spleen is associated with the splenomegaly, generalized lymphadenopathy and altered hepatic function. Subplantar administration of FCA significantly increased weight of spleen and decreased the weight of thymus which is in accordance with previous studies of decrease in spleen weight and increase in thymus weight might be due to immune-stimulatory effect of *Chloroxylon swietenia* Extract. The present study revealed that the paw volume increases with ankle stiffness in adjuvant- challenged animals. *Chloroxylon swietenia* administration delayed the onset and suppressed severity of clinical arthritis, as demonstrated by decreased both the paw volume. The decrease in plasma uric acid in arthritic animals might be due to its continuous utilization by the system during free radical quenching reaction. It has been reported that uric acid serves as antioxidant *in vivo*, scavenging singlet oxygen, peroxyl and hydroxyl radicals and hypochlorous acid [18]. However, it is degraded on continuous exposure to OH and HOCl. The concentration of uric acid oxidation products has reported to be increased in serum and synovial fluid (SF) from patients with RA. ESR level in rats treated with FCA was significantly increased as compared to Normal Control rats (NC). There was significant decrease in ESR in rat treated with *Chloroxylon swietenia* (100, 200mg/kg) as compared to Disease Control rats (DC). Whereas, treatment with Diclofenac sodium (10mg/kg) significantly attenuated this increased level of ESR as compared to Disease Control rats. As an indication of infectious and inflammatory disease WBC count and lymphocyte percentage is increased in arthritic rats [9]. There was significant increase in WBCs in Disease Control (DC) rats as Compared to Normal rats (NC). Rats treated with *Chlorooxylon swietenia* (100, 200mg/kg) showed significant decrease in WBCs when compared to Disease Control rats (DC). Whereas treatment with Diclofenac sodium (10mg/kg) significantly decrease WBCs as compared to control rats. Anemia is a commonly observed feature in chronic arthritis patients. A decrease in levels of RBC and hemoglobin were observed in arthritic rats. There was a significant improvement in the levels of hemoglobin and RBC in *Chloroxylon swietenia* treated rats. RBC membrane is analogous to liposomal membrane and its stabilization may also stabilizer lysosomal membrane. Stabilization of lysosomal membrane decreases the inflammatory response by preventing the release of lysosomal constituents of activated neutrophils such as bacteriocidal enzymes and protease which cause further tissue damage upon extra cellular release. The effect of ethanolic extract of *Chloroxylon swietenia* on inhibition of membrane stabilization: Membrane by hypo tonicity induced membrane lysis was studied to establish the mechanism of anti-inflammatory action of *Chloroxylon swietenia.* Membrane stabilization action was studied to establish the mechanism of anti-arthritic effect of *Chloroxylon swietenia.* Denaturation if tissue protein is one of the well-known causes of inflammation and arthritis disease. Production of auto antigen in certain arthritis disease may due to denaturation of proteins [18]. The effect of ethanolic extract of *Chloroxylon swietenia* on inhibition of protein denaturation. Extract of *Chloroxylon swietenia* at different dose levels provided significant protection against denaturation of proteins. In AIA treated group shows, mostly stroma composed of acute and chronic inflammatory cells mostly neutrophils and lymphocytes and the destruction of synovial membrane and bony trabaculae. We report that *chloroxylon swietenia* in established AIA protect the synovial membrane destruction at high dose than before dose. In synovial tissue, erosion of subchondral and cortical bone is common, leading to the characteristic erosions and also space between the joints seen on radiography. Here, we report that *Chloroxylon swietenia* treatment in established AIA markedly reduced bone erosions, examined by radio graphically.

CONCLUSION

From the results obtained in the present study, it may be concluded that *Chloroxylon swietenia* possess significant anti-arthritic activity. A significant increase in body weight, reduction in paw volume of both hind legs were observed in FCA induced arthritis in rats. A decrease in levels of RBC and hemoglobin were observed in arthritic rats. There was a significant improvement in the levels of hemoglobin and RBC in *Chloroxylon swietenia* treated rats. The increased levels of WBC, ESR were significantly suppressed in the extract administered arthritic group.

CONFLICTS OF INTEREST

None

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