

IN-VIVO STUDIES ON ANTI RHEUMATOID ACTIVITY OF INDIAN MEDICINAL PLANT

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ABSTRACT

The present work was undertaken in order to evaluate and anti-arthritis potential of whole plant of *Cuscuta reflexa*. The study was aimed at carrying out the pharmacological screening of ethanolic extract of whole plant of *Cuscuta reflexa* for anti-arthritis activity which was evaluated by in-vivo Adjuvant- induced arthritis model in rats by analyzing the some parameters. Bone destruction, which is a common feature of adjuvant arthritis, was examined by radiological analysis. Gross destruction of the joints of the untreated paws was observed in 5 out of 6 rats in the adjuvant control group. In rats given ethanolic extract of *Cuscuta reflexa* attenuate these abnormalities predominantly localized to the proximal areas of the paws (Table 5). Ethanolic extract at 200mg/kg dose alone failed to produce any significant improvement.

Ethanolic extract at 400 mg/kg dose alone should produce slightly good significant improvement than before dose. Total white blood cells (WBC) count increase with significantly increased in arthritic animals. ESR and RF were also significantly increased, while hemoglobin, red blood cells (RBC) was decreased in AIA animals. Results shown in below table 6 suggest that total WBC count, ESR and RF are significantly decreased, while hemoglobin, RBC was slightly increased in treated animals in dose-dependent manner as compared to disease control ($P < 0.01$).

KEYWORDS: Anti-arthritis activity, RBC, WBC, NSAIDs.

INTRODUCTION

Numerous physiological and biochemical processes in the human body may produce oxygen centered free radicals and other reactive oxygen species as byproducts. Overproduction of such free radicals can cause oxidative damage to biomolecules (e.g. lipids, proteins, DNA),

eventually leading to many chronic diseases, such as atherosclerosis, cancer, diabetes, aging, and other degenerative diseases in humans [Halliwell *et al.*, 1994, Poulson *et al.*, 1998]. Plants (fruits, vegetables, medicinal herbs, etc.) may contain a wide variety of free radical scavenging molecules, such as phenolic compounds (e.g. phenolic acids, flavonoids, quinines, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant activity [Zheng *et al.*, 2001]. The intake of natural antioxidants has been associated with reduced risks of cancer, cardiovascular disease, diabetes, and other diseases associated with ageing [Sun *et al.*, 2002].

Rheumatoid arthritis (RA) is a chronic, inflammatory, multisystem, autoimmune disorder. It commonly affects the joints in a polyarticular manner. It occurs worldwide, affecting approximately 1% of adults. This autoimmune disease can affect people of all ages, but typically targets those from 20–45 years old. Women are three times more likely to get RA than men [Ahlmen *et al.*, 2010].

The main categories of drugs used to treat rheumatoid arthritis are analgesics, disease-modifying anti-rheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and immunosuppressive drugs [Cambell *et al.*, 1988, Nandi *et al.*, 2008] but these drugs produce some unwanted side-effects such as gastrointestinal ulcerogenicity and renal morbidity. So, nowadays medicinal herb in the treatment and prevention of diseases is attracting attention by scientist's worldwide.

The present work was undertaken in order to evaluate and anti-arthritis potential of whole plant of *Cuscuta reflexa*. The study was aimed at carrying out the pharmacological screening of ethanolic extract of whole plant of *Cuscuta reflexa* for anti-arthritis activity which was evaluated by In-vivo Adjuvant- induced arthritis model in rats by analyzing the various parameters.

MATERIALS AND METHODS

Procurement and authentication of plant material

Based on the Ethnomedical information and literature survey *Cuscuta reflexa* (Covolvulaceae) plant was selected for the present study. The plant of *Cuscuta reflexa* was collected from Near Tekumatla village, Adilabad District, Andhra Pradesh, India. Identified and authenticated by Prof. V. Raju Department of Botany, Kaktiya University, Warangal.

Extraction procedure

a. Plant preparation and extraction

The plant was 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 °C. The extract was concentrated and the solvent was completely removed. They were freeze dried and stored in the vacuum desiccators until further use.

In-vivo method: Animals used

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±2 degree centigrade with 12hr. Rats have free access to standard chow diet and water *ad libitum*. The maintenance and the handling of animals were performed according to CPCSEA guidelines.

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⁻¹ *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 26G 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.

Hematological parameters

On the 28th day 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, Vaagdevi College of pharmacy.) 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 [Wintrobe *et al.*, 1961] and rheumatoid factor (RF) by turbid metric method Hemoglobin was also determined.

Histopathological examination

For Histopathological studies, the hind limbs were removed (knee joints) and presented in 10% formalin. The tissues were fixed in formalin, decalcified and embedded in paraffin

blocks. Sections prepared with the microtome were stained with hematoxylin and eosin and examined under microscope and photographs were taken [Narendhirakannan *et al.*, 2007].

Radiological analysis of bone destruction

After scarification on 28th day, knee joints were removed and certified radiologist from Vijetha Scans and Diagnostics, Warangal, Andra Pradesh. Who was 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, and marked); joint space destruction, 0–3 (none, minimal, extensive, ankylosis) [Van den berg *et al.*, 1994].

Histological processing and assessment of arthritis damage

After sacrifice on 28th day, knee joints were removed and fixed for 4 d in 5% formaldehyde. After decalcification in 5% formic acid, processed for paraffin embedding tissue sections (7 μ m thick) were stained with hematoxylin and eosin or safranin O. Certified pathologist from VBR Diagnostics, Warangal, Andra Pradesh, Who was unaware of the different drug treatments, scored the condition of tibiotarsal joint, 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)[Joosten *et al.*, 1994,1997, Taniguchi *et al.*, 1999].

RESULTS

Statistical analysis

All the data were presented as Mean \pm S.D for six animals in each group. Statistical analysis was performed with ANOVA and the differences among groups were determined by Dunnett's multiple comparison tests using Graph pad prism V 0.5 Values were considered to be significant when P value less than 0.05.

Toxicity study

In the present study the EECR subjected for toxicity studies for the LD50 dose determination. EECR administered up to a maximum dose level of 2 g/kg bd wt. and both extracts did not

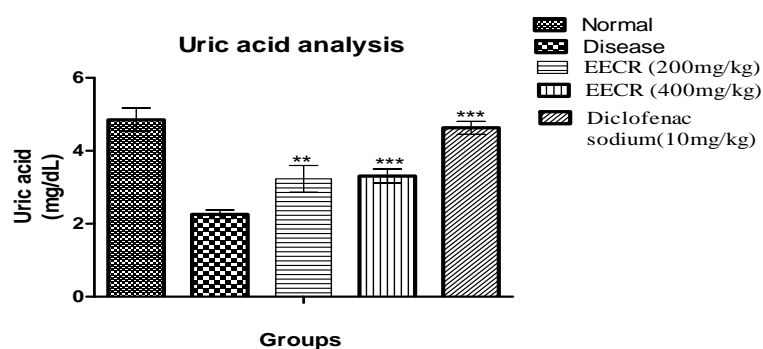
produce any mortality. $1/5^{\text{th}}$, $1/10^{\text{th}}$, $1/20^{\text{th}}$ of maximum dose tested for LD50 i.e. (2g/kg) were selected for the present study, LD50 of EECR 2000mg/kg.

Uric acid analysis

Table1: Effect of *Cusutareflexa* on serum uric acid parameter in control and experimental animals.

Parameter	Normal	Disease	Treatment 1	Treatment 2	Standard
Uric acid (mg/dL)	4.85±0.32	2.26±0.12	3.23±0.37 **	3.31±0.19 **	4.63±0.18 ***

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



Radiological analysis of bone destruction

Bone destruction, which is a common feature of adjuvant arthritis, was examined by radiological analysis. Gross destruction of the joints of the untreated paws was observed in 5 out of 6 rats in the adjuvant control group. Adjuvant-treated rats had developed definite joint space narrowing of the intertarsal joints, diffuse soft tissue swelling that included the digits, 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 *Cuscuta reflexa* attenuate these abnormalities predominantly localized to the proximal areas of the paws (Table 5). Ethanolic extract at 200 mg/kg dose alone failed to produce any significant improvement. Ethanolic extract at 400 mg/kg dose alone should produce slightly good significant improvement than before dose.

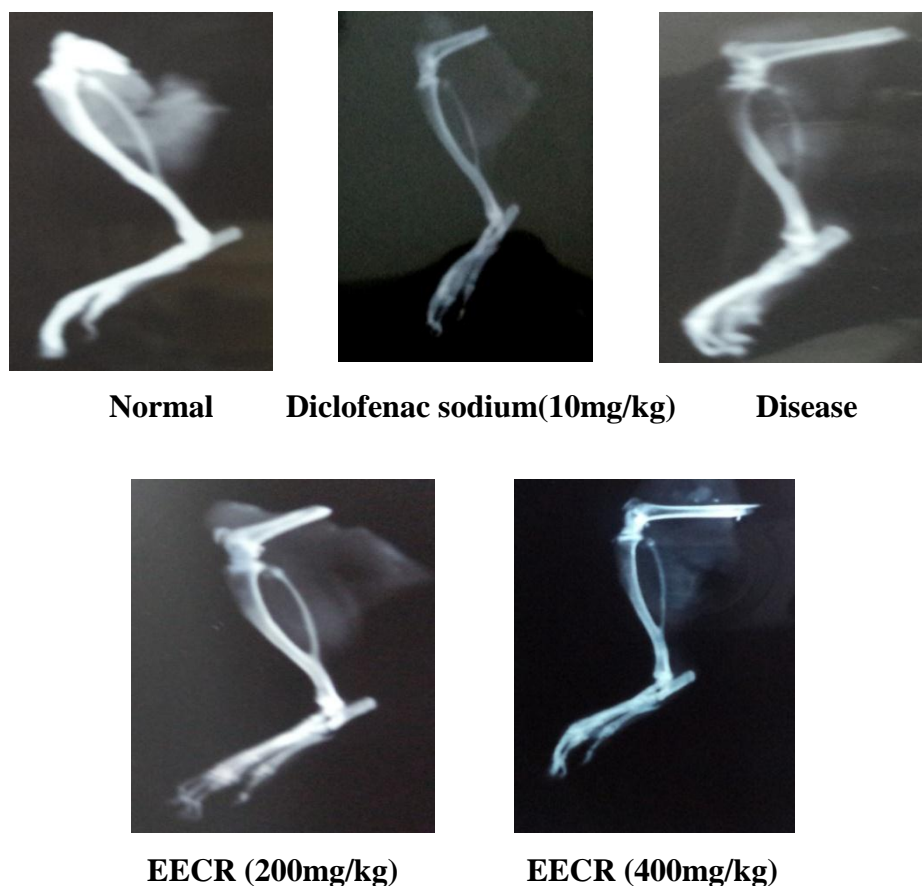


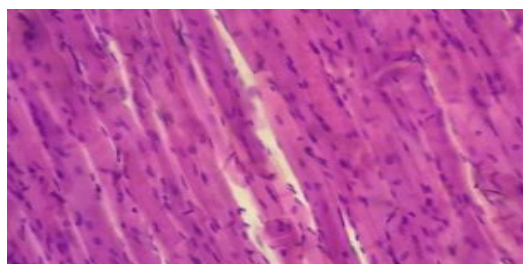
Table 2: Effect of Ethanolic extracts of *Cuscuta reflexa* extract and Diclofenac sodium on X-ray analysis in the adjuvant-treated rats.

Treatment	Disease	Standard	Treatment I	Treatment II
Dose(mg/kg/b.wt)	-	10	250	500
Radiology score	8	3 ^{**}	7	5 [*]

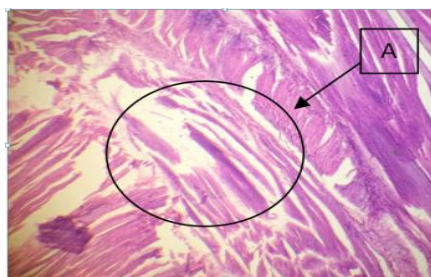
All values expressed as mean \pm SD, n=6, *p<0.05, **p<0.01, as compared to disease control group comparisons are done by one way ANOVA using Dunnett's test.

Histological analysis of bone destruction.

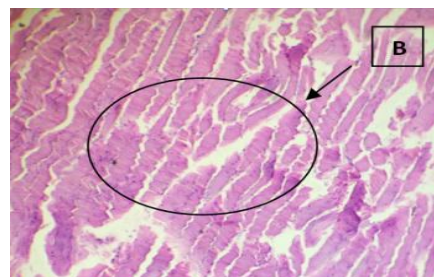
Histological changes like infiltration of a few neutrophils into mildly edematous synovium, destructive lesions in articular cartilage, vascularity formation into the joint space, more extensive shown in adjuvant-treated animals (Fig. below). Ethanolic extracts (400 mg·kg⁻¹) of *Cuscuta reflexa* produced knee joints protective effect compared to control in dose-dependent manner (Table 6). Ethanolic extract at 200 mg/kg dose alone failed to produce any significant improvement.



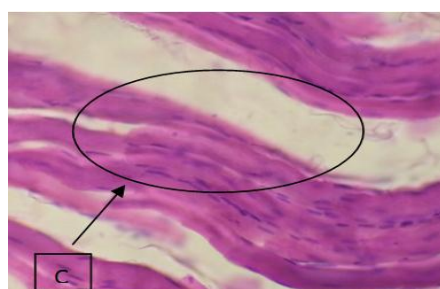
Normal



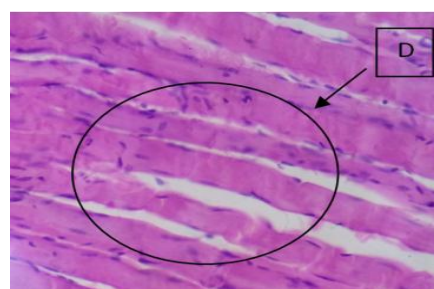
Disease



Diclofenac sodium (10mg/kg)



EECR (250mg/kg)



EECR (500mg/kg)

Table 3: Effect of Ethanolic extracts of *Cuscuta reflexa* and Diclofenac sodium on Histopathological analysis in the adjuvant-treated rat.

Treatment	Disease	Standard	Treatment I	Treatment II
Dose(mg/kg/b.wt)	-	10	250	500
Histology score	8	4 ^{**}	7	5 [*]

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

Hematological parameter

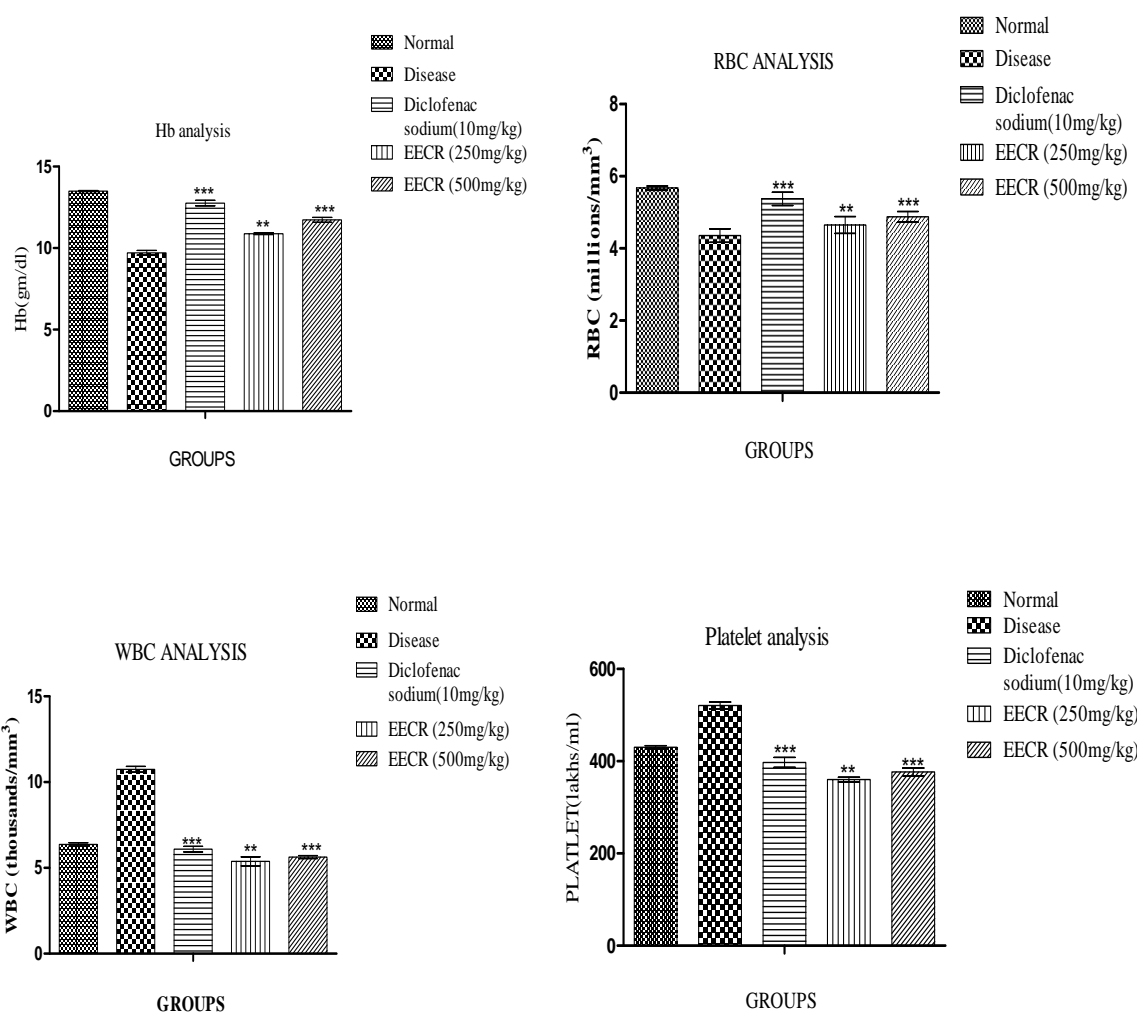
Total white blood cells (WBC) count increase with significantly increased in arthritic animals. ESR and RF were also significantly increased, while hemoglobin, red blood cells (RBC) was decreased in AIA animals. Results shown in below table 6 suggest that total WBC count, ESR and RF are significantly decreased, while hemoglobin, RBC was slightly

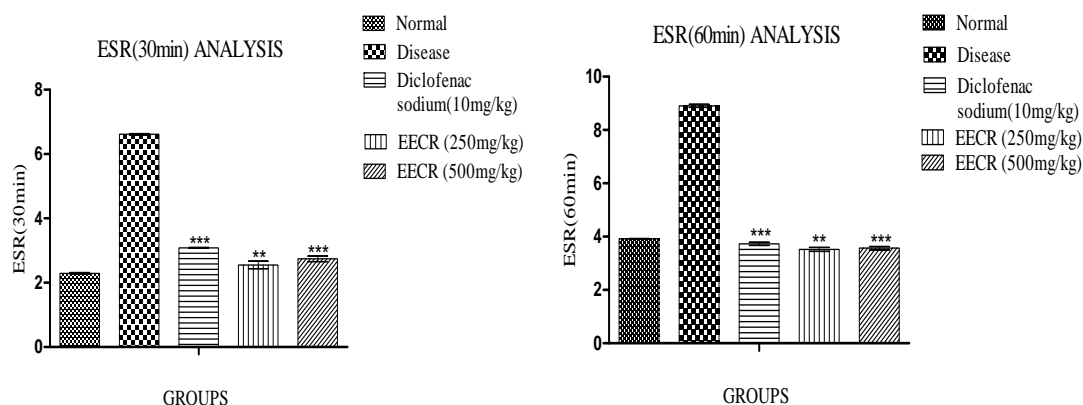
increased in treated animals in dose-dependent manner as compared to disease control ($P < 0.01$).

Table 4: Effect of *Cuscuta reflexa* on haematological parameters in control and experimental animals.

Groups	Hb (gm/dl)	RBC ($10^6/\text{mm}^3$)	WBC ($10^6/\text{mm}^3$)	Platelets ($10^6/\text{ml}$)	ESR	
					30min	60min
Control	13.49 \pm 0.03	5.67 \pm 0.05	5.38 \pm 0.26	43.00 \pm 3.76	2.29 \pm 0.02	3.91 \pm 0.01
Disease	9.70 \pm 0.14	4.35 \pm 0.18	10.74 \pm 0.17	52.08 \pm 7.35	6.61 \pm 0.01	8.90 \pm 0.05
Standard	12.76 \pm 0.16***	5.37 \pm 0.18***	6.09 \pm 0.16***	39.73 \pm 10.80***	3.08 \pm 0.01***	3.73 \pm 0.05***
Treatment I	10.87 \pm 0.5**	4.65 \pm 0.23**	5.38 \pm 0.26**	36.01 \pm 5.30**	2.55 \pm 0.11**	3.52 \pm 0.07**
Treatment II	11.73 \pm 0.14***	4.87 \pm 0.14***	5.63 \pm 0.08***	37.68 \pm 8.37***	2.74 \pm 0.08***	3.56 \pm 0.06***

All values expressed as mean \pm 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 Dunnett's test.





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. 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, peroxy and hydroxyl radicals and hypochlorous acid. However, it is degraded on continuous exposure to OH^\bullet and HOCl . The concentration of uric acid oxidation products has reported to be increased in serum and synovial fluid (SF) from patients with RA. This supports our present result. Thus, the increase in uric acid in drugs treated rats might exert protection to the joint cartilage against the deleterious effects of ROS/RNS [Bezerra *et al.*, 2004].

Alterations observed in the above parameter during arthritic conditions were normalized to a greater extent in *Cuscutareflexa* treated animals. In synovial tissue, erosion of subchondral and cortical bone is common, leading to the characteristic erosions seen on radiography. Osteoclasts can be seen in the areas of bone destruction during AIA. Here, we report that *Cuscutareflexa* treatment in established AIA markedly reduced bone erosions, examined by radiographical and Histopathological analysis. Neither bone destruction nor osteoclasts were noted in arthritic joints of animals treated with high-dose *Cuscutareflexa* indicating decreased formation of these cells.

As observed from the present study a similar decrease in Hb and increase in the WBC count and ESR levels in AIA rats were reported by Agarwal and Rangari [Agarwal *et al.*, 2003]. The decrease in Hb and RBC levels in arthritic rats reflects the presence of anemia in these rats. Anemia is the most common extracellular manifestation in RA [Hochberg *et al.*, 1988] and a

moderate hypo chromic; Normocytic anemia due to reduction in the RBC count with a modest reduction in the MCHC is a common feature of RA. The most important cause might be the decreased level of plasma iron due to sequestering of iron in the reticuloendothelial system and synovial tissue that lead to failure of bone marrow to respond to anemia. The decrease in plasma iron in turn was induced by IL-1 in association with the acute phase response [Connolly *et al.*, 1988, Klempner *et al.*, 1978]. Hence, it is provocative to speculate that the sequestration of less deformable erythrocytes by endothelial cells in the spleen plays a causative role in the shortened half-life of erythrocytes and subsequently anemia resulting in adjuvant arthritis. The increase in total WBC count in AIA rats falls in line with the reports. The increase in both WBC and platelet counts might be due to the stimulation of immune system against the invading pathogenic microorganism. This is evident by the infiltration of inflammatory mononuclear cells in the joints of AIA rats [Mythilypriya *et al.*, 2008].

ESR is an indirect measurement of acute phase response for determining the disease activity in RA [Marcelletti *et al.*, 2003]. Although CRP is a better marker for inflammation and though ESR is influenced by several factors such as the plasma concentration of fibrinogen, Immunoglobulins, RF and Hb, the increased level of ESR in arthritic rats adds information reflecting the chronicity and severity of the disease better than CRP [Skoghet *et al.*, 2003]. Hence, a combination of the tests might be worthwhile. In our study we were brought back to near normal levels upon *Cuscutareflexa* treatments, which emphasizes the beneficial effect of the drugs on AIA. Therefore, above study on *Cuscutareflexa* extracts demonstrate the significant anti-arthritic activity.

CONCLUSION

From the results obtained in the present study, it may be concluded that *Cuscutareflexa* possess significant anti-arthritic activity. Hence it could be beneficial for further work as active anti-arthritic agent.

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