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Ethanolic Extracts of leaves of *Alstonia scholaris* Linn. Modulates Chronic Inflammatory Immune Response in FCA Induced Arthritis

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Abstract

AIM- The aim of the present investigation is scientifically establishing the anti-arthritic activity of selected medicinal plant so that we are able to come up with a more effective and potent bioactive phytoconstituents with less side effects in comparison with existing synthetic drugs. **MATERIAL & METHODS-** The leaves of *Alstonia scholaris* were collected from campus of College of Pharmacy. All the plant materials were taxonomically identified by Dr. Gyanendra Tiwari, Senior Scientist, KNK College of Horticulture. All the plant materials were dried under shade and subjected to coarse powder for extraction process. Accurately weighed quantity of leaf powder of *Alstonia scholaris* were extracted using 95 % ethanol by soxhlet apparatus for 72 h. The ethanolic extracts were dried under the reduced pressure to get crude ethanolic extracts. The Wistar albino rats were divided into 10 groups of six animals in each. For the induction of chronic inflammatory response, FCA (0.1 ml) was injected through intra-articular injection in left ankle joint of rats on 0 day. The severity of adjuvant arthritis was quantified by measuring the volume of the hind paw using Plethysmograph. Body weight was measured of all groups at zero days before immunization and at 21st day after treatments over by using a single pan weighing balance. **RESULTS-** The preliminary phytochemical analysis revealed that different active constituent present in different extracts such as carbohydrates, proteins, amino acids, fat, oils, steroids, terpenoids, glycosides, alkaloids, tannins and other phenolics compounds. The assessment made on the 21st day showed that the *A. scholaris* treatments at both doses (low and high) had moderately significant and highly significant effect and reduced ($p< 0.01$& $p<0.001$) the adjuvant-induced lesions in the respective treatment groups as compared with the arthritis control group. However, standard, *A. scholaris* had highly significant effects ($p<0.001$) in recovery of RBCs and haemoglobin. They also showed highly significant effects on decrease in WBCs and ESR treated groups, also showed moderately significant effects as compared to arthritic group. **CONCLUSION-** Besides from the obvious therapeutic importance, these components would be useful in understanding the mechanism of diseases with higher levels of cellular and molecular level. These components could serve as lead molecules for development of prospective anti-arthritic agents.

Keywords: Anti-arthritic agent, Lead molecules, *Alstonia scholaris,* Shronic inflammatory response, Synthetic drugs, Plethysmograph

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INTRODCTION

The etiology of this disease is multi factorial. Genetic predisposition, environmental and hormonal affect has been reported to contribute in the initiation and progression of the disease (Cuzzocrea and Rosanna 2008). It is proposed that the disease is triggered by unknown antigens of infectious origins which when presented to immune cells in the joint tissue initiate an event that is followed by the induction of an immune response, resulting in inflammation in the lining of the joints. RA mainly represents a typical T-cell mediated disease (Cope, 2007). The most convenient evidence concerning the involvement of T cells in RA comes from immune genetics of RA (Macgregor *et al.,* 2000) and the fact that in experimental animal models such as adjuvant arthritis, the disease can be transferred by isolated T cell lines. Once T cells are activated in RA, it consequently leads to multiple effects, including activation and proliferation of synovial lining and recruitment of additional pro-inflammatory cells like mononuclear phagocytes, lymphocytes, and plasma cells from the bone marrow and circulation into the synovium subliming layer. These cells in the synovium next induce a hyperplasic reaction of the resident fibroblasts which eventually cause tissue destruction and cartilage and bone erosion as mediated by secretion of inflammatory mediators and pro-inflammatory cytokines (McInnes *et al.,* 2001).

As per the literature review, it has been observed that *Alstonia scholaris* (leaf), are listed among the various medicinal plants widely been used in the acute and chronic inflammatory conditions. In the absence of any scientific evidence for their anti-arthritic activity in chronic inflammatory conditions. Hence, there is a need in scientifically establishing the anti-arthritic activity so that we are able to come up with a more effective and potent bioactive phytoconstituents with less side effects in comparison with existing synthetic drugs.

MATERIALS AND METHODS

Plant Materials

The leaves of *Alstonia scholaris* were collected from campus of College of Pharmacy. All the plant materials were taxonomically identified by Dr. Gyanendra Tiwari, Senior Scientist, KNK College of Horticulture. The herbarium sheets were submitted in Department of Pharmacognosy, under voucher specimen.

Preparation of Total Crude Extract

All the plant materials were dried under shade and subjected to coarse powder for extraction process. Accurately weighed quantity of leaf powder of *Alstonia scholaris* were extracted using 95 % ethanol by soxhlet apparatus for 72 h. The ethanolic extracts were dried under the reduced pressure to get crude ethanolic extracts. After drying, the respective extracts were weighed and percentage yield was determined (Mukherjee, 2002).

Preliminary Phytochemical Tests

Qualitative chemical tests of ethanolic and Methanolic extracts were subjected to various chemical tests to detect various phytoconstituents (Khandelwal, 2006).

Selection of animals

Wistar albino rats of either sex between 2 and 3 months of age weighing 150-200 g were used which were procured from the central animal house of College of Pharmacy, India. All animals were housed in an animal room under normal condition of 25±1^{0}C, 12-h light and dark cycle. The animals were allowed free to access commercial rat pallet diet (Lipton India Ltd, Mumbai, India) and water *ad libitum.* The bedding materials of the cages were changed every day. All the experimental procedures were carried out in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines.

Acute toxicity studies

The acute oral toxicity studies were carried out according to the guidelines set by the Organization for Economic Co-operation and Development (OECD), revised draft guideline 423 (OECD guidelines, 2001).

Evaluation of anti-arthritic activity

The Wistar albino rats were divided into 10 groups of six animals in each. For the induction of chronic inflammatory response, FCA (0.1 ml) was injected through intra-articular injection in left ankle joint of rats on 0 day. Pre-induction baseline was taken prior to the injection of Freund's Complete Adjuvant (FCA) measured by left paw volume of each animal at 0 day for the induction of arthritis in Wistar rats. The treatments with all plant extracts were given once daily from day of injection to 21st day. A suspension of the test extracts were prepared in 1% Tween 80.The animal groups are as follows (Arulmozhi *et al*., 2011).

Group-I: Arthritic control, treated with 0.1 mL of FCA on zero day.

Group-II: Standard control: treated with prednisolone (10 mg/kg, p.o.) + FCA

Group-III: Treated with ethanolic extracts of *A. scholaris* (200 mg/kg, p.o.) + FCA

Group-IV: Treated with ethanolic extracts of *A. scholaris* (400 mg/kg, p.o.) + FCA

Measurements of paw volume

The severity of adjuvant arthritis was quantified by measuring the volume of the hind paw using Plethysmograph. Paw volume (ml) was measured at 0 days and thereafter 4, 8, 12, 16 and 21 days of FCA post-inoculation. Data were expressed as the increase in paw volume with respect to day 0 paw volume. The percentage inhibition of paw volume was measured by following formula (Arulmozhi *et al*., 2011; Bandara *et al*., 1989).

$$\text{Percentage inhibition} = Vc - Vt / Vt \times 100$$

Where,

Vc-Paw volume of control animals

Vt-Paw volume of treated animals

Measurements of body weight

Body weight was measured of all groups at zero days before immunization and at 21st day after treatments over by using a single pan weighing balance (Jalalpure *et al*., 2011).

Measurements of hematological parameters

On the 21st 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 (celldyn-1200, Abbott Carepam). Erythrocyte sedimentation rate (ESR) was determined using the Wintrobe method. RBCs and Haemoglobin were determined by routine laboratory method (Jalalpure *et al*., 2011).

RESULTS

Extractive Value Determination

Dried leaves of *scholaris* were extracted using ethanol. The percentage yields of all dried extracts were determined by using the following formula.

$$\text{Percentage yield} = \frac{\text{Weight of Extract}}{\text{Weight of powder drug Taken}} \times 100$$

Table 1: Different extracts with their appearance and % yield (in gm)

S. No.	Extracts	Color of dried extracts	Consistency of dried extracts	% Yield (W/W)
1	Ethanolic extracts of *Alstonia scholaris*	Dark Green	Sticky	28 %

Preliminary Phytochemical Screening

The preliminary phytochemical analysis revealed that different active constituent present in different extracts such as carbohydrates, proteins, amino acids, fat, oils, steroids, terpenoids, glycosides, alkaloids, tannins and other phenolics compounds.

Acute Toxicity Studies of Plant Extracts

No toxic effects were observed at a higher dose of 2000 mg/kg body weight of Wistar rats. Hence, 1/ 10th dose was selected as effective dose or therapeutic dose. The cut off value of 200 and 1/5 dose double of 400 mg/kg were selected for anti-arthritic and anti-inflammatory activity.

Freund's complete adjuvant induced rat paw edema

Observations of paw volume were recorded on 4th, 8th, 12th, 16th, 21st day after adjuvant injection. The assessment made on the 21st day showed that the *A. scholaris* treatments at both doses (low and high) had moderately significant and highly significant effect and reduced (p< 0.01& p<0.001) the adjuvant-induced lesions in the respective treatment groups as compared with the arthritis control group.

Table No 2: Effects of extracts on paw volume in FCA induced arthritis in rat

S. No.		Paw Volume in mL					
	Groups & Treatments	**Zero Day**	**4th Day**	**8th Day**	**12th Day**	**16th Day**	**21st Day**
1	Normal Control	0.29±0.08	0.31±0.03	0.31±0.04	0.31±0.01	0.31±0.06	0.31±0.14
2	Arthritic Control, 1% Tween 80, p.o.	0.30±0.02	0.50±0.04**	0.85±0.01***	0.92±0.02***	1.31±0.06***	1.63±0.02***
3	Prednisolone 10 mg/kg, p.o.	0.31±0.07	0.33±0.06*	0.40±0.18**	0.45±0.03***	0.52±0.04***	0.55±0.08***
6	*A. scholaris*, 200 mg/kg, p.o.	0.31±0.06	0.44±0.07	0.52±0.02*	0.63±0.05*	0.70±0.09**	0.77±0.14**
7	*A. scholaris*, 400 mg/kg, p.o.	0.33±0.04	0.42±0.12	0.50±0.01*	0.58±0.03**	0.62±0.02**	0.68±0.03***

Values are expressed as mean±SEM, n=6 in each group; * p <0.05, compared to disease control ** p<0.01, compared to disease control. *** p <0.001, compared to disease control

Effects on body weight

In arthritic group, decrease in body weight were observed on the subsequent days, whereas groups treated with standard, extracts of *A. scholaris* showed improvements in body weight. This effect was observed from 14th day to last day of the experiment as compared to arthritic rats. All the extracts had moderately and highly significant increase in body weight (p<0.01 & p<0.001) as compared to arthritic rats.

Table 3: Effects of plant extracts on body weight in FCA induced arthritis in rat

S. No.	Groups & Treatments	Days	
		Zero	**21st**
1	Normal Control	190.20±0.78	191.47±0.20
2	Arthritic Control, 1% Tween 80, p.o.	191.40±0.18	165.18±0.20***
3	Prednisolone, 10 mg/kg, p.o.	191.80±0.20	216.30±0.16***
4	*A. scholaris*, 200 mg/kg, p.o.	190.18±0.12	208.40±0.12**
5	*A. scholaris*, 400 mg/kg, p.o.	192.25±0.40	211.60±0.02***

Values are expressed as mean±SEM, n=6 in each group; *p<0.05, compared to disease control ** p<0.01, compared to disease control. ***p<0.001, compared to disease control

Effects on haematological parameters

FCA-induced arthritic rats at 21st day showed elevation in the total WBC count and reduction in RBC. However, significantly *(p<0.001)* increased ESR while the haemoglobin was significantly *(p<0.001)* reduced in the control group when compared with normal group. However, standard, *A. scholaris* had highly significant effects (p<0.001) in recovery of RBCs and haemoglobin. They also showed highly significant effects on decrease in WBCs and ESR treated groups, also showed moderately significant effects as compared to arthritic group.

Table No 4: Effects of extracts on haematological parameters in arthritis in rat

S. No.	Groups & Treatments	Haematological Parameters			
		Total WBCs count×10^3 cells/mm^3	RBCs (Million/$mm)^2$	Haemoglobin (g/dl)	ESR (mm/h)
1	Normal Control, 1% Tween 80, p.o.	8.18±0.90	7.88±0.11	14.82±0.13	11.40±0.42
2	Arthritic Control, 1% Tween 80, p.o.	14.70±1.12***	5.36±0.16**	10.46±0.12**	15.80±0.18***
3	Prednisolone 10 mg/kg, p.o.	8.48±0.75***	7.90±0.08**	15.20±0.31***	11.90±0.12***
4	*A. scholaris*, 200 mg/kg, p.o.	11.58±0.26*	6.10±0.09**	12.10±0.31*	13.72±0.10**
5	*A. scholaris*, 400 mg/kg, p.o.	10.86±0.21**	6.58±0.19*	12.64±0.82**	13.18±0.32**

Values are expressed as mean±SEM, n=6 in each group; *p<0.05, compared to disease control ** p<0.01, compared to disease control. ***p<0.001, compared to disease control

DISCUSSION

Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of diseases, although relatively modest acquaintance about their mode of action is existing. There is an emergent interest in the pharmacological evaluation of various plants used in Indian traditional systems of medicine. Thus, in the present investigation, an attempt was made to evaluate the anti-arthritic activity of selected medicinal plants (*Alstonia scholaris*) on the basis of ayurveda and their traditional uses in a suitable experimental animal model and to isolate the active constituents from the most active fractions.

CFA-induced experimental model for arthritis is considered closest to simulating human rheumatoid arthritis and therefore it is the most widely used chronic test model in which the associated clinical and Histopathological changes are comparable to those seen in human form (Billingham & Davies, 1979; Butler *et al.,* 1992). In this model, dead tubercle bacilli in liquid paraffin initiate an immune-mediated inflammatory reaction which ultimately culminates in chronic inflammation and poly arthritis (Cai *et al*., 2006). Initially the injection of CFA into the right hind-foot produces an inflamed swelling in the paw which reaches to its maximum during the first 3 days. Thereafter, the swelling is slow until the 8th day when the foot begins to swell again. As the disease progresses, on day 10th more severe oedema develops in the injected paw and inflamed lesions termed as secondary lesions are detected in the uninjected paw. The secondary lesions then begin to appear and increase in thickness, in the fore-paws, ears and tail. After the 13th day further swelling of the feet or joints occurs and by the 21st day the inflammation starts to subside leaving pale granulomatous swellings around the joints (Newbould *et al.,* 1963). The initial reaction of edema and soft-tissue thickening at the depot site is considered to be caused by the irritant effect of the adjuvant used while as the late-phase arthritic events are presumed to be due to immunologic events (Ward *et al.,* 1966).

The assessment of paw edema, an apparently simple, sensitive and quick procedure for evaluating the degree of inflammation in arthritic rats was done on alternate days in both the hind paws after adjuvant injection. Treatment with all test extracts of selected plants showed dose dependent suppression in edema of the injected paw (primary lesions). Maximal effects were observed at the dose of 400 mg/kg body weight of all test extracts. Reduction of the primary could be attributed to immunological protection rendered by our test extracts. All the test extracts were found to effectively reduce the primary lesions in arthritic rats. The suppression of this response therefore suggests any immunosuppressive activity for our test extracts. Moreover, this effect of test extracts was comparable to that of prednisolone. Our study results reveal that extracts of *Alstonia scholaris* treated rats significantly reduced the paw volume.

During the development of arthritic syndrome, the body weight of rats used as an indirect index in restoration of health. The body weight was significantly decreased in arthritic rat as compared to normal rat, but in the test extracts and standard drug treated groups, the body weights of the rats did not decline. The results of our study therefore indicated that there is a relationship between the extent of inflammation and loss of body weight. As the incidence and severity of arthritis increase, the changes in the body weights of the rats also occur during the course of the experimental period (Winder *et al*., 1969). Previous findings suggest that absorption of ^{14}C- glucose and ^{14}C-leucine in rat's intestine was reduced in the case of inflamed rats (Zwerina *et al*., 2005) but on the treatment with anti-inflammatory drugs, the decrease in absorption is neutralized. In our study, the body weight was significantly increased in the groups treated with prednisolone and all the extracts treated groups and this may be due to the restoration of absorption capacity of intestine. From our results, it can be concluded that all the extracts possess potentially useful anti-arthritic activity since they give a positive result in controlling inflammation in adjuvant-induced arthritis in rats.

With the development of arthritic conditions, there was a significant alteration of haematological parameters i.e. red blood cells (RBCs), white blood cells (WBCs), Haemoglobin (Hb) and erythrocyte sedimentation rate (ESR). As the disease progressed, RBCs and haemoglobin were decreased whereas; WBCs and ESR were significantly increased in arthritic control group when treated with normal control.

It was proposed that the reduction in the Hb and RBC count during arthritis results from premature destruction of red blood cells, reduced erythropoietin levels and also due to abnormal storage of iron in the reticuloendothelial system and synovial tissue (Mowat *et al.,* 1971). Further reports of a significant rise of WBC count, in arthritic control group is possibly due to the stimulation of immune system against the invading antigen and also to an IL-1β mediated rise in the respective colony-stimulating factors. In addition an increase in ESR is a common feature in rheumatoid arthritis (Glenn *et al.,*1971) and this increase in the ESR is attributed to the accelerated formation of endogenous proteins such as fibrinogen and a/b globulin, and such a rise in the ESR indicates an active but obscure disease process (Hu *et al.,* 2005).

The results of our study revealed that all the extracts treated group's causes significant alterations in the hematological parameters and maximal effects were observed at 400 mg/kg. The reversal of RBC counts and Hb levels observed in case of test extract treated groups could be attributed to the protective effects on tissue repair and suppression of disease progression. By modulation of immune system, all the extracts and prednisolone treated groups normalize the WBCs and ESR.

CONCLUSION

Besides from the obvious therapeutic importance, these components would be useful in understanding the mechanism of diseases with higher levels of cellular and molecular level. These components could serve as lead molecules for development of prospective anti-arthritic agents. Further detailed studies are required to elucidate the exact mechanism based on molecular and genetic level responsible for anti-arthritic activity. The present findings are significant for the development of alternative, inexpensive and perhaps safer strategies for the treatment of diseases.

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