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Anti-inflammatory & Anti-arthritic Activity of Different Bioactive Fractions of Ethanolic Extract of *Alstonia scholaris* Linn.

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ABSTRACT

AIM- The aim of the present investigation is to study the anti-inflammatory and anti-arthritic activity of different fractions. **MATERIAL & METHODS-** For the fractionization, dried 50 g ethanolic extracts of *Alstonia scholaris* extracts were taken and dissolved into distilled water. The water extract was filtered to remove all insoluble components. The water extracts was taken into separating funnel and fractionated with petroleum ether, chloroform and finally with butanol, according to polarity of solvents. Wistar albino rats of either sex between 2 and 3 months of age weighing 150-200 g were used. All animals were housed in an animal room under normal condition of 25 ± 1^{0}C, 12-h light and dark cycle. Inflammation was induced by 0.1 mL of 1% suspension of carrageenan into hind paw of rat by sub planter route. Treatments of all fractions were given 1 h prior to administration of carrageenan. Paw volume was measured with digital plethysmograph at 0, 1, 2, 3, 4 and 5th h after injection. Arthritis was induced by single injection of 0.1mL of FCA in left hind paw intradermaly. Before immunization, paw volume of rats of all groups were measured. Severe arthritic symptoms i.e. inflammation, pain, deformity and erosion were developed in 12-14th days of immunization. **RESULTS-** The chloroform fraction (100 mg/kg) significantly (p<0.001) inhibited carrageenan induced rat paw edema as compared to control group. Maximum inhibition of rat paw edema was observed with chloroform fraction (100 mg/kg) at the end of 5th h by 34.16 when compared to control group. Indomethacin (10 mg/kg) inhibited the paw edema by 45.12 %. The treatment of chloroform fraction was initiated at the onset stage of Polyarthritic development i.e., day 14th. The articular index of the arthritic control group rapidly increased from day 14th which indicated that the Polyarthritic symptoms had certainly developed. **CONCLUSION-** By comparing the results, we concluded that chloroform fraction of *Alstonia scholaris* showed best activity in FCA induced arthritis and bioactive fractions showed strong presence of terpenoids so these may be strong candidate of anti-arthritic activity.

KEYWORDS-

Anti-inflammatory, Anti-arthritic Activity, Different Bioactive Fractions, Ethanolic Extract, *Alstonia scholaris* Linn.

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INTRODUCTION

Clinically, RA is characterized by Polyarthritic, swelling and, in many cases, manifests extra-articular involvement. In the early stage of the disease, typical signs and symptoms are swelling and pain of the proximal interphalangeal and metacarpophalangeal joints. Later, the larger joints become affected, especially those of the arms, feet and knees. In addition, RA can affect other systems of the body, and this may range from rheumatoid nodules to life-threatening vasculitis (Smolen & Steiner 2003). 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 (Kuiper *et al.,* 2001; Symmons *et al.,* 2002). 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 (McInnes *et al.,* 2001).

As per the literature review, it has been observed that *Alstonia scholaris* (leak and bark) is 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 B. R. Nahata College of Pharmacy, Mandsaur.

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. The extracts were dried completely under reduced pressure. After drying, the respective extracts were weighed and percentage yield was determined (Mukherjee, 2002).

Fractionization of Extracts

In our previous study, in Freund's Complete Adjuvant model of all plant extracts, ethanolic extracts of *Alstonia scholaris* shown best activity as compared to another plants. Hence on the basis of the results of activity so selected for the further fractionization.

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For the fractionization, dried 50 g ethanolic extracts of *Alstonia scholaris* extracts were taken and dissolved into distilled water. The water extract was filtered to remove all insoluble components. The water extracts was taken into separating funnel and fractionated with petroleum ether, chloroform and finally with butanol, according to polarity of solvents. All the bio fractions were collected and dried under reduced pressure and their percentage yields were calculated respectively (Shukla *et al*., 2012).

Preliminary phytochemical screening

Preliminary phytochemical screening was performed of all fractions according to methods described (Kokate, 2003; Khandelwal, 2006).

Selection of animals

Wistar albino rats of either sex between 2 and 3 months of age weighing 150-200 g were used. 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 of all bioactive fractions

All the fractions were dissolved in 1% Tween 80. The bioactive fractions were administered orally to Wistar rats in increasing dose (50, 250, 500, 750 and 1000 mg/kg) (OECD guidelines, 2001).

Anti-inflammatory activity of fractions in carrageenan induced rat paw edema

Animals were divided into 7 groups and each group contains 6 animals. The animal groups and treatment schedule are as follows.

Group-I: Normal control treated with normal saline (5 ml/kg p.o.)

Group-II: Disease control treated with 0.1 mL of 1% carrageenan in 0.9% saline

Group-III: Standard control treated with Indomethacin (10 mg/kg p.o.) + Carrageenan

Group-IV: Pet ether fraction of *A. scholaris* (100 mg/kg p.o.) + Carrageenan

Group-V: Chloroform fraction of *A. scholaris* (100 mg/kg p.o.) + Carrageenan

Group-VI: Butanolic fraction of *A. scholaris* (100 mg/kg p.o.) + Carrageenan

Group-VII: Aqueous fraction of *A. scholaris* (100 mg/kg p.o.) + Carrageenan

Inflammation was induced by 0.1 mL of 1% suspension of carrageenan into hind paw of rat by sub planter route. Treatments of all fractions were given 1 h prior to administration of carrageenan. Paw volume was measured with digital plethysmograph at 0, 1, 2, 3, 4 and 5th h after injection. The inhibitory activity was calculated using the following formula (Mali *et al*., 2013).

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

Where, Vc- Paw volume of control rat

Vt- Paw volume of treated rat

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Anti-arthritic activity of bioactive fraction

Animals were divided into nine groups and each group containing six animals. The animal groups are as follows.

Group-I: Normal control treated with (5 ml/kg p.o. normal saline) + mineral oil

Group-II: Arthritic control treated with (5 ml/kg p.o. normal saline) + FCA

Group-III: Standard control treated with (prednisolone 10 mg/kg) + FCA

Group-IV: Treated with chloroform fraction of *A. scholaris* (50 mg/kg) + FCA

Group-V: Treated with chloroform fraction of *A. scholaris* (100 mg/kg) + FCA

Arthritis was induced by single injection of 0.1mL of FCA in left hind paw intradermaly. Before immunization, paw volume of rats of all groups were measured. Severe arthritic symptoms i.e. inflammation, pain, deformity and erosion were developed in 12-14th days of immunization. The treatments were started from 14th day after adjuvant injection and for 35th day, once daily, by oral route and paw volume was measured on 0, 7, 14, 21, 28 and 35th day as described in 5.5.4 (Arulmozhi *et al*., 2011).

Arthritis assessment

The severity of the arthritis in each paw was quantified daily by a clinical score measurement from 0 to 4 as follows: 0 – no macroscopic signs of arthritis (swelling or erythema), 1 – swelling of one group of joints (namely, wrist or ankle joints), 2 – swelling of two groups of swollen joints, 3-swelling of three groups of swollen joints, 4 welling of the entire paw (Arulmozhi *et al*., 2011).

Estimation of various biochemical parameters.

Estimation of alkaline phosphatase (ALP)

0.5 ml of 0.4% p-nitro phenol phosphate substrate and 0.5 ml of glycine buffer (pH 10.5) were added in tubes marked as blank and test. The tubes were placed in water bath at 37^{0}C for 5 min. The reaction was initiated by the addition of 0.1 ml of liver homogenate and distilled water to the test and blank tubes, respectively and the time was noted (Walter & Schutt, 1974; Kandaswamy *et al*., 2007).

Estimation of acid phosphatase (ACP)

Into clear glass tubes marked blank and test, 0.5 ml of 0.4% p-nitro phenyl phosphate substrate, 0.5 ml of 1.0 M citrate buffer (pH 4.85) and 0.2 ml of 0.2M DL-tartarate were added. The tubes were incubated at 37^{0}C in a water bath for 5 min. The reaction was initiated by the addition of 0.1 ml of serum or liver homogenate and water to the test and blank tubes, respectively and the time was noted (Walter & Schutt, 1974; Kandaswamy *et al*., 2007).

Estimation of lactate dehydrogenase (LDH)

The incubation mixture contained 1.0 ml of buffered substrate and 0.2 ml of enzyme. 0.2 ml of NAD was added to test, mixed and incubated at 37^{0}C for 15 min. exactly after 15 min, 1.0 ml of DNPH was added to each (test and control) tubes and 0.2 ml of NAD was added to control (King, 1965b; Kandaswamy *et al*., 2007).

Estimation of Malonaldehyde (MDA)

The thiobarbituric acid-reactive substance was measured as a marker of lipid peroxidation in the articular cartilage. The homogenized tissue was added to 1.5 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetate buffer (pH

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3.5) and 1.5 ml of 0.8% TBA (thiobarbituric acid) solution. The mixture was heated at 95 ◦C for 1 h. After cooling, 5ml of n-butanol pyridine (14:1) was added for extraction and the absorbance of n-butanol–pyridine layer at 532 nm (Shimadzu UV Vis 1700) was measured for determination of TBA reactive substance (Kumar *et al*., 2009; Arulmozhi *et al*., 2011).

RESULTS

Preliminary Phytochemical Screening of Fractions

Fractionization was carried out by separating funnel and water was used to separate Petroleum ether, chloroform, butanolic and water soluble fractions. Preliminary phytochemical screening of various fractions from *A. scholaris* showed presence of various active phytoconstituents.

Table No 1: Qualitative chemical analysis of fractions by chemical tests

S. No.	Name of Plants	Phytoconstituents	Pet. ether fraction	Chloroform fraction	Butanolic fraction	Water fraction
1	*Alstonia scholaris*	Alkaloids	--	+	--	--
		Steroids	+	+	--	--
		Triterpenoids	+	+	--	--
		Fatty Acids	+	--	--	--
		Flavonoids	--	+	+	--
		Tannins	--	--	+	+
		Glycosides	--	--	+	+

Where, (+) - Present, (-) – Absent

Acute Toxicity Studies of Fractions

No toxic effects were observed at a higher dose of 500 mg/kg body weight of Wistar female rats. Hence, 1/ 10th and $1/5^{th}$ dose was selected as effective dose or therapeutic dose. The cut off value was 50 and 100 mg/kg were selected for *Alstonia scholaris* in anti-arthritic and anti-inflammatory activity.

Anti-Inflammatory Activity of Fractions in Rat Paws Edema

The chloroform fraction (100 mg/kg) significantly (p<0.001) inhibited carrageenan induced rat paw edema as compared to control group. Maximum inhibition of rat paw edema was observed with chloroform fraction (100 mg/kg) at the end of 5th h by 34.16 when compared to control group. Indomethacin (10 mg/kg) inhibited the paw edema by 45.12 %.

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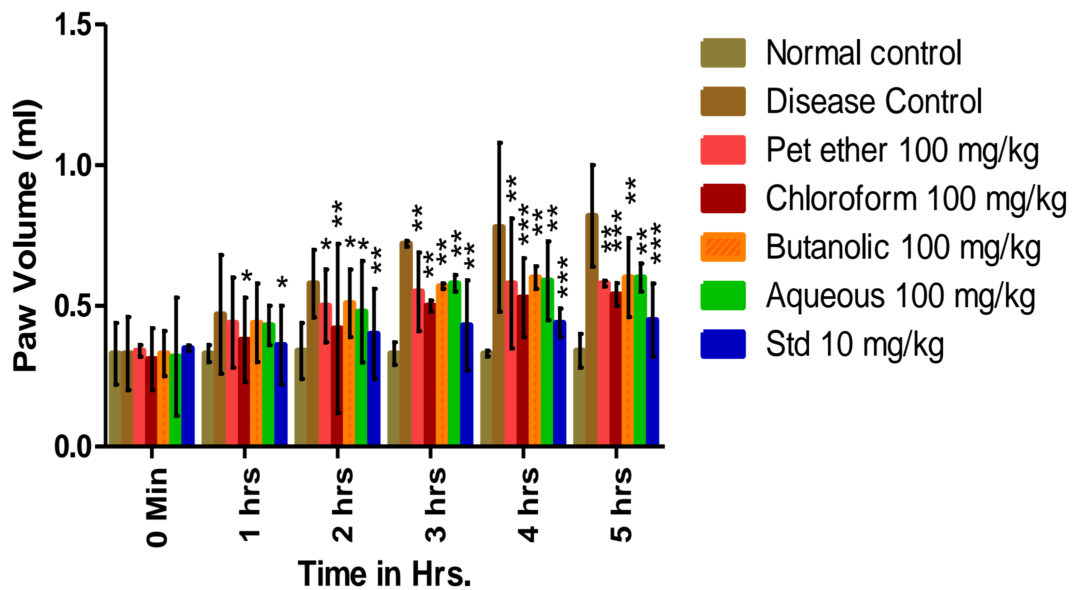

Figure No 1: Effects of different fractions on paw volume in rat

ANTI-ARTHRITIC ACTIVITY OF CHLOROFORM FRACTIONS

Freund's complete adjuvant induced rat paw edema

In adjuvant induced arthritic animals, a dose dependent reduction in paw swelling was exhibited in chloroform fraction of *Alstonia scholaris*. At the doses of 50 and 100 mg/kg of chloroform fraction, arthritic swelling was inhibited by 72.71 and 74.35% (p <0.001), respectively, compared to the adjuvant control on 35th day. Prednisolone treated group showed an inhibition of 78.63%.

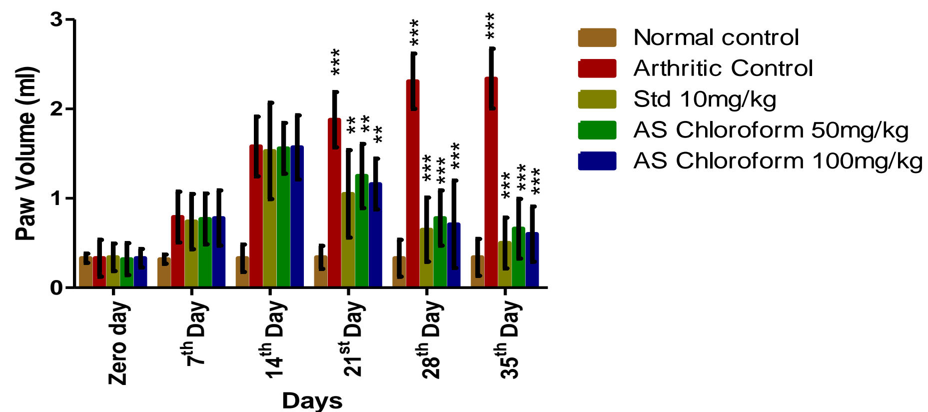

Figure No 2: Effect of chloroform fraction on paw volume in arthritic rat

Effects of chloroform fractions on arthritic assessment in arthritic rats

The treatment of chloroform fraction was initiated at the onset stage of Polyarthritic development i.e., day 14th. The articular index of the arthritic control group rapidly increased from day 14th which indicated that the Polyarthritic

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symptoms had certainly developed. During the initial phase of treatment, the articular indexes of the treated groups showed moderately significant (p<0.01) difference with those of arthritic control group.

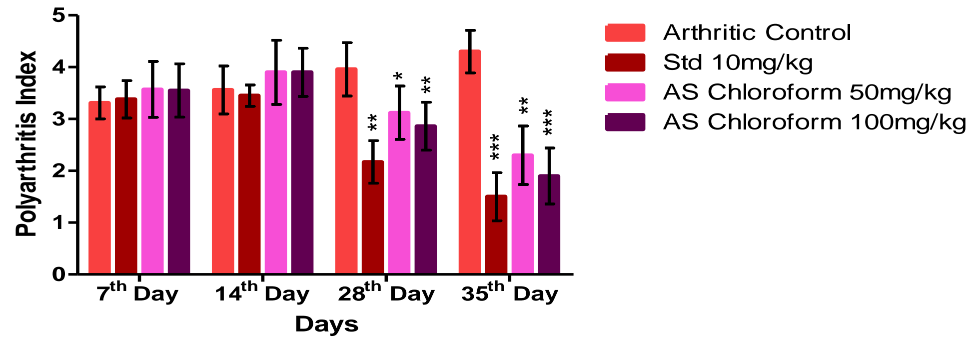
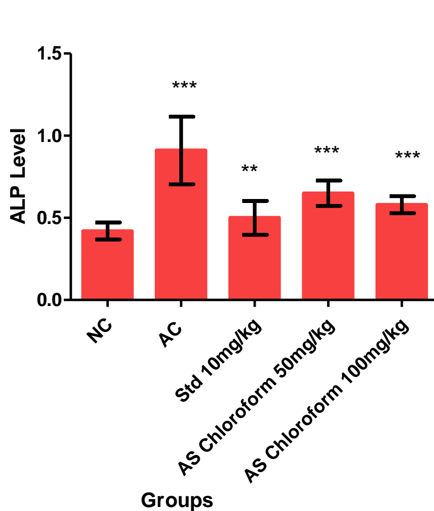


Figure No 3: Effect of chloroform fraction on arthritic assessment in arthritic rat

Membrane marker enzymes

A marked increase in the activity of membrane marker enzymes (ALP, LDH and ACP) was observed in the liver tissues of arthritic rats when compared to control rats. There is significant increase in lysosomal enzyme of arthritic rats. Treatment with chloroform fraction showed a highly significant (p<0.001) decrease in the activity of membrane marker enzymes was seen in treated animals at dose of 100 mg/kg.



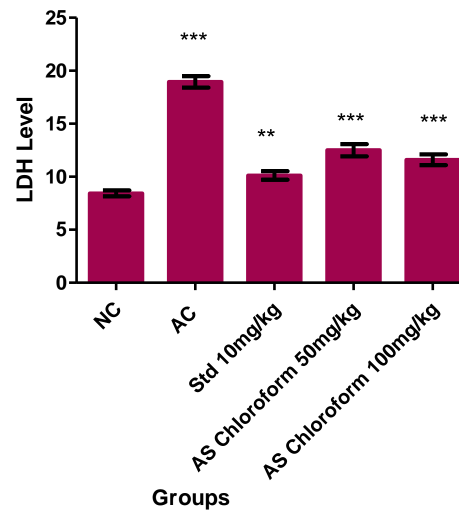


Figure No 4: Effect of chloroform fraction on LDH level

Figure No 5: Effect of chloroform fraction on ALP level

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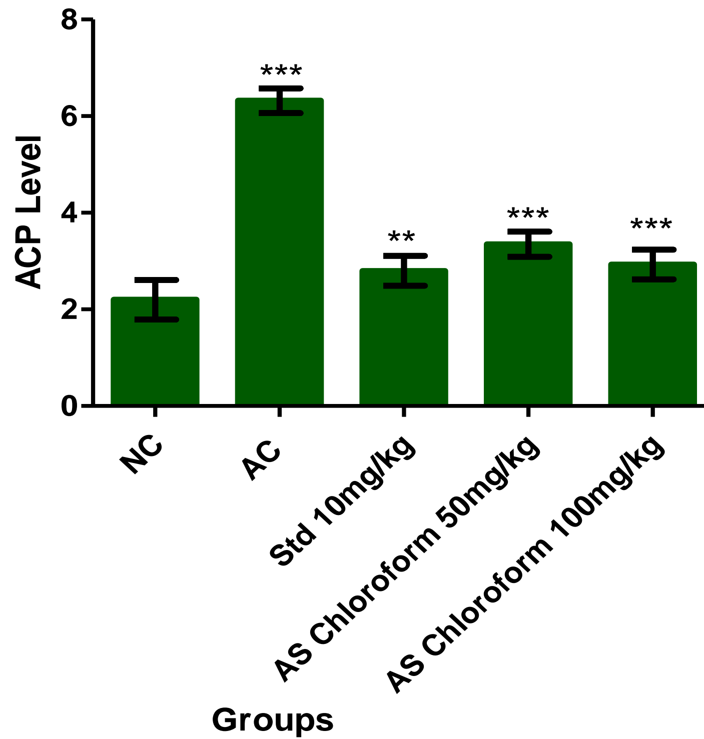

Figure No 6: Effect of chloroform fraction on ACP level

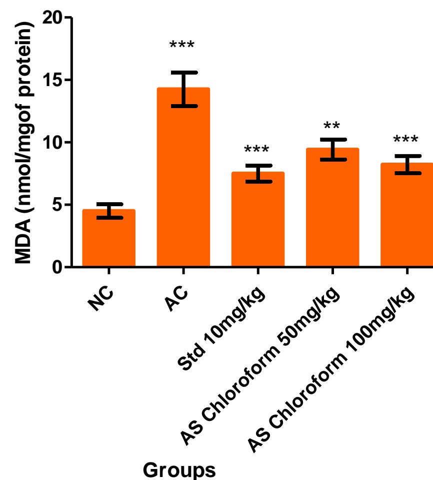

Figure No 7: Effect of chloroform fraction on MDA level

DISCUSSION

Fractionation of ethanolic extract of *Alstonia scholaris* were carried out by using various solvent such as petroleum ether, chloroform, n-butanol and aqueous on the basis of polarity. Phytochemical screening of all fractions was performed. Petroleum ether fraction showed the presence of fatty acids, terpenoids and steroids. In chloroform fraction, alkaloids and terpenoids were present. Butanol fraction showed the presence of flavonoids, irioid

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glycosides, tannins and phenolic compounds. Flavonoids, irioid glycosides, tannins and phenolic compounds were present in aqueous fraction. Availability of these phytocompounds in the various fractions may be responsible for anti-arthritic activity shown by these fractions. For the acute inflammatory condition, all the fractions were evaluated by carrageenan induced rat paw edema model. In case of chloroform fraction from *Alstonia scholaris* shown significant activity when compared with another fractions. Carrageenan is a family of linear sulphated polysaccharides extracted from the red seaweed marine alga *Chondrus crispus* and involved in the contribution of mediators involved in vascular changes associated with acute inflammation. Edema formation by the carrageenan involves three phases; first, second and third caused by the release of histamine and serotonin, bradykinin and prostaglandins respectively. The inflammatory edema reached its maximum level at the third hour and after that it started declining. In our study, fractions in all doses and Indomethacin showed significant anti-inflammatory effects in carrageenan induced rat paw edema. A previous study indicated that the 3rd hour of the edema induced by carrageenan, in which the edema effect reached its maximum (Kirkova *et al.*, 1992), is characterized by the presence of prostaglandins and other compounds of slow reaction (Spector & Willoughby, 1963).

However, in the carrageenan-induced rat paw edema model, the production of prostanoids has been through the serum expression of COX-2 by a positive feedback mechanism. In the present investigation, all fractions were evaluated by paw edema model and significantly showed anti-inflammatory activity form second hour and maximum activity at 5 h, suggesting that the anti-inflammatory activity may be due to inhibition of mediators of the inflammation such as histamine, serotonin released during the first phase of inflammation and prostaglandins and bradykinin which released during the second phase of inflammation. Maximum activity was shown by chloroform fraction of *Alstonia scholaris* at dose of 100 mg/kg as comparable to control. From the results of carrageenan induced rat paw edema model, it was concluded that chloroform fraction of *Alstonia scholaris* showed potent anti-inflammatory activity in acute model of inflammation. Hence, these bioactive fractions were evaluated by chronic inflammatory model i.e. FCA induced arthritis. Polyarthritic index was associated with an immune system mediated inflammatory reaction and after CFA treatment, experimental animal developed Polyarthritic index (Cai *et al.*, 2006). The Polyarthritic index was an initial reaction of edema and soft-tissue thickening at the site and the irritant effect of the adjuvant and the disease progression in the injected foot are presumed to be immunologic events (Ward & Cloud, 1965). The appearance of secondary lesions is a manifestation of cell-mediated immunity. In disease control group, arthritic Index was significantly higher compared to normal control group while prednisolone and fractions treated groups showed significantly less score as compared to model control group. This indicates the protective effect and immunosuppressant properties of test drugs against adjuvant-induced arthritis.

Acid phosphatase seems to be an important index for the examination of the integrity of the lysosomal membrane and is responsible for the tissue damage and necrosis of hepatic tissue (Yasuda *et al.*, 2000). One of the characteristic features of adjuvant induced arthritis in rats is the correlation between the development of inflammatory process and the release of lysosomal enzymes in to the extra-cellular compartment.

Lactate dehydrogenase (LDH) is cytoplasmic cellular enzymes and is present in the extra cellular space and serve as indicators suggestive of disturbances of the cellular integrity induced by pathological conditions. They don't play any other metabolic function in this space. Other cellular enzymes, such as alkaline phosphatase (ALP) are membrane bound indicator of type II cell secretary activity, an indicator of phagocytic activity, can also be used as sensitive markers of cellular integrity and cellular toxicity induced by pathological conditions. Increased activities of these enzymes were seen in liver (Olsen *et al*., 1990) of arthritic rats.

In our study, the activity of lysosomal enzymes were significantly increased in liver of arthritic rats and significantly reduced by treatments of bioactive fractions. An important mechanism of anti-arthritic activity is the membrane stability modulating effect. The treatment of chloroform fraction of ethanolic extract of leaf extract may exert its effects by modifying the lysosomal membrane in such a way that it is capable of fusing with the plasma membrane and thereby preventing the discharge of acid hydrolyses or by inhibiting the release of lysosomal enzymes (Chakraborthy, 2009).

CONCLUSION

By comparing the results, we concluded that chloroform fraction of *Alstonia scholaris* showed best activity in FCA induced arthritis and bioactive fractions showed strong presence of terpenoids so these may be strong candidate of anti-arthritic activity.

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