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Original Article

Chromatographic finger print analysis and lysosomal membrane stabilisation activity of active fraction of *Alstonia scholaris* leaf extract in arthritic rats

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Object: The present study was aimed to assess the anti-arthritic activity of chloroform fraction of *Alstonia scholaris* leaf extract against Freund's complete adjuvant (FCA)-induced arthritis in rats. **Materials and Methods:** The anti-inflammatory activity of various fractions of ethanolic extract of *Alstonia scholaris* at concentration of 100 mg/kg was studied using the carrageenan-induced inflammatory models. The chloroform fraction shows significant anti-inflammatory activity. The chloroform fraction was further studied for anti-arthritic activity and HPTLC fingerprint analysis. For anti-arthritic activity, the active chloroform fraction was administered at the concentrations of 50 and 100 mg/kg body weight. The effect of chloroform fraction on liver ALP, ACP and LDH levels of lysosomal enzymes of FCA arthritic animals were studied. Indomethacin and prednisolone (10 mg/kg) was used as standard. HPTLC studies were carried out using CAMAG HPTLC system equipped with linomat IV applicator, TLC scanner; Reprostar 3 and WIN CATS-4 software were used. **Results:** The chloroform fraction at 100 mg/kg, showed maximum inhibition (34.16%) of inflammation induced by carrageenan. In FCA-induced arthritis, the chloroform fraction showed a highly significant reduction in paw volume (50 mg/kg-72.71%; 100 mg/kg-74.35%). The levels of lysosomal enzymes were significantly decreased in the chloroform fraction-treated groups. **Conclusion:** The possible mechanism of action of the chloroform fraction of *Alstonia scholaris* leaf extract may be through its stabilising action on lysosomal membranes. Future studies will provide new insights into the anti-arthritic activity of *Alstonia scholaris* and isolation of compound from it may eventually lead to development of a new class of anti-arthritic agent.

Key words: *Alstonia scholaris*, athritis, chloroform fraction, lysosomal membrane stabilisation

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, destructive disorder of the joints.[1] Despite extensive research the pathogenesis of RA has remained elusive. It is well-accepted that the local production of pro-inflammatory cytokines in inflamed synovial tissue, TNF-α and IL-1b in particular contribute to the pathogenesis of RA.[2-4] However, the reasons for the overproduction of cytokines and their cellular origin are less clear.

Alstonia scholaris Linn. R. Br. a tree belonging to the family Apocynaceae, has been used since time immemorial in the folklore and traditional systems of medicine in India, to treat several diseases. It grows wild throughout in deciduous, evergreen forests and even in plains. Folklore use include application of milky juice of leaves on wounds, ulcers, and for rheumatic pain, as well mixed with oil and applied for ear ache.[5,6] Extracts of *A. scholaris* is reported to possess several pharmacological activities that include antiplasmodial activity,[7] antimutagenic effect,[8] immunostimulatory effect,[9] hepatoprotective activity,[10] antidiabetic and antihyperlipidemic activities.[11]

The plant is reported to relieve inflammation and chronic rheumatic pains in folklore medicine.[5] In our previous study ethanolic extracts of *A. scholaris* leaves showed significant anti-inflammatory activity (data not shown here). Based on the above perspective, the present study is designed to investigate anti-flammatory activity of various non-polar and polar fractions and to develop HPTLC finger print profile of active chloroform fraction and to use of effective fraction for its anti-arthritic activity in Freund's complete adjuvant (FCA)-induced arthritic animals.

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MATERIALS AND METHODS

Collection and Authentification of Plant

The leaves of *A. scholaris* (Apocynaceae) were collected in the months of July from the campus of Mandsaur Institute of Pharmacy, Mandsaur, India. The plant material was taxonomically identified by Dr. Gynendra Tiwari, Scientist, KNK College of Horticulture, Mandsaur, India and the voucher specimen is submitted in Department of Pharmacognosy for future reference.

Chemicals and Reagents

Freund's Complete Adjuvant, carrageenan (Sigma Aldrich, USA) and Prednisolone (Wheth Pvt. Ltd., India) were used. Other chemicals and reagents used for the study were of analytical grade and procured from approved organisations.

Fractionisation of Ethanolic Extract of *A. Scholaris* Leaves

The dried ethanolic extract (30 g) was suspended in water and filtered to remove the insoluble material. The filtrate was taken in separating funnel and fractionated by using petroleum ether, chloroform, butanol and water. All the fractions were dried under vacuum. Preliminary phytochemical studies of pet ether showed presence of steroids and fatty acids, chloroform showed terpanoids and steroids, butanol showed presence of flavonoids, tannins and aqueous fraction showed flavonoids and glycosides.

Animals

Wistar albino rats (150 – 200 g.) of either sex were used for the study. The animals were maintained under environmental condition and fed with standard pellet diet and water *ad libitum*. The study protocol was approved by Institutional Animal ethical Committee (IAEC). CPCSEA guidelines were adhered to during maintenance and experiment.

Acute Toxicity Studies

Acute toxicity studies were carried out for pet ether, chloroform, butanolic and aqueous fractions of ethanolic extract of *A. scholaris* leaves according to OECD guidelines 423.[12] The pet ether, chloroform, butanolic and aqueous fractions were administered orally in dose of 500 mg/kg body weight. The animals (n = 3) were observed 24 hrs for the signs of toxicity. The attention was directed on convulsion, diarrhoea, coma, respiratory depression, salivation and perspiration.

Carrageenan-Induced Rat Paw Oedema

Six groups of six rats were included in this experimental model of acute inflammation. Oedema was induced by injection of 1% suspension of carrageenan in 0.9% sterile saline solution into the right plantar region of the rat. The pet ether, chloroform, butanol and aqueous fraction of ethanolic extracts of the *A. scholaris* (50 and 100 mg/kg), prednisolone (10 mg/kg body weight), or vehicle were administered orally 1 h before injection of carrageenan. Paw volume was measured with digital plethysmograph at 0, 1, 2, 3, 4 and 5th hour after injection.[13] The inhibitory activity was calculated using the following formula.

$$\text{Percentage inhibition} = \frac{\text{Control (Co)} - \text{Treated (Ct)}}{\text{Control}} \times 100$$

where Ct = paw volume after carrageenan injection and Co = paw volume before carrageenan injection.

HPTLC Profile of Active Chloroform Fraction

Chromatographic Method

HPTLC studies were carried out following the method of Harborne[14] and Wagner *et al*.[15] The protocol for preparing sample solutions was optimised for high-quality fingerprinting. The fingerprinting of active chloroform fraction was executed by spotting 4 µL of suitably diluted sample solution on a HPTLC plate. The plates were developed and scanned. The peak areas were recorded. Chromatography was performed on 20 × 10 cm precoated HPTLC silica gel 60 F_{254} plates. The sample of active fraction was applied as bands 6-mm wide and 8-mm apart by means of CAMAG (Muttenz, Switzerland) Linomat IV sample applicator equipped with a 25-µL syringe.

Solvent System Development

A number of solvent system were tried, but the best resolution was obtained with the solvents, Pet ether: ethyl acetate (6.8:3.2).

Detection of Spots

The developed plate was dried at 100°C in hot air oven for 3 min to evaporate solvents from the plate. The plate was kept in photo-documentation chamber (CAMAG reprostar 3) and images captured under UV light at 254 and 364 nm, respectively. The R_f values and finger print data were recorded by WIN CATS software.

Fruend's Complete Adjuvant-induced Arthritis

Chronic arthritis was induced by the injection of 0.1 mL of FCA containing 10 mg of heat killed *Mycobacterium tuberculosis*, in 1 mL of paraffin oil into the right hind paw of the rat intradermally. The animals were divided into five groups and each group containing six animals. Group I served as normal control; Group II served as arthritic control; Group III was treated with prednisolone 10 mg/kg, standard anti-arthritic drug; Group IV and V were treated with *A. scholaris* chloroform fraction in dose of 50 and 100 mg/kg. Treatment was given orally daily after 14 days from the day of adjuvant injection for 35 days. The volume of the paw was measured before induction, before treatment and after treatment; the percentage inhibition was

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determined. Animals were then euthanised on the 36th day by cervical decapitation; blood was collected in plain and ethylene diamine tera-acetic acid containing tubes for plasma/serum separation.[16]

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 - swelling of the entire paw.[17]

Biochemical Estimation

At the end of the experimental period, rats were fasted overnight and the anaesthetised rats were sacrificed by cervical decapitation. Liver homogenates were centrifuged at 600 g for 10 min. The sediment which containing nuclei, unbroken cells and plasma membranes (nuclear fraction) were separated and the supernatant was subjected to centrifugation at 16,000 g for 30 min. Enzyme activity in the supernatant was determined. The marker enzymes alkaline phosphatase (ALP),[18] acid phosphatase (ACP)[18] lactate dehydrogenase (LDH)[19] were estimated by liver.

Statistical Analysis

All the results were expressed as mean ± SEM of six animals. Analysis of variance was performed ANOVA followed by Students "*t*" test comparison test. $P < 0.05$ was considered as significant.

RESULTS

Preliminary Phytochemical Screening

The preliminary phytochemical screening of the fractions strongly indicated the possible presence of fatty acids, steroids, triterpanoids, flavonoids, polyphenolics, glycosides and saponins with the absence of tannins and anthraquinones.

Acute Toxicity Studies

As suggested by OECD guidelines, the tested animals were observed individually for 24 hr after single dosing. The animals did not exhibit any symptoms and survived beyond the recommended duration of observation with dose of 500 mg/kg of pet ether, chloroform, butanolic and aqueous fractions. Therefore, 100 mg/kg dose was selected for the anti-inflammatory and 50 and 100 mg/kg for anti-arthritic activity.

Carrageenan-induced Rat Paw Oedema

The anti-inflammatory effect of *A. scholaris* leaves fractions was evaluated using carrageenan-induced paw oedema in rats. One hour after, the administration of fractions, they were inflamed with carrageenan, and the oedema volume was increased, showing its maximum value at 4 h. In our study, the chloroform fractions of *A. scholaris* inhibited the oedema formation at 100 mg/kg concentration at the third hour by 30.55 ($P < 0.01$), respectively. This effect also extended and significantly increased up to the fifth hour by 34.16 ($P < 0.001$). The reference drug significantly inhibited oedema formation by 40.27% and 45.12% at the third and the fifth hour, respectively, ($P < 0.001$) [Table 1].

HPTLC of Active Chloroform Fraction of *A. scholaris* Ethanolic Extract

Table 2 shows HPTLC fingerprint of active chloroform fraction which revealed several peaks. HPTLC profile of fraction under UV was recorded. The corresponding HPTLC chromatograms are represented in Figures 1 and 2. The active chloroform fraction revealed four major spots, respectively, with R_f values in range of 0.02 to 0.79 for 4-µL application volume, respectively. The purity of sample was confirmed by comparing the absorption spectra at the start, middle and end position of the bands.

Effect of Chloroform Fraction on Arthritis Assessment

It was observed that arthritis had been successfully created in all the rats by the administration of CFA. In this study, the arthritic rat joints exhibited the typical swelling patterns of

Table 1: Effect of various fractions of *Alstonia scholaris* ethanolic leaf extracts on carrageenan-induced paw oedema

Groups	0 Min.	1 hr	2 hr	3 hr	4 hr	5 hr
Normal control	0.32±0.11	0.33±0.03	0.34±0.10	0.33±0.04	0.33±0.01	0.34±0.06
Disease control	0.33±0.13	0.47±0.21	0.58±0.12	0.72±0.01	0.78±0.30	0.82±0.18
Pet ether fraction (100 mg/kg)	0.34±0.02	0.44±0.16 (6.83)	0.50±0.13* (13.79)	0.55±0.14** (23.61)	0.58±0.23** (25.64)	0.58±0.01** (29.26)
Chloroform fraction (100 mg/kg)	0.31±0.11	0.38±0.15* (19.14)	0.42±0.30** (27.58)	0.50±0.02** (30.55)	0.53±0.14*** (32.05)	0.54±0.04*** (34.16)
Butanol fraction (100 mg/kg)	0.33±0.08	0.44±0.14 (6.83)	0.51±0.12* (12.06)	0.57±0.01** (20.83)	0.60±0.04** (23.07)	0.60±0.14** (26.82)
Aqueous fraction (100 mg/kg)	0.32±0.21	0.43±0.07 (8.51)	0.48±0.18* (17.24)	0.58±0.03** (19.44)	0.59±0.14** (24.35)	0.60±0.05** (26.82)
Indomethacin (10 mg/kg)	0.35±0.01	0.36±0.14* (23.40)	0.40±0.16**	0.43±0.09** (40.27)	0.44±0.05*** (43.58)	0.45±0.13*** (45.12)

* – $P<0.05$; ** – $P<0.01$; *** – $P<0.001$

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rat polyarthritis. The treatment of chloroform fraction was initiated at the onset stage of polyarthritis development i.e., day 14. The articular index of the arthritic control group rapidly increased from day 14 which indicated that the polyarthritic 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. However, after this phase, the indexes started to significant decrease ($P < 0.001$) and finally reached 56% of those of the arthritic control group [Figure 3].

Table 2: HPTLC profile of chloroform fraction of *Alstonia scholaris*

Volume (μL)	Peak	R_f values	Height area	% area
4	1	0.02	179.1	1.51
4	2	0.63	2393.3	20.15
4	3	0.73	2445.5	20.59
4	4	0.79	6860.6	57.76

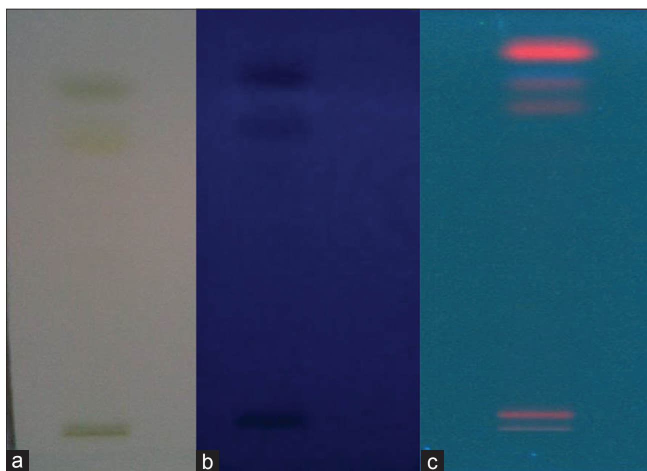

Figure 1: HPTLC profile of chloroform fraction of Alstonia scholaris ethanolic extract of leaf. (a) Visible light; (b) UV (254 nm); (c) UV (366 nm)

Effect of Chloroform Fraction on FCA-induced Arthritis

In adjuvant-induced arthritic animals, a dose-dependent reduction in paw swelling was exhibited in chloroform fraction of *Alstonia scholaris*. Swelling and redness developed over 24 h period in the foot injected with adjuvant in the control animals and prolonged, whereas in the chloroform fraction-treated animals the swelling and redness were suppressed. 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 4].

Effect of Chloroform Fraction on Lysosomal 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 chloroform fraction-treated animals at 100 mg/kg [Table 3].

DISCUSSION

The FCA-administered rats showed soft tissue swelling around the ankle joints during the development of arthritis, which was considered as oedema of the particular tissues.

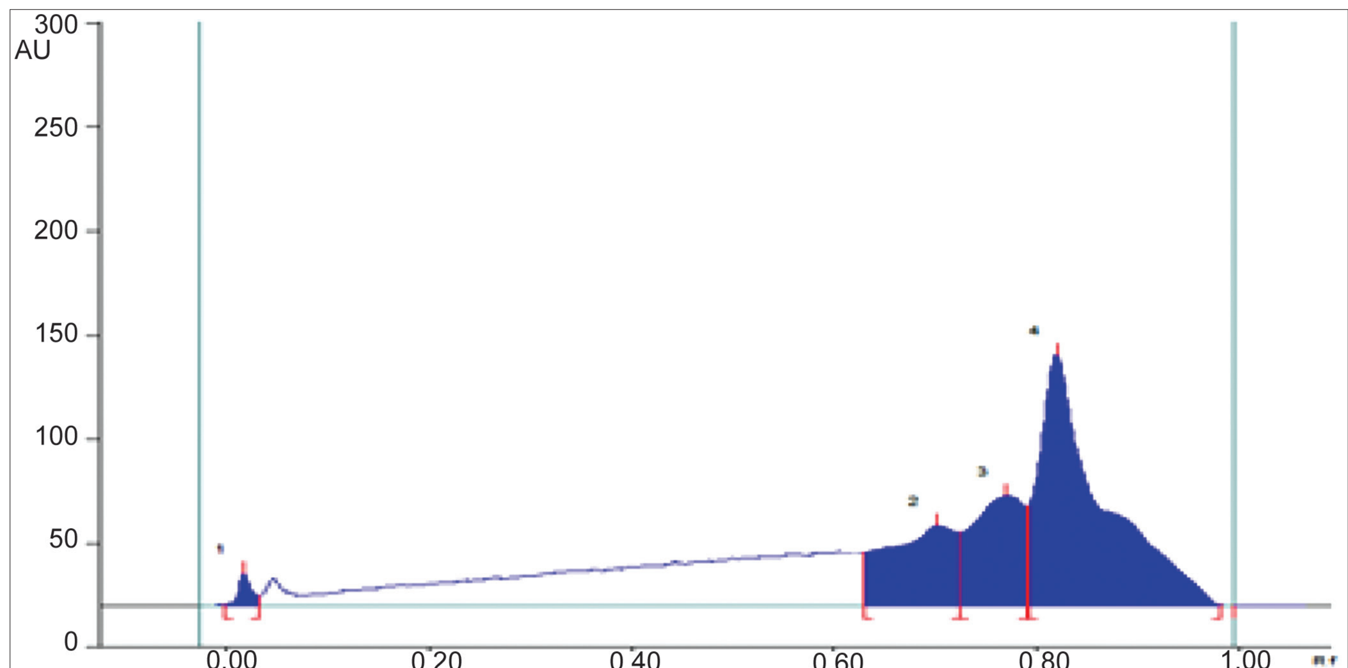

Figure 2: HPTLC chromatogram of chloroform fraction of *Alstonia scholaris* (4 μL) showing different peaks of phytoconstituents

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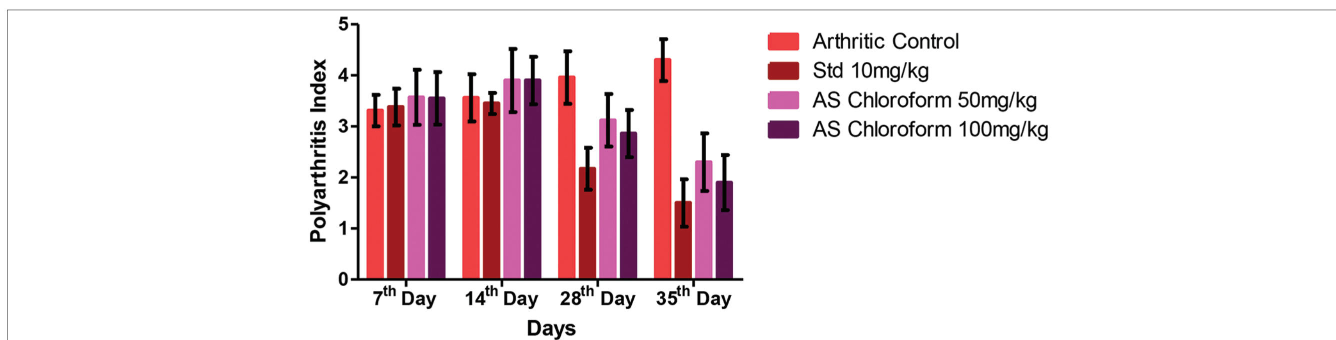

Figure 3: Effect of chloroform fraction of Alstonia scholaris ethanolic extract on polyarthritic index of adjuvant induced arthritic rats

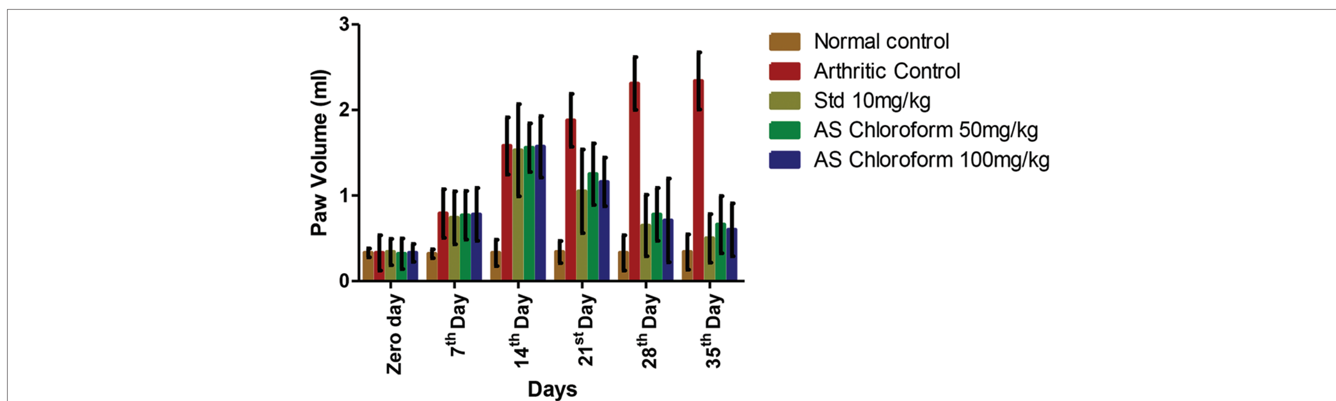

Figure 4: Effect of chloroform fraction of Alstonia scholaris ethanolic extract on paw volume of adjuvant induced arthritic rats

Table 3: Effect of chloroform fractions of *Alstonia scholaris* ethanolic leaf extracts on membrane marker enzymes of control and experimental animals in liver

Groups	Alkaline phosphatase (μmoles of phenol formed/h/ mg protein)	Lactate dehydrogenase (μmoles of pyruvate liberated/min/ mg protein)	Acid phosphates ($\times 10^{-2}$ μmol of phenol formed/min/ mg protein)
Normal control	0.42±0.02	8.43±0.11	2.20±0.16
Arthritis control	0.91±0.08***	18.95±0.21***	6.32±0.10***
Chloroform fraction (50 mg/kg)	0.65±0.03**	12.50±0.22**	3.35±0.10**
Chloroform fraction (100 mg/kg)	0.58±0.02***	11.60±0.20***	2.93±0.12***
Prednisolone (10 mg/kg)	0.50±0.04***	10.12±0.16***	2.80±0.12***

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

As the disease progressed, a more diffused demineralisation developed in the extremities.[20] Many medicinal plants provide relief comparable to that of conventional medicinal agents in the treatment of diseases. *A. scholaris* is one of the well-known medicinal plants reported to relieve rheumatic pains in folklore medicine[5] with an additional antioxidant property.

In the present study, dried ethanolic extract of *A. scholaris* leaves were sequentially fractionated into petroleum ether, chloroform and butanol solvents. All the fractions were evaluated for acute anti-inflammatory activity by carrageenan-induced inflammatory models. Chloroform fraction of ethanolic extract showed significant anti-inflammatory activity when compared to other fractions. The chloroform fraction was further processed for its anti-arthritic activity and HPTLC fingerprinting. HPTLC profile of chloroform fraction revealed the presence of 4 spots for 4 μL sample application, respectively, in range of R_f 0.02 to 0.79.

The carrageenan-induced inflammatory process involves three phases: first, second and third phases caused by the release of histamine and serotonin, bradykinin and prostaglandins respectively.[21] Both histamine and serotonin are characterised by the increase of vascular permeability. Prostaglandins mediate maximum vascular responses during the third phase of inflammation.

In the present study, the anti-inflammatory activity of the four fractions of ethanolic extract of *A. scholaris* was assessed with the use of carrageenan-induced paw oedema. The chloroform fraction was able to significantly reduce the paw oedema at 3rd and 5th hour of the experiment. This may be due to the inhibition of cyclo-oxygenase enzymes that are involved in the formation of prostaglandins.[22]

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On the other hand, for a chronic anti-inflammatory effect of chloroform fraction, CFA-stimulated induced chronic arthritis was accessed in rats. FA is an antigen solution emulsified in mineral oil that is used to potentiate the immune response. The complete form (CFA) is composed of inactivated and dried mycobacterium and is effective in stimulating cell-mediated immunity and may lead to the potentiation of the production of certain immunoglobulins. Injections of CFA in the hind paw-induced primary and secondary chronic arthritis.[23] In our study, chloroform fraction exhibited the anti-arthritic effect as evidenced by the decrease in polyarthritic index and decrease in oedema formation when compared with arthritic control animals. In arthritic animals there id over production of TNF-α and it is closely related to the increase in signs of arthritic syndrome.[24] The administration of chloroform fraction of *A. scholaris* causes decreased polyarthritic index and oedema formation might possibly be linked to its modulatory role on TNF-α. The ability of the fraction to reduce oedema formation may be related to its inhibitory action on prostaglandin synthesis.

Lysosomes are membrane enclosed cytoplasmic organelles, which possess an acidic interior that contain many hydrolytic enzymes. The altered enzyme activities in arthritis can be regarded as an index of lysosomal enzyme activation occurring in response to metabolic need of degrading various constituents of cells such as mucopolysaccharides and glycoproteins accumulated in tissues due to arthritis associated with vasculopathies.[25]

Acid phosphates (ACP) found to be an important index for the examination of the integrity of the lysosomal membrane and are responsible for the tissue damage and necrosis of hepatic tissue.[26] Cytoplasmic cellular enzymes, like lactate dehydrogenase (LDH) in the extracellular space, although of no other metabolic function in this space, are of benefit because they serve as indicators suggestive of disturbances of the cellular integrity induced by pathological conditions. Other cellular enzymes, such as alkaline phosphates (ALP) is membrane bound indicator of type II cell secretary activity or 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 liver lactate dehydrogenase, alkaline phosphates and acid phosphates[27] were observed in arthritic rats. This may be attributed towards persistent inflammation. These changes are in agreement with the decreased lysosomal stability in adjuvant-induced arthritis.

In the present study, the activity of lysosomal enzymes in liver was markedly increased in the adjuvant induced arthritic rats and significantly reduced after treatment with chloroform fraction of *A. scholaris*. An important mechanism of anti-inflammatory activity is the membrane stability modulating effect.[28] 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 hydrolases or by inhibiting the release of lysosomal enzymes.[29]

In accordance with previous studies, phytoconstituents like steroids, flavonoids, alkaloids, terpenoids and tannins have been shown to possess anti-inflammatory, analgesic and membrane-stabilising activity.[30] The preliminary phytochemical analysis of chloroform fraction confirmed the presence of steroids, triterpanoids and flavonoids. Hence, anti-arthritic potential of chloroform fraction may be due to either steroids or triterpanoids.

CONCLUSIONS

The development of inflammation during adjuvant induced arthritis is due to the biochemical changes of lysosomal enzymes. The chloroform fraction of ethanolic extract of *A. scholaris* might exert anti-inflammatory activity by modifying the lysosomal membrane in such a way that it is capable of fusing with the plasma membrane and thereby, preventing the release of lysosomal enzymes, and could retard complications and spread of the inflammatory process. The presence of biologically active ingredients in the chloroform fraction is responsible for the observed anti-arthritic and membrane stability-modulating effect. Further work is in progress to isolate the active anti-arthritic compound(s) for detailed studies.

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