ORIGINAL ARTICLE

Systemic administration of fractions from *Nyctanthes arbor-tristis* attenuates chronic inflammatory response in Freund's-complete-adjuvant-induced arthritis in rats

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Object: The present study was aimed to assess the anti-arthritic activity of chloroform and n-butanolic fraction of *Nyctanthes arbor-tristis* leaf extract against Freund's-complete-adjuvant (FCA)-induced arthritis in rats. **Material and Method:** Ethanolic extract of *Nyctanthes arbor-tristis* leaves were fractionated in various organic solvents and on the basis of results of anti-inflammatory activity, chloroform and n-butanolic fractions were selected for anti-arthritic activity. The active chloroform and n-butanolic fraction were administered at the dose of 50 and 75 mg/kg body weight. The effects of both fractions on liver alkaline phosphatase (ALP), acid phosphatase (ACP) and lactate dehydrogenase (LDH) levels and malonaldehyde (MDA), glutathione (GSH) and superoxide dismutase (SOD) from articular cartilages in arthritic animals were studied. Prednisolone (10 mg/kg) was used as standard. **Results:** In FCA-induced arthritis, the chloroform and n-butanolic fraction showed a highly significant reduction in paw volume (75 mg/kg–76.49%; 70.94%). The levels of various membrane marker enzymes and oxidative free radicals were significantly decreased in the both fraction treated groups and GSH and SOD activities were significantly increased compared with the arthritic control. The chloroform fraction showed most prominent activity as compared to n-butanolic fraction. **Conclusion:** The possible mechanism of action of the chloroform fraction of *Nyctanthes arbor-tristis* leaf extract may be due to either stabilizing action of membrane marker enzymes or inhibition of oxidative free radicals and thereby preventing the spread of inflammation. Future studies will provide new insights into the anti-arthritic activity of *Nyctanthes arbor-tristis* and isolation of compound and its possible mechanism of action.

Key words: Arthritis, chloroform and n-butanolic fraction, *Nyctanthes arbor-tristis*, membrane marker enzymes, oxidative free radicals

INTRODUCTION

An inflammatory reaction implicates macrophages and neutrophils, which secrete a number of mediators (eicosinoids, oxidants, cytokine and lytic enzymes) responsible for the initiation, progression and persistence of the acute or chronic state of inflammation.[1]

Non-steroidal anti-inflammatory drugs (NSAIDs) reduce the pain and inflammation by blocking the metabolism of arachidonic acid by isoforms cyclo-oxygenase enzyme (COX-1 and/or COX-2), and thereby reduce the production of prostaglandin. However, due to the high gastric lesion risks of NSAIDs,[2] there is much hope for finding anti-inflammatory drugs from traditional medicinal plants without side-effects. These drugs are potent in action but show wide range of adverse effects, whereas herbal drugs bear comparatively less side effects. Herbal drugs can therefore be considered as a better alternative to synthetic anti-inflammatory drugs.[3]

Nyctanthes arbor-tristis L. (Oleaceae) belongs to such a group of medicinal plant and are shrubs or small trees with soft white hairs, young branches sharply quadrangular. *Nyctanthes arbor-tristis* L. is said to have a wide range of medicinal benefits to mankind. The leaves of the plant have been used by ayurvedic physicians for arthritis and obstinate sciatica.[4] Various parts of *Nyctanthes arbor-tristis* reported to have immunostimulant, hepatoprotective, anti-lieshmanial, antiviral and antifungal activities.[5] The leaves have been used in ayurvedic medicine to treat sciatica, arthritis, fevers and as laxative.[6] Flower of *Nyctanthes arbor-tristis* is shown to have antibacterial activity against many gram-positive and gram-negative micro-organisms.[7] Three iridoid glucosides (arbortristosides A, B, C) and 6β-hydroxyloganin have been isolated from the plant and tested as antilieshmanial agents.[8]

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In our previous study, the chloroform and butanolic fractions from ethanolic extract of plant leaves has illustrated potent anti-inflammatory activity in carrageenan-induced rat paw edema method in rats.[9] But there is no scientific evidence of these fractions in chronic inflammatory condition i.e. arthritis. Based on the above perspectives, the present study was designed to investigate the most promising fraction of *Nyctanthes arbor-tristis* responsible for anti-arthritic activity in chronic inflammatory and immunological reactions. Our findings may also provide scientific evidence to support the folk medicinal utilization of *Nyctanthes arbor-tristis* for the treatment of arthritis.

MATERIAL AND METHODS

Collection and Authentification of Plant

The fresh leaves of *Nyctanthes arbor-tristis* (Oleaceae) were collected in the months of September from the campus of College of Krishi Vigyan Kendra (KVK), College of Horticulture, Mandsaur, India. The plant material was taxonomically identified by Dr. Gyanendra Tiwari, Scientist, KNK College of Horticulture, Mandsaur, India and the voucher specimen is submitted in Department of Pharmacognosy, Mandsaur Institute of Pharmacy, Mandsaur for future reference.

Fractionization of Ethanolic Extract of *Nyctanthes arbor-tristis* Leaves

The dried ethanolic extract (50 g) was suspended in water and filtered to remove the insoluble material. The water fraction was taken in separating funnel and fractionated by various organic solvents to get petroleum ether, chloroform, butanol and water soluble layer. Each fraction was dried under vacuum to obtain petroleum ether (0.9 g), chloroform (4.5 g), n-butanolic (5.2 g) and water fractions (1.8 g). Preliminary phytochemical studies of chloroform and n-butanolic fractions were performed for the presence of steroids, fatty acids, terpanoids, flavonoids, tannins and glycosides.[10]

Animals

Wistar albino rats (200-250 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). Committee for the purpose of control and supervision on experimental animals (CPCSEA) guidelines were adhered to during maintenance and experiment.

Acute Toxicity Studies

Acute toxicity studies were carried out for chloroform and butanolic fractions of ethanolic extract of *Nyctanthes arbor-tristis* leaves according to Organization for Economic Co-operation and Development (OECD) guidelines 423.[11] The chloroform, butanolic fractions were administered orally in dose of 500 mg/kg body weight. The animals ($n = 3$) were observed 24 hours for the signs of toxicity. The attention was directed on convulsion, diarrhoea, coma, respiratory depression, salivation and perspiration.

Freund's Complete Adjuvant induced Arthritis

Chronic inflammatory reaction was induced by the injection of 0.1 mL of FCA (Sigma Aldrich, USA) 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 seven 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 (Wyeth Pvt. Ltd., India) 10 mg/kg, standard anti-arthritic drug; Group IV and V were treated with chloroform fraction in dose of 50 and 75 mg/kg; Group VI and VII were treated with n-butanolic fraction in dose of 50 and 75 mg/kg of ethanolic extract of *Nyctanthes arbor-tristis* leaves. 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 determined.[12]

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.[13]

Biochemical Estimation

At the end of the experimental period, rats were fasted overnight and the anaesthetized 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),[14] acid phosphatase (ACP),[14] lactate dehydrogenase (LDH)[15] were estimated by liver. Malonaldehyde (MDA)[16,17], glutathione (GSH)[13,16] and superoxide dismutase (SOD)[13,16] were estimated by articular cartilages.

Data Analysis

All values are presented as means ± SEM. Differences between the drug-treated groups and the control group were evaluated by independent unpaired sample t-tests using the prism software 5.0 version. $P < 0.05$ was considered significant.

RESULTS

Preliminary Phytochemical Screening

The preliminary phytochemical screening of the chloroform fraction strongly indicated the presence of steroids, triterpanoids and n-butanolic fraction showed presence of flavonoids, polyphenolics and glycosides.

Acute Toxicity Studies

As suggested by OECD guidelines, the tested animals were observed individually for 24 hour 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 chloroform and n-butanolic fractions. Therefore, 50 and 75 mg/kg for anti-arthritic activity.

Freund's Complete Adjuvant -induced Arthritis

In adjuvant induced arthritic animals, a dose-dependent reduction in paw swelling was exhibited in chloroform and n-butanolic treated fraction of *Nyctanthes arbor-tristis*. As shown in the Table 1, at the doses of 75 mg/kg of chloroform fraction and n-butanolic fraction, arthritic swelling was inhibited by 76.49 and 70.94% ($P < 0.001$, 0.01), respectively, compared to the adjuvant control on 35th day. Prednisolone treated group showed an inhibition of 78.63%.

Arthritis Assessment

The treatment of chloroform and n-butanolic fraction was initiated at the onset stage of polyarthritis development i.e., day 14. 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 highly significant decrease ($P < 0.001$) in chloroform treated rats and moderately significant decrease ($P < 0.01$) in n-butanolic fraction-treated rats [Table 2].

Oxidative Stress Parameters

As shown in Table 3, MDA levels were observed to increase in Group II when compared with Group I. However, GSH levels and SOD activities were observed to decrease in Group II when compared with Group I. Administration of chloroform fraction at dose of 75 mg/kg causes highly significant decrease ($P < 0.001$) in MDA levels and increase in GSH and SOD activities however, n-butanolic fraction showed moderate decrease ($P < 0.01$) in MDA level and increase in GSH and SOD activities.

Membrane Marker Enzymes

A liver tissue was used to access the marked increase in the activity of membrane marker enzymes (ALP, LDH and ACP) in the arthritic rats when compared to control rats.

Table 1: Effect of chloroform and n-butanolic fractions of ethanolic extract of *Nyctanthes arbor-tristis* leaves on paw volume

Groups and treatments	Paw volume in (ml)					
	Zero Day	7th Day	14th Day	21st Day	28th Day	35th Day
Normal control	0.33±0.02	0.32±0.02	0.33±0.06	0.34±0.05	0.33±0.08	0.34±0.08
Arthritic control	0.33±0.08	0.79±0.11	1.58±0.13	1.88±0.12***	2.31±0.12***	2.34±0.13***
Prednisolone 10 mg/kg	0.34±0.06	0.74±0.12	1.53±0.21	1.05±0.19** (44.14)	0.65±0.14*** (71.86)	0.50±0.11*** (78.63)
Nyctanthes arbor-tristis, chloroform fraction 50 mg/kg	0.32±0.10	0.76±0.10	1.55±0.10	1.20±0.12* (36.17)	0.72±0.12** (68.83)	0.63±0.10** (73.07)
Nyctanthes arbor-tristis, chloroform fraction 75 mg/kg	0.33±0.11	0.78±0.10	1.56±0.14	1.10±0.12** (41.48)	0.68±0.13*** (70.56)	0.55±0.10*** (76.49)
Nyctanthes arbor-tristis, n-butanolic fraction 50 mg/kg	0.32±0.09	0.77±0.11	1.58±0.14	1.30±0.16* (30.85)	0.80±0.12* (65.36)	0.74±0.12** (68.37)
Nyctanthes arbor-tristis, n-butanolic fraction 75 mg/kg	0.33±0.04	0.78±0.14	1.57±0.15	1.25±0.18* (33.51)	0.74±0.12** (67.96)	0.68±0.10*** (70.94)

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

Table 2: Effect of chloroform and n-butanolic fractions of ethanolic extract of *Nyctanthes arbor-tristis* leaves on polyarthritic index

Groups and treatments	Polyarthritic index			
	7th Day	14th Day	28th Day	35th Day
Arthritic control	3.31±0.12	3.56±0.18	3.96±0.20	4.30±0.16
Prednisolone 10 mg/kg	3.38±0.14	3.45±0.08	2.17±0.16**	1.50±0.18***
Nyctanthes arbor-tristis, chloroform fraction 50 mg/kg	3.34±0.11	3.48±0.10	3.10±0.20*	2.20±0.21**
Nyctanthes arbor-tristis, chloroform fraction 75 mg/kg	3.35±0.18	3.63±0.12	2.85±0.18**	1.85±0.16***
Nyctanthes arbor-tristis, n-butanolic fraction 50 mg/kg	3.39±0.11	3.70±0.18	3.20±0.22*	2.50±0.18**
Nyctanthes arbor-tristis, n-butanolic fraction 75 mg/kg	3.40±0.19	3.80±0.20	2.90±0.18**	2.10±0.20**

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

Table 3: Effect of chloroform and n-butanolic fractions of ethanolic extract of *Nyctanthes arbor-tristis* leaves on oxidative stress and membrane marker enzymes

Groups and treatments	Oxidative stress parameters			Membrane marker enzymes		
	MDA nmol/mg of protein	Glutathione µmol/g of protein	SOD U/mg of protein	Alkaline phosphatase (ALP) (µmoles of phenol formed/h/mg protein)	Lactate dehydrogenase (LDH) (µmoles of pyruvate liberated/min/mg protein)	Acid phosphatase (ACP) (×10–2 µmol of phenol formed/min/mg protein)
Normal control	4.50±0.21	7.48±0.26	7.85±0.34	0.42±0.02	8.43±0.11	2.20±0.16
Arthritic control	14.25±0.51***	2.30±0.11***	3.10±0.11***	0.91±0.08***	18.95±0.21***	6.32±0.10**
Prednisolone 10 mg/kg	7.50±0.25***	6.58±0.20***	5.85±0.20**	0.50±0.04***	10.12±0.16***	2.80±0.12**
Nyctanthes arbor-tristis, chloroform fraction 50 mg/kg	9.30±0.22**	5.40±0.22**	4.98±0.22*	0.61±0.05**	12.44±0.21**	3.30±0.12*
Nyctanthes arbor-tristis, chloroform fraction 75 mg/kg	8.10±0.24***	6.10±0.30***	5.78±0.21***	0.53±0.01**	11.50±0.14***	2.90±0.16**
Nyctanthes arbor-tristis, n-butanolic fraction 50 mg/kg	9.40±0.28*	5.35±0.29*	4.85±0.26*	0.68±0.02*	12.58±0.20**	3.40±0.13*
Nyctanthes arbor-tristis, n-butanolic fraction 75 mg/kg	8.28±0.24**	5.90±0.23**	5.68±0.22**	0.56±0.03**	11.90±0.16**	2.98±0.11**

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

There is significant increase in membrane marker enzymes of arthritic rats. Treatment with chloroform and n-butanolic fraction showed a moderately significant ($P < 0.01$) decrease in the activity of membrane marker enzymes was seen in animals treated at 75 mg/kg [Table 3].

DISCUSSION

As a result of pathological destruction of collagen in bone and cartilage cross links, mature collagen is resorbed more rapidly. This causes a rise in their excretion. In arthritic conditions, apart from the crosslink resorption at the site of inflamed joints, there may be increased resorption due to general bone loss associated with disease activity.[18]

In FCA model, the affected cartilages are infiltrated by blood-derived cells, mainly neutrophils, macrophages and dendritic cells.[19] In response to activation, these cells generate ROS released in large amounts surrounding environment. This released ROS overcomes endogenous antioxidant defenses and induces impairment and destruction of the affected joint constituents such as synovial fluid, cartilage and other articular constituents. The tissues are damaged by the overproduction of reactive oxygen species.[20,21] One of the several approaches for the treat treatment of rheumatoid arthritis is to employ various membrane marker enzymes and antioxidants. *Nyctanthes arbor-tristis* is the well-documented medicinal plant reported to relieve rheumatic pains in traditional system of medicine.

Preliminary phytochemical analysis of ethanolic extract of *Nyctanthes arbor-tristis* leaves showed presence of fatty acids, steroids, terpenoids, tannins, flavonoids and glycosides. To separate these active principles responsible for the activity, fractionation of ethanolic extract of *Nyctanthes arbor-tristis* leaves was performed using two solvents namely chloroform and n-butanol. Phytochemical analysis showed the presence of steroids and terpenoids in chloroform fraction and flavonoids and glycosides in n-butanolic fractions.

It has been reported that adjuvant diseases can be induced by either FCA supplemented by mycobacterium or N, N-dioctylddecyl-N′, N-bis (2-hydroxy-ethyl) propanediamine. In the present study, chloroform and n-butanolic fractions were accessed in FCA-induced arthritis. Chloroform fractions highly significantly reduced paw swelling on 35th day as compared to n-butanolic fractions. This effect may be due to inhibitory effects on prostaglandin-mediated pathways. Activation of polymorphonuclear neutrophils is primary response against invading pathogens. Hence, due to activation of neutrophils, an inflammatory response is suppressed.[22]

The severity of arthritis was expressed as the arthritic score for each individual animal, being the sum of the measures of the four paws subtracted by the measures recorded before the immunization. The arthritic score of chloroform fraction was significantly lower as compared to n-butanolic fractions, indicating their anti-arthritic activity.

Lipid peroxidation is a critical mechanism of the injury that occurs during rheumatoid arthritis, which is often measured by analysis of tissue MDA. The large amount of MDA in arthritic control group is consistent with the occurrence of damage mediated by free radicals. Treatment with chloroform and butanolic fractions of *Nyctanthes arbor-tristis* produced a significant attenuation of MDA and effect is more significant with chloroform fraction as compared to n-butanolic fraction. The decrease in neutrophil accumulation by chloroform fraction treatment

might be due to the inhibition of lipid peroxidation and the consequent decrease in the chemo tactic decrease of peroxide.[23,24] The production of oxygen free radicals that occurs with the development of arthritis in the articular cartilage leads to decreased GSH and SOD levels as a consequence of their consumption during oxidative stress and cellular lysis,[25,26] which is evident by decreased levels of GSH and SOD in arthritic control group. Chloroform fraction causes significantly inhibited the decrease of GSH and SOD, probably by competing for scavenging of free radicals, which in turn resulted in recuperation of antioxidant enzyme levels.

The altered enzyme activities in arthritis can be regarded as an index of membrane marker enzyme activation occurring in response to metabolic need of degrading various constituents of cells such as mucopolysaccharides and glycoprotein accumulated in tissues due to arthritis associated with vasculopathies.[27]

Various membrane marker enzymes like ACP, LDH and ALP were used to be an important index for the examination of the integrity of the membrane and are responsible for the tissue damage and necrosis of hepatic tissue. These enzymes are also serving as indicators suggestive of disturbances of the cellular integrity and phagocytic activity induced by pathological conditions.[28,29] Increased activities of liver LDH, alkaline phosphates and ACP were observed in arthritic rats. This may be ascribed towards persistent inflammation.

In the present study, the activity of membrane marker enzymes was markedly increased in the arthritic rats and significantly reduced after treatment. Chloroform fraction had significantly lowered the enzymatic activity as compared to n-butanolic fraction and it may be due to inhibition of rupture of membrane and release of membrane marker enzymes.

Chloroform fraction of ethanolic extract of *Nyctanthes arbor-tristis* leaves shown more potent anti-arthritic activity as compared to n-butanolic fraction. As in phytochemical investigation, chloroform fraction showed the presence of steroids and terpenoids. It is previously reported that various Phytoconstituents like alkaloids, terpenoids, steroids and flavonoids have potent anti-inflammatory, analgesic and anti-arthritic activity. Hence, the anti-arthritic potential of chloroform fraction may be due to presence of steroids or terpenoids.

CONCLUSION

The arthritic disease progression was correlated with the fragility of the lysosomal membranes, subsequent discharge of the lysosomal enzymes and generation of reactive oxygen species.

Our results suggest that treatment of chloroform fraction has prominent anti-arthritic effect on adjuvant-induced arthritis in rats. The mechanism of the effect might be due to either 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 spread of the inflammatory mediators or inhibition of generation of oxidative free radicals. However, further studies are underway to isolate the lead molecule(s) responsible for the activity and also to identify on the mechanism of action of the same.

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