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Comparative Studies of Different Organs of *Nyctanthes arbortristis* in Modulation of Cytokines in Murine Model of Arthritis

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Objective To study the modulation effect of pro- and anti-inflammatory cytokines following long term use of water soluble ethanol extracts from different organs of *Nyctanthes arbortristis* (NAT) in mouse model of arthritis. **Methods** Arthritis was induced in mice by two injections of Freund's complete adjuvant on days 0 and 12 in the sub-planter surface of the right hind paw. **Results** Injection of adjuvant resulted in a maximum primary edema of the footpad with erythema, and edema and distortion of joints of the right hind paw after 24-48 hours. Second injection of FCA led to the formation of secondary swellings persisting more than four weeks that spread onto the other hind limb but to a lesser extent. Histological analysis of the ankle on day 47 showed marked evidence of cartilage destruction in association with pannus formation and moderate bone resorption. Proinflammatory cytokine levels in the inflamed joint homogenate were elevated on days 2, 14, and 47. Oral administration of leaf and fruit extracts in arthritic mice reduced joint homogenate levels of tumor necrosis factor-α, interleukin-1β, and interleukin-6 on days 2, 14, and 47 in comparison to untreated arthritic mice. Interleukin-10 level was elevated in the inflamed joint on days 2, 14, and 47 in comparisons to untreated arthritic mice. **Conclusion** Evidence of lesser inflammation of the footpad and joint and associated histological observation support the therapeutic benefit of leaf and fruit extracts from *Nyctanthes arbortristis*. This study helps in understanding the mechanism of anti-inflammatory action of *Nyctanthes arbortristis* in the light of pro- and anti-inflammatory cytokine balance.

Key words: *Nyctanthes arbortristis;* Arthritis; TNF-α; IL-1β; IL-6; IL-10

INTRODUCTION

Arthritis is a disease characterized by joint pain followed by bone and joint destruction[1]. Cytokines play a major role in arthritis[2]. Dysregulated expression of tumor necrosis factor-α (TNF-α) in experimental animals has been shown to cause destructive arthritis[3]. The development of arthritis is markedly suppressed in interleukin-β (IL-1β) deficient collagen-induced arthritis (CIA)[4]. Interleukin-6 (IL-6) gene disrupted mice are resistant to antigen and collagen-induced arthritis[5-7]. These studies indicate that pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) play a role in arthritis and are potential targets for therapy. Miagkov and coworkers[8] have characterized arthritis as an overabundance of pro-inflammatory cytokines, and inadequate anti-inflammatory cytokines. Therefore, the different strategies explored to treat arthritis include blocking of TNF-α with high affinity antibodies and soluble receptors[9-10] or blocking of IL-1β receptors[11-12] or the use of anti IL-6 antibody[13]. Herbal medicine, especially the extracts from *Nyctanthes arbortristis* (NAT) leaves has been shown to possess anti-arthritic properties. In addition, decoction of the leaves of NAT has been also shown to possess hepatoprotective, anti-leishmanial, anti-viral, and anti-fungal activities, as well as analgesic, antipyretic, and ulcerogenic activities. The leaves have been found to contain tannic acid, methyl salicylate, amorphous glucosides, mannito, resin, ascorbic acid, carotene, and traces of a volatile oil[14]. Iridoid glucosides, arbortristosides A-C and 6β-hydroxyloganin isolated from NAT have anti-leishmanial activity whereas arbortristosides A-C are anti-allergic. The effect of water soluble fraction of ethanol extract from NAT on TNF-α level in plasma of arthritic and soluble protein A-treated

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mice studied by us could consistently deplete TNF-α from the host plasma[15]. NAT extract could also ameliorate silica-induced early fibrogenic reaction in lung of mice by reducing TNF-α levels in the bronchoalveolar lavage fluid[16]. The anti-inflammatory activity of NAT has been demonstrated by several other groups[17-19]. In spite of its wide clinical use, particularly in inflammatory conditions, very little is known about the effect of long term use of NAT extracts on the pro-inflammatory and anti-inflammatory cytokine profile.

Although leaves of NAT have been extensively studied for their anti-inflammatory property, little is known about the efficacy of other parts of the plant and the consequence of its prolonged use. In the present study, we made a comparative assessment of different parts of NAT for their anti-inflammatory property for a prolonged period.

MATERIALS AND METHODS

Experimental Animals

Female Balb/c mice weighing 25-30 g were used in the study. Permission was sought from the Animal Ethics Committee of the Institute for the work. Animals were fed with pellet diet and water *ad libitum*. Mice were divided into 5 groups of 15 animals each. Group I comprised of sham control mice, group II of arthritic mice, groups III-V of arthritic mice receiving leaf, fruit, and seed extract daily, 5 days a week till day 47. The NAT extract was administered by oral gavage using a blunt ended steel canula.

Induction of Arthritis

Arthritis was induced in mice by injecting 10 μL Freund's complete adjuvant (FCA) (Sigma, USA: Lot #8048808) containing heat-killed *Mycobacterium tuberculosis* (H37Ra, ATCC, 25177) in sub-planter surface of the right hind paw[15, 20]. A booster dose of 10 μL FCA was given to animals in sub-planter surface of the same hind paw on day 12[20].

Preparation of Extracts From NAT

Fruits, seeds, and leaves of NAT were collected in January and February, from the trees growing in the premises of Industrial Toxicology Research Centre, Gheru Campus. A voucher specimen (Bnp101) of the plant was deposited to National Botanical Research Institute herbarium (LWG), Lucknow, India. Fruits, seeds, and leaves were dried in shade and powdered. The powder was macerated with 95% ethanol, the extract was filtered and the solvent was evaporated using a lyophilizer. The residue was stirred vigorously with sterile distilled water. The mixture was allowed to stand for 30 min and then filtered. The filtrate was again lyophilized. The yield for fruits, seeds, and leaves was 6.02%, 22.34%, and 6.28% of the plant organ, respectively. The residues were suspended appropriately in sterile distilled water to prepare a stock solution at a concentration of 20 mg/mL. The stock solution was appropriately diluted in sterile distilled water and administered at an optimal dose of 25 mg of water soluble ethanol extract/kg body weight/100 μL to each mouse.

Administration of NAT Extract

Treatment with extract was started on day 0, orally (25 mg/kg per day), simultaneous with FCA injection. Earlier, an acute oral NOAEL of 2000 mg/kg was established based on body weight, relative organ weight, gross necropsy, hematology, clinical chemistry and cage side observation. The present dose was 1/80 of NOAEL. On days 2, 12, and 47, animals from each group were sacrificed to collect inflamed joint and blood serum for different analysis. These three time points were selected as day 2 represented the peak time point showing primary edema, day 14 the progression phase of secondary edema and day 47 the well defined arthritis phase.

Footpad Swelling

Footpad swelling was measured with the help of geometric formula of eclipse circumference: $2\pi \times \sqrt{(a^2+b^2)}/2$, where a and b are measures of diameter at two different planes taken with the help of a Vernier caliper.

Preparation of Joint Homogenates

Inflammatory sites on the joints ranging 4-5 mm were dissected from arthritic and treated mice and identical sites in normal mice, weighed and a 10% homogenate was prepared in ice cold phosphate buffered saline (PBS) containing 0.5% Tween-20. The homogenate was centrifuged at 2000×g for 10 min and the supernatant was filtered using a 0.2 μmol/L millipore filter and used for cytokine assay[20].

Cytokine Enzyme Linked Immunosorbent Assay

TNF-α, IL-1β, IL-6, and IL-10, were evaluated in the inflamed joint homogenates using solid phase sandwich ELISA[20]. The protocols laid in the technical bulletin of the manufacturers were adopted (R&D, Minneapolis, USA). The coating antibody for TNF-α assay was monoclonal antibody against mouse TNF-α. Monoclonal antibody against IL-1β, IL-6, and IL-10 was used for evaluating IL-1β, IL-6, and IL-10, respectively. The different second antibodies

used for the detection of TNF-α, IL-1β, IL-6, and IL-10 were biotin-conjugated goat anti mouse TNF-α, goat anti-mouse IL-1β, IL-6, and IL-10, respectively. The detection reagent containing streptavidin-conjugated horse radish peroxidase and tetramethylene benzidine (TMB) was used as a substrate.

Histopathology

Inflamed joints from the region of ankle were collected, digits were removed and tissues were weighed. Tissues were placed in 10% neutral buffered formalin at the ratio of 1:10 (W/V) for 48 hours to allow fixation. Further tissues were kept in Gooding's Stewart decalcifying solution (a mixture of formaldehyde and formic acid in distilled water at the ratio of 4:7:29) in the ratio of 1:100 (W/V) for 5 days to facilitate decalcification. Decalcification was confirmed by pinching the tissue gently using a sharp needle, and processed for paraffin embedding. Tissues were sectioned at 5 microns and stained with hematoxylin and eosin.

RESULTS

We induced arthritis in mice by giving one injection of FCA on day 0 and the second injection on day 12. First injection led to the maximum formation of primary footpad edema, joint edema and distortion of phalanges between days 2 and 4 in the right hind limb. Between days 10 and 12, the primary swelling attained a minimum size but never reverted back to normal size. However, a pale granulomatous appearance persisted. On day 12 a second injection of FCA at the same site was given that led to the formation of secondary swellings persisting more than four weeks that spread on the other hind limb but to a lesser extent. Between days 45 and 47, well defined arthritic symptoms were observed, such as secondary swellings on the right and left hind limbs with mild distortion of the phalanges. The degree of footpad swellings is presented in Fig.1. The tail looked crocked in shape. Histology of the ankle of arthritic mice showed marked cartilage destruction in association with pannus formation and moderate bone resorption (Fig. 2b in comparison to normal Fig. 2a). Leaf and fruit extract-treated arthritic mice showed mild bone resorption, cartilage destruction and pannus formation, indicating the anti-arthritic property of the extract from leaves and fruits of NAT (Figs. 2c and 2d). Seed extract in contrast, failed to ameliorate FCA-induced histopathological changes (Fig. 2e).

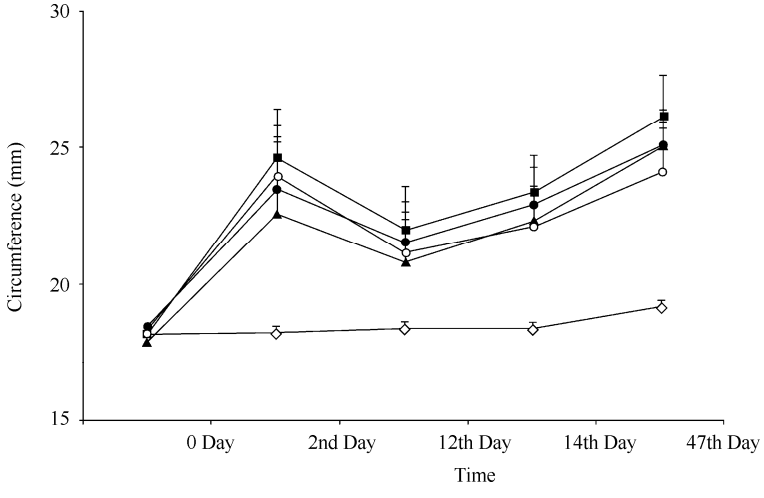

FIG. 1. Effect of fruit, seed and leaf extracts of *Nyctanthes arbortristis* on paw edema in adjuvant induced arthritic mice. (<>——<>) Normal mice; (▪——▪) Adjuvant induced arthritis (AIA); (▲——▲) AIA+ fruit extract; (●——●) AIA+ seed extract; (o——o) AIA+ Leaf extract. The values shown at different time points are the $\bar{x} \pm s$ of five animals.

The pro-inflammatory cytokine (TNF-α, IL-1β, and IL-6) levels in inflamed joint homogenate of FCA-injected mice were elevated in comparison to normal joint homogenate on day 2. The normal levels of TNF-α, IL-1β, and IL-6 were 31.6±3.24 pg/mL, 15±1.41 pg/mL, and 17.5±4.22 pg/mL, respectively. The levels of TNF-α, IL-1β, and IL-6 were elevated on days 14 and 47 in mice receiving FCA on days 0 and 12 in comparison to normal joint homogenate (Tables 1-3)

Daily oral administration of extracts from leaves and fruits of NAT in FCA-injected mice reduced the IL-1β and IL-6 levels in the inflamed joint homogenate on days 2, 14, and 47 in comparison to the untreated arthritic mice (Tables 1 & 2). TNF-α levels were reduced on days 14 and 47 but not on day 2 in comparison to the untreated mice (Table 3). Extracts from leaves and fruits of NAT were efficient in reducing IL-1β, IL-6, and TNF-α while extract from NAT seeds could not.

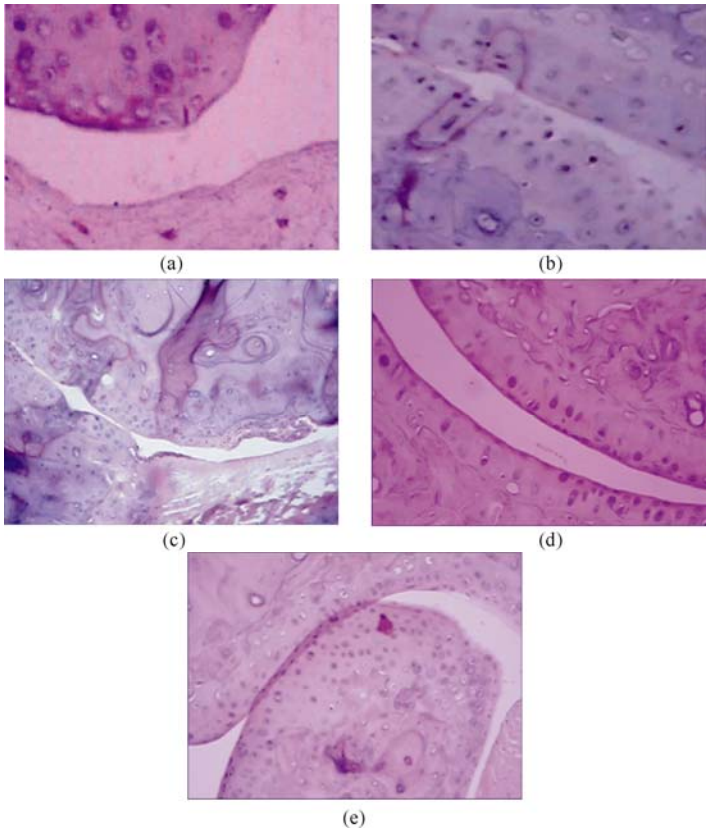

FIG. 2 Histology of the inflamed ankle of arthritic mice. a. Normal ankle joint of mice showing defined synovial space and cartilage (200×); b. The ankle joint of adjuvant induced arthritic mice showing reduced synovial space, cartilage destruction and neutrophilic infiltration; c. *Nyctanthes arbortristis* leaf extract treated ankle joint of AIA mice showing partial reduction in synovial space and minimal cartilage destruction; d. *Nyctanthes arbortristis* fruit extract treated ankle joint of AIA mice showing less infiltration of neutrophils and near normal synovial space. e. *Nyctanthes arbortristis* seed extract treated ankle joint of AIA mice showing reduced synovial space and cartilage destruction.

TABLE 1

TNF-α Level (pg/mL) in AIA Mice Treated for Different Time Period With Different Organ Extracts of *Nyctanthes arbortristis*

Experimental Groups	Day 2	Day 14	Day 47
Normal	32 ± 9	33 ± 8	35 ± 8
AIA	122 ± 14 [a]	531 ± 31 [a]	591 ± 47 [a]
AIA+ Fruit Extract	144 ± 16	297 ± 27	218 ± 30
AIA+ Seed Extract	128 ± 20	500 ± 35	490 ± 38 [c]
AIA+ Leaf Extract	133 ± 18	271 ± 14 [b]	259 ± 19 [b]

Note. Fifteen animals were used in each group, out of them 5 animals were sacrificed at each study point. [a]significant (P<0.01) in comparison to TNF-α levels in normal mice on the respective day. [b]significant (P<0.01) in comparison to TNF-α levels in arthritic mice on the respective day. [c]significant (P<0.05) in comparison to TNF-α levels in arthritic mice on the respective day.

TABLE 2

IL-1β Level (pg/mL) in AIA Mice Treated for Different Time Period With Different Organ Extracts of *Nyctanthes arbortristis*

Experimental Groups	Day 2	Day 14	Day 47
Normal	15 ± 4	17 ± 5	18 ± 4
AIA	691 ± 48 [a]	835 ± 23 [a]	1031 ± 32 [a]
AIA+ Fruit Extract	198 ± 22 [b]	591 ± 27 [b]	672 ± 26 [b]
AIA+ Seed Extract	496 ± 38 [c]	680 ± 28 [b]	883 ± 31 [c]
AIA+ Leaf Extract	306 ± 14 [b]	514 ± 22 [b]	279 ± 24 [b]

Note. Fifteen animals were used in each group, out of them 5 animals were sacrificed at each study point. [a]significant (P<0.001) in comparison to IL-1β levels in normal mice on the respective day. [b]significant (P<0.001) in comparison to IL-1β levels in arthritic mice on the respective day. [c]significant (P<0.01) in comparison to IL-1β levels in arthritic mice on the respective day.

TABLE 3

IL-6 Level (pg/mL) in AIA Mice Treated for Different Time Period With Different Organ Extracts of *Nyctanthes arbortristis*

Experimental Groups	Day 2	Day 14	Day 47
Normal	17 ± 5	18 ± 6	22 ± 8
AIA	574 ± 43 [a]	1533 ± 59 [a]	1799 ± 75 [a]
AIA+ Fruit Extract	297 ± 27 [b]	672 ± 33 [b]	425 ± 13 [b]
AIA+ Seed Extract	349 ± 29 [b]	1050 ± 60 [b]	1505 ± 32 [b]
AIA+ Leaf Extract	223 ± 15 [b]	627 ± 53 [b]	337 ± 17 [b]

Note. Fifteen animals were used in each group, out of them 5 animals were sacrificed at each study point. [a]significant (P<0.001) in comparison to IL-6 levels in normal mice on the respective day. [b]significant (P<0.001) in comparison to IL-6 levels in arthritic mice on the respective day.

TABLE 4

IL-10 Level (pg/mL) in AIA Mice Treated for Different Time Period With Different Organ Extracts of *Nyctanthes arbortristis*

Experimental Groups	Day 2	Day 14	Day 47
Normal	117 ± 18	130 ± 12	143 ± 10
AIA	73 ± 12 [a]	85 ± 16 [a]	67 ± 24 [a]
AIA+ Fruit Extract	92 ± 10	148 ± 22 [b]	155 ± 18 [b]
AIA+ Seed Extract	87 ± 12	94 ± 29	76 ± 23
AIA+ Leaf Extract	69 ± 10	81 ± 24 [b]	186 ± 21 [b]

Note. Fifteen animals were used in each group, out of them 5 animals were sacrificed at each study point. [a]significant (P<0.01) in comparison to IL-10 levels in normal mice on the respective day. [b]significant (P<0.01) in comparison to IL-10 levels in arthritic mice on the respective day.

Anti-inflammatory cytokine level (IL-10) in inflamed joint homogenate of AIA mice was reduced on day 2 as compared to normal joint homogenate (Table 4). The level of IL-10 in normal joint homogenate was 117±18.01 pg/mL. Elevated levels of IL-10 were found in the joint tissue homogenate of arthritic mice treated with extracts from NAT leaves, seeds and fruits on days 14 and 47.

DISCUSSION

Decoction of NAT leaves has been used to treat various diseases for a very long time[21]. In the present investigation, we made a comparative study on the therapeutic and preventive efficacy of different organs of NAT in adjuvant-induced arthritis. AIA mouse is a model of rheumatoid arthritis that has sensitivity to various anti-inflammatory agents[15, 22]. TNF-α, IL-1β, and IL-6 are the major proinflammatory cytokines with high concentrations in the inflamed joint synovium[23-26]. Our results re-establish these observations in mice receiving 2 doses of FCA: one on day 0 and the other on day 12. Daily treatment with extracts from leaves and fruits of NAT reduced the TNF-α, IL-1β, and IL-6 from day 14 while seed extracts were ineffective, suggesting that the active component is in the hard covering of the fruit. In terms of histopathological observation and measurements of footpad swelling, seed extracts were ineffective in controlling arthritis although the IL-10 level was elevated, suggesting that the anti-inflammatory cytokine alone is incapable of modulating the progression of arthritis. In case of treatment with extracts from leaves and fruits of NAT, depletion of pro-inflammatory and elevation of anti-inflammatory cytokine occurred in the joint homogenate of arthritic mice, finally leading to moderate control on progression of arthritis, suggesting that a balance between proinflammatory and anti-inflammatory cytokines is necessary for controlling the progression of arthritis. Alternatively, a shift in the balance between pro- and anti-inflammatory cytokines occurred in the adjuvant-induced mice, favoring inflammation and the extract from leaves and fruits bears anti-arthritic properties and is suggestive of therapeutic and preventive benefit.

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