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CROCUS SATIVUS AND *NYCTANTHES ARBORTRISTIS* EXTRACT MODULATES ANTI-INFLAMMATORY CYTOKINE IN EXPERIMENTAL ARTHRITIS

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ABSTRACT: Rheumatoid arthritis (RA) is a chronic inflammatory disease, affecting 1-2% population worldwide. RA is common among the women in comparison to men. The disease mostly affects the age group 45 onwards, while juvenile cases are also reported. Exact pathogenesis of the disease is still a question. Studies indicate RA as an overabundance of pro-inflammatory cytokines, and inadequate anti-inflammatory cytokines. TNF-α, IL-1β, and IL-6 are established pro-inflammatory cytokines and IL-10 as anti-inflammatory cytokine in several autoimmune and inflammatory diseases. *Crocus sativus* is considered as an anticonvulsant, antidepressant, antispasmodic and diaphoretic agent and *Nyctanthes arbortristis* has been known for its anti-helmenthic, anti-bacterial activity etc. The present study was designed to evaluate the effectiveness of *Crocus sativus* (CS) and *Nyctanthes arbortristis* (NAT) towards anti-inflammatory property in inflamed animal tissue. We induced arthritis by injecting Freund's complete adjuvant in the paw of mice. Water soluble ethanolic extract of *Nyctanthes arbortristis* (leaf) and *Crocus sativus* (stigma) was administered orally to adjuvant induced arthritic mice at the dose of 23.72 and 100 mg/kg body weight, respectively for 47 days. Paw edema and anti-inflammatory cytokine were assessed in adjuvant induced arthritic mice. Daily administration of extracts for 47 days significantly reduced the paw edema and elevated the IL-10 levels in arthritic mice. Our results indicate the efficacy of simultaneous oral dose CS and NAT extracts towards anti-inflammatory property in the experimental mice.

INTRODUCTION: Rheumatoid arthritis (RA) is a common inflammatory disease affecting a large population worldwide [1]. Disease results in the inflammation in diarthrodial joint tissue [2], followed by progressive destruction of bone and cartilage [3]. The exact etiology of RA remains still unknown.

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It has been reported that either a foreign agent or some alteration in control of cellular responses is involved in the synovial inflammation [4]. Cytokines are responsible to initiate and regulate immune responses. Cytokines also play a major role in arthritis [5].

The presence of various cytokines in the cells of synovial lining and sublining, including type a synoviocytes and other macrophage- like populations have been demonstrated [6]. Dysregulated expression of tumor necrosis factor-α (TNF-α) in experimental animals has been shown to cause destructive arthritis [7].

The development of arthritis is markedly suppressed in interleukin-β (IL-1β) deficient collagen-induced arthritis (CIA) [8]. Miagkov and coworkers have characterized arthritis as an overabundance of pro-inflammatory cytokines, and inadequate anti-inflammatory cytokines [9].

Various animal models have been developed to elucidate the pathogenesis of the disease and to evaluate the efficacy of possible therapeutics. Induced RA models include adjuvant arthritis [10], collagen induced arthritis [11], pristane induced arthritis [12] and streptococcal cell wall induced arthritis [13]. Gene manipulated mouse models include HTLV-1 transgenic mouse [14], Human TNF-α transgenic mouse [15], Human IL-1β transgenic mouse, and Balb/c IL-1 Ra deficient mouse [16]. All these models developed autoimmunity and the pathology of the joints closely resembled that of human RA.

Nyctanthes arbortristis (*Family*: *Oleaceae*) is one of the Indian medicinal plant. *Nyctanthes arbortristis* is a shrub or a small tree growing to 10 m tall, with flaky grey bark. It has its distribution mainly in East Asia. It is also known by other names such as Night-flowering Jasmine, Harshingar, Parijat, Bruscia, Pariaticu, Parilium and Scabrita [17] etc. The plant has been extensively used for the preparation of decoction of leaves by Ayurvedic physicians for the treatment of obstinate sciatica, malaria, intestinal worms and as a tonic, cholagogue and laxative [18].

Crocus sativus L. (Family: Iridaceae) commonly known as saffron, is a perennial stemless herb which is cultivated in several countries like India, Iran and Greece. In India, it is mainly cultivated in Kashmir and Uttaranchal. The dried red stigmas of the flower are mainly used for therapeutical purposes such as anticonvulsant, antidepressant and antitumor activities [19]. The *Crocus sativus* stigmas are mainly used for therapeutic purposes. Saffron extract has also been shown to have protective effects on genotoxin induced oxidative stress in swiss albino mice [20].

In last two decades, alternative medicine has been practiced as an effective tool for therapeutic purposes. Among alternative medicine methods, herbal remedies are now widely accepted therapy to arthritis [21]. Recent studies from our laboratory have revealed the repair of oxidative stressed state using anti-oxidants [22], and the control of this stress using herbal preparations [23]. Also, *Nyctanthes arbortristis* extract has been proven to reduce inflammatory cytokines in adjuvant induced arthritis [24]. In the present study, we have tried to assess simultaneous dosing of NAT leaf extract and *Crocus sativus* extract for anti-inflammatory property. This study will help in understanding the mechanism of action of simultaneous dosing of different herbal extracts in an experimental disease.

MATERIALS AND METHODS:

Preparation of *Nyctanthes arbortristis* leaf extract (NLE): For our experiment, leaves of *Nyctanthes arbortristis* (NAT) were collected in the month of January and February, from the tree growing in the premises of Institute (**Fig.1**). A sample specimen (Bnp101) of the plant was deposited to National Botanical Research Institute herbarium (LWG), Lucknow, India for species authentication. Further, leaves were dried in shade and powdered. The powder was macerated with 95% ethanol, the extract was filtered and the solvent was evaporated using lyophilizer. The residue was stirred vigorously with distilled water; the mixture was allowed to stand for 30 min and filtered. The filtrate was again lyophilized. The yield of extract from leaves was 6.28%. The stock solution was appropriately diluted in sterile distilled water and administered as oral dose of 23.72mg/kg body weight to each mouse. The dose was selected as effective one from our previous study [24].

FIG. 1: *NYCTANTHES ARBORTRISTIS* PLANT

Preparation of *Crocus sativus* extract (CSE):
Crocus sativus L. stigmas were procured from Sigma, USA (**Fig. 2**). 4.0 grams of stigmas were macerated in 70% ethanol for three days. The mixture was then filtered and collected. The solution thus obtained was lyophilized. As a result, the extract yield was 48.2%. The dried powder was then reconstituted to get desired concentration for experimentation i.e, 100 mg/kg body weight. The dose was selected as effective one from our previous study [23].

FIG. 2: *CROCUS SATIVUS* STIGMA

Experimental animals: Female Balb/c mice weighing 25-30 g were used throughout the studies. Prior permission for the experiment was sought from the Institutional Animal Ethics Committee. Animals were kept in separate cages under standard conditions of the animal house, and fed pellet diet and water *ad libitum*. Mice were divided into 4 groups of 12 animals each. The group I comprised of normal mice, group II comprised of arthritic mice receiving distilled water, group III comprised arthritic mice receiving NAT leaf and CS extract daily till day 47. The NAT leaf extract (NLE) was administered in morning and CS extract (CSE) was administered in evening, the time interval of 6 hrs was maintained. Group IV comprised arthritic mice receiving acetylsalicylic acid (ASA) at the dose of 200mg/kg body weight for 47 days.

Induction of arthritis: 10 µL Freund's Complete Adjuvant (FCA) (Sigma, USA) containing heat killed Mycobacterium tuberculosis (H37Ra, ATCC, 25177) was injected in the sub planter surface of the right hind paw of mice to induce arthritis. A booster dose of 10µL FCA was given to animals in sub planter surface of the same hind paw on 12th day. Thus, adjuvant induced arthritis animals were prepared.

Treatment: NLE and CSE treatment was started on the day 0, orally (23.72 and 100 mg/kg body weight/day respectively), simultaneous with the FCA injection. NLE was administered in morning and CSE was administered in evening, the time interval of 6 hrs was maintained.

Measurement of Footpad Swelling (edema): Footpad swelling in mouse 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 Homogenate: On day 2, ankle joint from normal animals were dissected and homogenate was prepared. On day 47, Inflammatory site on the joints ranging from 4-5mm was dissected out, weighed and a 10% homogenate was prepared in ice cold phosphate buffered saline (PBS) containing 0.5% Tween-20. The homogenates was centrifuged at 2000g for 10 min and the supernatant was filtered using 0.2 µM Millipore filters and used for cytokine assay.

Quantitative estimation of anti-inflammatory cytokine: ELISA based IL-10 cytokine analysis kit was procured from Ray Biotech, Inc. USA, and IL-10 was estimated, in the joint homogenates, using solid phase sandwich ELISA. The protocols laid in the technical bulletin of the manufacturers were followed. Plates were read on iMark microplate reader (Bio-Rad, CA).

Statistical analysis: Student 't' test was performed to evaluate the significance of the difference in the mean values of extract treated and untreated group. $P<0.05$ was considered as significant.

RESULTS:
Effect of NLE and CSE on footpad swelling: We induced arthritis in mice by one injection of FCA on day 0 and the second injection on day 12. First injection resulted in formation of primary footpad edema. Between day 10 and 12, the primary swelling appeared reduced in size but didn't reverted to normal size. However, a pale granulomatous appearance was observed.

On day 12, second injection of FCA at the same site was given which led to the formation of secondary swelling, 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. On day 47, we observed non-significant change in the footpad swelling in normal mice, while, the simultaneous dose of NLE and CSE resulted in significant reduction in the footpad swelling as compared to AIA mice (**Table 1**).

TABLE 1: EFFECT OF NLE AND CSE ON FOOTPAD SWELLING IN AIA MICE

Group	Day 2	Day 14	Day 47
Normal	18.2 ± 0.3	18.4 ± 0.2	18.8 ± 0.4
AIA	19.5 ± 1.8 [a]	21.9 ± 1.6 [a]	26.1 ± 1.5 [a]
AIA+(NLE+CSE)	19.2 ± 0.8	21.3 ± 0.8	21.9 ± 0.8
AIA+ASA	19.1 ± 0.3[b]	19.8 ± 0.4 [b]	21.2 ± 0.9 [b]

[a]significant ($p<0.05$) in comparison to normal mice on the respective time point;
[b]significant ($p<0.05$) in comparison to arthritic mice on the respective time point;

Effect of NLE AND CSE on anti-inflammatory cytokine IL-10 levels in AIA mice: On day 2, IL-10 levels were recorded as 117pg/ml in normal mice while 85pg/ml in AIA mice. We observed non-signifcant change in IL-10 levels in simultaneous dose (NLE+CSE) receiving AIA mice group. We also recorded no change in IL-10 levels in standard drug dose group. On day 47, we found significant reduction in IL-10 levels in the AIA mice as compared to normal mice while simultaneous dose (NLE+CSE) resulted in elevation of IL-10 levels as compared to AIA mice. This elevation was comparable to standard drug receiving mice group (**Table 2**).

TABLE 2: EFFECT OF NLE AND CSE ON ANTI-INFLAMMATORY CYTOKINE IL-10 LEVELS (pg/ml) IN AIA MICE

Group	Day 2	Day 47
Normal	117 ± 18	143 ± 10
AIA	85 ± 12 [a]	67 ± 24 [a]
AIA+(NLE+CSE)	72 ± 8	107 ± 16
AIA+ASA	83 ± 34[b]	112 ± 10 [b]

[a]significant ($p<0.05$) in comparison to normal mice on the respective time point;
[b]significant ($p<0.05$) in comparison to arthritic mice on the respective time point.

DISCUSSION: The present study is an attempt to observe the anti-inflammatory effect of simultaneous dose of NLE and CSE in experimentally induced arthritic mice. In adjuvant induced arthritis, there is a series of reactions executed by host in effort to prevent tissue damage and destroy the infective organism by activating the repair process that are necessary to bring organism to normal functioning [25].

This process is commonly known as acute phase response (APR). A sequence of events is thus initiated, leading to the release of soluble mediators that mobilize the metabolic response of organism. Bacterial products activate macrophage, which in turn release different cytokines such as TNF and IL-1. These cytokines possess pleiotropic activity and have been implicated as major players in arthritis [26]. The release of these cytokines enhances the inflammatory response and worsens the tissue. Thus, the APR follows a sequence of events in which macrophages and platelets are activated and thus cytokines are released [27].

In present experiment, we have observed a visible as well as quantifiable reduction in footpad edema of AIA mice receiving simultaneous dose of NLE and CSE. This indicates that both the extracts possess anti-inflammatory potential even when they are administered on the same day. This may be contributed to their synergistic action. In our previous study, we have observed the anti-inflammatory action of NLE [24] and CSE [23], individually. Various workers have reported the anti-inflammatory activity of Chinese and Korean herbal plants in arthritis. There are several studies that support the use of herbal preparation for reducing arthritic symptoms.

A research reported the anti-inflammatory activity of *Celastrus orbiculatus* Thunb. in carrageenan-induced mouse where significant reduction in paw edema was seen, when the compound (100 mg/kg) was orally administrated [28]. Furthermore, studies on active component of Tripterygium wilfordii hook F (TWHF), triptolide produced promising immunosuppressive and anti-inflammatory action [29]. Later on, a polyherbal preparation resulted in increased mobility and decrease in paw edema after 90 days treatment [30].

IL-10 is an anti-inflammatory cytokine. It is believed that a balance between the pro-inflammatory and anti-inflammatory cytokines decides the direction of the immune reaction either in favor of inflammation or a therapy [31]. A balanced Th1 and Th2 cytokines in the body fluids defines a normal healthy immune response while a shift to either Th1 and Th2 cytokines induce a disease processes. IL-10 [32] and IL-4 counteract the effects of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 [33]. In our present study, we have found a significant increase in IL-10 levels in simultaneous dose (NLE+CSE) receiving AIA mice, which is comparable to standard drug (ASA). This indicates that our extracts are capable of maintaining anti-inflammatory cytokine in a balanced state which inturn alters the progression of the disease.

Several researchers have been working on the combined dose of herbal preparation for their therapeutical effect in various diseases. Wang and Tao demonstrated the clinical efficacy of YTR combined MTX + LEF in the treatment of RA as better than using Western medicine alone [34]. In another study, Jie and researchers demonstrated that Biqi Capsule (BQC) combined with MTX showed better clinical efficacy than use of BQC or MTX alone [35]. Similarly Chen and coworkers observed that Yiqi Fumai Injection (YFI) combined hydroxychloroquine sulfate tablet showed better effects in treating Sjogren's syndrome patients than using Chinese medicine or allopathic medicine [36].

In another study, Wang and his team evaluated the clinical efficacy and safety of bushen quhan zhiwang decoction (BQZD) combined methotrexate (MTX) in treating rheumatoid arthritis (RA) and found that BQZD could enhance the efficacy and reduce adverse reactions of MTX through synergistic effects with MTX [37].

The anti-inflammatory potential of NLE and CSE may be attributed to the presence of active ingredients in the *Nyctanthes arbortristis* leaves and *Crocus sativus* stigma. There are many phytochemical investigations of NAT revealing the presence of iridoid glycosides (such as arbortristoside A, arbortristoside B and arbortristoside C), Nyctanthic acid, Lupeol and alkaloids in leaves [38], Oleanolic acid and β-sitosterol in seeds of this plant [39]. Several researchers have demonstrated the presence of various active constituents such as crocin, crocetin, safranal, flavonoids [40, 41] etc. Thus, anti-inflammatory property of saffron extract may be attributed to synergistic action of these active constituents [42].

CONCLUSION: Rheumatoid arthritis is an autoimmune, chronic inflammatory disease that targets the synovial membrane, as well as extra-articular tissues. Although RA is a disease of unknown etiology, yet it is quite well established that lowering of anti-inflammatory cytokines perpetuates the progression of arthritis, due to impaired cytokine system in the tissue. In the present study, on the one hand, significant reduction in footpad edema was recorded, while on the other hand, elevated levels anti-inflammatory cytokine IL-10 was observed in the NLE and CSE treatment receiving inflammed joints of arthritic mice. Thus, present study reveals the effectiveness of NLE and CSE for its anti-inflammatory potential during experimental arthritis. However, further studies are needed to completely understand the anti-inflammatory mechanism of these herbal extracts.

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CONFLICT OF INTEREST: None

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