Immunomodulatory activity of aqueous extract of *Nyctanthes arbor-tristis* flowers with particular reference to splenocytes proliferation and cytokines induction

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**Abstract:**

**Objectives:** To investigate the immunomodulatory activity of aqueous extract of *Nyctanthes arbor-tristis* flowers (NAFE) with particular reference to splenocytes proliferation and induction of cytokines.

**Materials and Methods:** Antibody titer was determined by tube agglutination and indirect ELISA assay in four groups of mice-control, antigen alone, and NAFE-treated (400 and 800 mg/kg for 21 days) after immunization with *Salmonella* antigen while cellular immunity was studied in three groups of rats (control and NAFE-treated - 400 and 800 mg/kg) following DNBC application. Splenocytes from untreated and NAFE-treated rats were stimulated using concanavalin-A (Con-A) and optical density (OD) and stimulation index were determined. Splenocytes from control rats were also treated in vitro with NAFE (50–1600 µg/ml) and Con-A to determine the effect on splenocytes proliferation. Interleukin-2 (IL-2) and IL-6 levels in splenocytes supernatant from control and NAFE-treated rats and following in vitro treatment of splenocytes with NAFE (50–1600 µg/ml) were determined using ELISA kits.

**Results:** Marked to a significant increase in antibody titer by both the methods in NAFE-treated mice and a significant increase in skin thickness in rats after challenge with DNBC, respectively suggested humoral and cell-mediated immunostimulant potential of NAFE. Significant increase in OD and stimulation index following *ex vivo* and *in vitro* exposure of splenocytes and sensitization with Con-A and significant elevation in IL-2 and IL-6 levels in splenocytes supernatant was also observed after their *ex vivo* and *in vitro* exposure to NAFE.

**Conclusion:** Humoral and cell-mediated immunostimulant activity of NAFE seems to be mediated through splenocytes proliferation and increased production of cytokines, especially IL-2 and IL-6.

**Key words:** Antibody titer, cell-mediated, cytokines, interleukin-2, interleukin-6, *Nyctanthes arbor-tristis* flowers, splenocytes proliferation

*Nyctanthes arbor-tristis*, family-Oleaceae, is commonly known as harsingaar or night jasmine. *Nyctanthes* means “night flowering” and *arbor tristis* means the “sad tree” as it loses its brightness during daytime. Its flowers open at dusk and finish at dawn and have pleasant fragrance. Several studies on phytoconstituents of extracts of leaves, stem bark, seeds, roots, and flowers of *N. arbor-tristis* have been taken up, but leaves are the most studied ones and have been reported to contain nyctanthine, an alkaloid, in addition to mannitol, resinous substance, ascorbic acid, coloring agent, sugar, traces of oily substances, tannic acid, methyl salicylate, carotene, etc.[3-5] Flavonoid and iridoid glycosides have also been detected in the leaves of *N. arbor-tristis*. Seed kernels of *Nyctanthes* contain 12–16% of pale yellow fixed oil containing glycosides, linoleic acid, oleic acid, lignoceric acid, stearic acid, myristic acid, salicylic acid, palmitic acid, and β-sitosterol[5-6] apart from phenyl propanoid glycoside, nycoside-A[6] and water soluble glucosamman.

[7] Phytochemical examination of the stem of *N. arbor-tristis* resulted in isolation and identification of β-sitosterol a new glycoside naringenin-4-0-β glucopyranosyl-a-xylopyranoside.[8] The bark of this plant contains a glycoside and two alkaloids, one soluble in water and the other in chloroform[9] while roots contain an alkaloid, tannin, and glycosides.[6,9-10]

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Flowers of this plant contain numerous phytoconstituents, namely - diterpenoid nycanthin, flavonoids, anthocyanins, essential oils, beta-monogentiobioside, beta-digentiobioside d-mannitol, nycanthoside, rengyolone, astragalin, arborside C, carotenoid apart from tannin, and glucose. N. arbor-tristis is bestowed with numerous pharmacological activities which include antipyretic, analgesic, anti-inflammatory, anti-anemic, hepatoprotective, tranquilizing, hypotonic, anticonvulsant, local anesthetic, antihistaminic, antioxidant, antispermatogenic, antibacterial, antifungal, antiviral, antimalarial, antitrypanosomal, anti-amoebic, anthelmintic, leishmanicidal and anti-cancer. Especially, flowers of this plant have been found to useful as sedative and effective as stomachic, carminative, astringent, expectorant, hair tonic, and also in the treatment of piles and skin diseases. Although immunobioactivity of N. arbor-tristis flowers extract against sheep red blood cells (RBCs) and heat-killed Salmonella antigen, and cyclophosphamide-induced myelosuppression in mice has been reported but the cellular signaling molecules of immunostimulant activity with special reference to splenocytes proliferation, and cytokines production is yet to be elucidated. Therefore, this study was undertaken to unravel the of immunomodulatory activity of this test plant with particular reference to splenocytes proliferation and cytokines induction.

Materials and Methods

Plant Material
Flowers of N. arbor-tristis were collected from Veterinary College Campus, Mathura and got authenticated from Prof. A. K. Agrawal, Head Department of Botany, B. S. A Degree College, Mathura, UP, India based on taxonomic features of the whole plant material including flowers.

Preparation of Extract
Hot aqueous extract of shade dried and coarsely powdered N. arbor-tristis flowers (NAFE) was prepared in soxhlet apparatus by hot percolation method, and it was concentrated to dryness using rotatory evaporator under reduced pressure and low temperature (<40°C). The extract was kept in air-tight containers and stored at 4°C for further studies.

Experimental Animals
Swiss albino mice (18–25 g) and Wistar rats (100–120 g) of either sex were procured from Laboratory Animal Resource Section, Indian Veterinary Research Institute, Izatnagar and maintained in laboratory animal house. An acclimatization period of 15 days was allowed before the start of the experiment. Animals had free access to clean drinking water and pelleted laboratory animals feed and light and dark cycle of almost 12 h was maintained. The experimental protocol was approved by the Institutional Animal Ethics Committee.

Humoral immune response
Experimental mice were divided into four groups of eight animals each. Mice of Group I (negative control) received only distilled water, Group II served as positive control and immunized with Salmonella typhimurium “O” antigen. Mice of Group III and IV were orally administered NAFE at 400 and 800 mg/kg, respectively for 21 days. Thereafter, mice of both the groups were immunized with Salmonella “O” antigen along with continuance of NAFE administration at 400 and 800 mg/kg. The first dose of S. typhimurium “O” antigen (0.5 ml) was administered subcutaneously at 3–4 sites on day 1. The first booster of same antigen was administered on 7th day and the second booster on 15th day by the same procedure as described earlier.

Blood samples from mice of all the groups were collected by retro-orbital plexus puncture 15 days after the second booster dose, and serum was separated for determining antibody titer by tube agglutination test and indirect ELISA method using commercially available kit. Optical density (OD) of each well was measured at 450–570 nm using ELISA reader (SPAN, India) and the titer was determined as per the method described by Trusfield.

Cell-mediated immune response
Cell-mediated immune (CMI) response in rats was determined following the method of Tiwary and Goel. Rats of either sex were randomly divided into three groups of five animals each. Group I served as control and received only distilled water. Group II and III animals were orally administered NAFE at 400 and 800 mg/kg body weight (b.wt.), respectively and simultaneously, DNCB (1-Chloro, 2,4-dinitrochlorobenzene), a well-established control antigen (allergen) was applied locally. The site of DNCB application was examined for erythema, inductions, and vesicle formation. Thickness of the skin at the DNCB application site was measured at 0, 6, 12, 24, 36, 48, 60, and 72 h post-DNCB challenge using a Vernier caliper. Comparing the pre- and post-DNCB application skin thickness, change in skin thickness was determined and expressed in mm.

Splenocytes proliferation assay
Ex vivo and in vitro effect of NAFE on splenocytes proliferation was determined employing the procedure described earlier.

Ex vivo studies
Adult rats of either sex were randomly divided into three groups of six animals each. Rats of Group I (control) were administered triple glass distilled water while those of Group II and Group III were orally administered NAFE at 400 and 800 mg/kg b.wt., respectively for 21 days. After 21 days, animals of all the three groups were sacrificed, and splenocytes were harvested. Two hundred microliter of the cell culture was transferred to each well in a flat bottom culture plate. Two micrograms of concanavalin-A (Con-A) was added to each well except blank which contained only cell culture. Culture plate was incubated at 37°C in CO₂ incubator (5% CO₂; 80% relative humidity for 72 h. After incubation, the supernatant was removed, and the plate was air-dried. Cell culture grade dimethyl sulfoxide (50 µl) was added in each well to dissolve the formazone crystals, formation of which is directly proportional to the number of viable cells. Live cells reduce tetrazolium salts to colored formazone compound while the dead cells do not form formazone crystals. OD of each well of the culture plate was measured at dual wavelengths of 560–670 nm using ELISA reader and percent stimulation index was calculated using the following equation and compared with the OD of control well having no extract.

\[
\text{Stimulation index} = \left( \frac{\text{OD of the well with Con-A}}{\text{OD of the well without Con-A}} \right) \times 100
\]
**In vitro studies**

For evaluation of in vitro effect of NAFE, test extract was filtered through 0.2 micron membrane filter, and different dilutions (50, 100, 200, 400, 800, and 1600 µg) of NAFE were added into different wells of the culture plate containing 200 µl of spleen cells (2 x 10^6 cells/ml) in RPMI-1640 medium, except the control which contained only splenocytes culture. Con-A (2 µg) was also added in all the wells except blank, and rest of the procedure was same as described in ex vivo studies.

**Cytokines (interleukin-2 and interleukin-6) induction**

**Ex vivo studies**

For ex vivo studies, adult rats of either sex weighing 100–120 g were randomly divided into three groups of eight animals each. Rats of Group I (control) were administered triple glass distilled water while those of Group II and Group III were orally administered NAFE at 400 and 800 mg/kg b.wt., respectively for 21 days. Animals of all the three groups were humanely sacrificed on the 22nd day, and spleen was aseptically collected for harvesting splenocytes. 200 µl spleen cells (2 x 10^6 cells/ml) in RPMI-1640 medium were placed in each well of culture plate and 2 µg of Con-A each was added in all the wells except blank. Interleukin (IL-2) and IL-6 cytokine levels were determined in the supernatant collected from cultured splenocytes of different treatment groups using the commercially available ELISA kits (Thermo Scientific).

**In vitro studies**

For in vitro studies on the effect of NAFE on cytokines (IL-2 and IL-6) induction in splenocytes, splenocytes were isolated from healthy untreated rats. Nyctanthes flowers extract was filtered through 0.2 micron membrane filter and different concentrations (50, 100, 200, 400, 800 and 1600 µg/ml) of NAFE were added into different wells of culture plate containing 200 µl of spleen cells (2 x 10^6 cells/ml) in RPMI-1640 medium while the control well contained only splenocytes. 2 µg of Con-A each was added in all the wells except blank and levels of cytokines (IL-2 and IL-6) were determined in supernatants collected from cultured splenocytes as described above.

**Results**

**Humoral Immune Response**

Antibody titers in mice of different groups against S. typhimurium “O” antigen using tube agglutination test and indirect ELISA test are summarized in Table 1. Perusal of antibody titers presented in Table 1 revealed that the negative control group, serum antibody titer was markedly, but nonsignificantly, higher in lower NAFE dose (400 mg/kg) group. However, compared to the control and 400 mg/kg NAFE-treated groups, it was significantly (P < 0.01) higher in higher dose NAFE group (800 mg/kg) by both the assay procedures.

**Cell-mediated Immune Response**

Skin thickness data of mice of control and NAFE-treated groups (400 and 800 mg/kg) at 0 and 6, 12, 24, 36, 48, 60, and 72 h after last DNCB application are presented in Table 2. Statistical analysis of the data revealed that skin thickness in NAFE-treated (800 mg/kg) group differed significantly (P < 0.01) from that of control group as skin thickness was significantly more at 6, 24, and 48 h and also between 400 and 800 mg/kg NAFE-treated groups at certain time intervals as shown in Table 2.

**Table 1: Effect of simultaneous administration of Nyctanthes arbor-tristis flowers (400, 800 mg/kg) for 21 days and Salmonella typhimurium O antigen on humoral immune response in mice by tube agglutination test and indirect ELISA assay in mice**

| Groups/treatment | Tube agglutination test | Antibody titer |
|------------------|-------------------------|----------------|
| Control negative | 720.00±293.16a          | 1650.00±373.69a|
| Control positive | 920.00±141.02a          | 3400.00±705.08a|
| (S. typhi O antigen) |                        |                |
| NAFE (400 mg/kg) | 2000.00±546.78b         | 6000.00±1021.22b|
| NAFE (800 mg/kg) | 3600.00±453.80b         | 22000.00±7089.63b|

**Table 2: Effect of oral administration of Nyctanthes arbor-tristis flowers (400, 800 mg/kg) for 21 days on di-nitrochloro-benzene-induced delayed type hypersensitivity response in rats**

| Skin thickness in (mm) | Control | NAFE 400 mg/kg | NAFE 800 mg/kg |
|------------------------|---------|----------------|----------------|
| Before DNCB application| 0.98±0.02a | 1.08±0.11a  | 1.00±0.00a     |
| Before last DNCB application (0 h) | 1.15±0.12a | 1.27±0.19a  | 1.38±0.17a     |
| 6 h after last DNCB challenge | 1.64±0.10a | 1.76±0.13a | 2.56±0.19a     |
| 12                     | 2.18±0.09a | 2.25±0.17a  | 2.51±0.18a     |
| 24                     | 1.60±0.14a | 2.54±0.20a  | 2.12±0.10a     |
| 36                     | 1.40±0.10a | 1.52±0.15a  | 1.58±0.14a     |
| 48                     | 1.22±0.08a | 1.24±0.11a  | 1.68±0.07a     |
| 60                     | 1.04±0.04a | 1.30±0.12a  | 1.28±0.10a     |
| 72                     | 0.98±0.02a | 1.12±0.05a  | 1.24±0.11a     |

**Effect of Nyctanthes arbor-tristis Flowers on Splenocytes Proliferation**

**Ex vivo effects**

Data on the effect of oral feeding of rats with NAFE (400 and 800 mg/kg) for 21 days on splenocytes proliferation without sensitization with Con-A or after sensitization with Con-A are presented in Table 3. Compared to the control group, NAFE significantly (P < 0.01) stimulated splenocytes proliferation at both the dose levels (400 mg/kg and 800 mg/kg) as there was significant (P < 0.05) increase in OD and stimulation index in both groups and it was similar to that observed after sensitization with Con-A.

**In vitro effects**

Effect of in vitro exposure of splenocytes to different concentrations of NAFE (50–1600 µg/well) and Con-A (2 µg) and resultant alteration in OD and stimulation index compared to those in control groups are summarized in Table 4. Perusal of results revealed that compared to the OD of control
group, there was significant \( P < 0.05–0.01 \) increase in OD in all the NAFE-treated splenocytes wells and it was almost comparable to that induced by Con-A. Similarly, stimulation index following NAFE-exposure was also found to be almost comparable to that of Con-A, but stimulation index in different NAFE-treated groups did not differ significantly from each other, i.e. no concentration-dependent effect was observed as evident from data presented in Table 4.

### Effect of Nyctanthes arbor-tristis Flowers on Cytokines (Interleukin-2 and Interleukin-6) Induction

#### Ex vivo studies

Data on cytokines (IL-2 and IL-6) levels in splenocytes of rats of NAFE-treated (400 and 800 mg/kg) groups and control group are presented in Table 5. IL-2 levels (pg/ml) in splenocytes of NAFE-treated groups (400 mg/kg and 800 mg/kg) were found to be 985.81 ± 7.85 and 1012.41 ± 7.39, respectively and these values were significantly \( (P < 0.001) \) higher from those of control group (948.58 ± 6.39). Even the IL-2 value in higher dose treatment group (800 mg/kg) was significantly \( (P < 0.01) \) higher than in 400 mg/kg treatment group \([23,24]\). Similarly, compared to control group, IL-6 values were also significantly \( (P < 0.01) \) higher in NAFE-treated groups (400 and 800 mg/kg), but contrary to IL-2, effect on IL-6 values was not dose-dependent \([23,24]\).

#### In vitro studies

Data on the effect of in vitro treatment of rats splenocytes with different concentrations of NAFE (50, 100, 200, 400, 800, and 1600 µg/well) on IL-2 and IL-6 levels in splenocytes are presented in Table 6. Perusal of data revealed that compared to control, there was a significant increase in IL-2 and IL-6 IL-2 levels (pg/ml) at almost all the used concentrations, but the effect was not concentration-dependent.

### Discussion

Marked to significant \( (P < 0.01) \) increase in serum antibody titer in mice against S. typhimurium “O” antigen following treatment with NAFE is suggestive of the promising immunomodulatory potential of aqueous extract of Nyctanthes flowers. Our findings are in confirmation with the results of other workers\([15,16,21]\) who have reported an increase in humoral immune response to sheep RBCs and macrophage migration index in Balb/c albino mice following treatment with ethanolic extract of Nyctanthes flowers, seeds, and roots and also increase in antibody titers, numerical values of immunocytes, and functions of phagocytes following treatment with Nyctanthes leaves extracts.\([16,21]\) Immunoimmunomulatory potential of Nyctanthes leaves, seeds, and flowers have also been reported based on the carbon clearance test in cyclophosphamide-induced myelosuppression in mice,\([21]\) against systemic candidiasis\([21]\) and pesticides toxicity.\([22]\)

Cytokines are immunological signals between cells and amplify both the local and systemic host responses; therefore, increase in interleukins is considered as a biomarker of increased immune response. Cytokines are necessary for stimulation of T and B lymphocytes. T-helper lymphocytes (Th) differentiate into Th1 and Th2 cells; Th1 are responsible for pro-inflammatory cellular immunity and express IL-2 and mediate humoral immunity while Th2 cells express IL-6 and mediate humoral immunity,\([23,24]\) by differentiating B-cells to plasma cells to generate antibodies. Comparison of the cytokines levels in NAFE treatment groups with those in control group revealed significant \( (P < 0.05–0.001) \) increase in IL-2 and IL-6 levels by NAFE, which corroborates well with significant increase in skin thickness and antibody titers, respectively. IL-2 activates T-cells, especially Th cells and provokes action of large granular lymphocytes and natural killer cells\([23]\) while IL-6, being a multifunctional lymphokine, regulates humoral immune response by stimulating B-lymphocytes and their differentiation into plasma cells.\([23,24]\)

Compared to controls, significant \( (P < 0.05–0.001) \) increase in IL-2 and IL-6 levels in NAFE-treated splenocytes (50–1600 µg/well) in this study further substantiates the immunomodulatory potential of NAFE even at very low concentrations (50 µg).

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**Table 3:** Effect of oral administration of *Nyctanthes arbor-tristis* flowers (400, 800 mg/kg) for 21 days on *ex vivo* splenocytes proliferation in rats

| Parameters       | Control | NAFE (400 mg/kg) + Con-A (5 mg/ml) | NAFE (800 mg/kg) + Con-A (5 mg/ml) | NAFE (mg/kg) |
|------------------|---------|-----------------------------------|-----------------------------------|--------------|
| OD               | 0.65±0.01† | 4.65±0.08b, †              | 4.21±0.035a, †              | 4.78±0.04†  |
| Stimulation index | -       | 4.35±0.025†                | 0.69±0.001b                 | 4.35±0.025  |

\( *P<0.001 \), Data presented are mean±SE of six animals in each group. Different superscripts in row differ significantly. Con-A=Concavalin A, OD=Optical density, SE=Standard error, NAFE=Nyctanthes arbor-tristis flowers

**Table 4:** Effect of *in vitro* treatment of splenocytes with *Nyctanthes arbor-tristis* flowers at 50, 100, 200, 400, 800, and 1600 µg/well on rats splenocytes proliferation

| Treatment (without Con-A) | OD | Stimulation index |
|---------------------------|----|------------------|
| Control                   | 0.71±0.06† | -               |
| Con-A (2 µg/well)         | 0.57±0.004** | 4.08±0.03**      |
| NAFE (50 µg/well)         | 0.63±0.025c** | 4.45±0.018       |
| NAFE (100 µg/well)        | 0.61±0.024d** | 4.33±0.17        |
| NAFE (200 µg/well)        | 0.60±0.011e** | 4.00±0.098      |
| NAFE (400 µg/well)        | 0.66±0.022c,d,e | 4.68±0.15       |
| NAFE (800 µg/well)        | 0.68±0.020c,d,e | 4.79±0.134     |

\( *P<0.05, *P<0.001 \). Data presented are mean±SE of six observations. Different superscripts in column differed significantly. Con-A=Concavalin A, OD=Optical density, SE=Standard error, NAFE=Nyctanthes arbor-tristis flowers
Table 5: Effect of oral administration of *Nyctanthes arbor-tristis* flowers (400, 800 mg/kg) to rats for 21 days on interleukins-2 and interleukins-6 induction in splenocytes

| Parameters | Control | NAFE-treated group |
|------------|---------|---------------------|
|            | 400 mg/kg | 800 mg/kg |
| IL-2 (pg/ml) | 948.58±6.39<sup>a</sup> | 985.81±7.85<sup>b</sup> |
| IL-6 (pg/ml) | 715.67±10.94<sup>b</sup> | 778±15.30<sup>bc</sup> |

<sup>a</sup>P<0.05, <sup>b</sup>P<0.01, <sup>c</sup>P<0.001. Data presented are mean±SE of six animals in each group. Different superscripts in a row differed significantly. IL=Interleukin(s), SE=Standard error, NAFE=*Nyctanthes arbor-tristis* flowers

Table 6: Effect of *in vitro* exposure of rat splenocytes to different concentrations of *Nyctanthes arbor-tristis* flowers on interleukins-2 and interleukins-6 cytokines induction

| Treatment | IL-2 (pg/ml) | IL-6 (pg/ml) |
|-----------|--------------|--------------|
| Control   | 948.88±6.39<sup*a</sup> | 715.69±10.94<sup*a</sup> |
| NAFE (50 µg/well) | 983±6.11.64<sup>bc</sup> | 764.81±11.09<sup>bc</sup> |
| NAFE (100 µg/well) | 979.26±5.02<sup>c</sup> | 793.68±8.94<sup>c</sup> |
| NAFE (200 µg/well) | 1015.96±6.99<sup>bc</sup> | 837.98±17.43<sup>d</sup> |
| NAFE (400 µg/well) | 1003.55±7.73<sup>a</sup> | 833.65±8.77<sup>d</sup> |
| NAFE (800 µg/well) | 993.83±6.64<sup>b</sup> | 782.22±4.83<sup>β</sup> |
| NAFE (1600 µg/well) | 998.67±5.86<sup>β</sup> | 773.85±10.43<sup>β</sup> |

Therefore, results of our *ex vivo* and *in vitro* studies evidently suggest that cytokines signal/modulate immunostimulant activity of NAFE. *Ex vivo* and *in vitro* studies on splenocytes proliferation also revealed significant increase in optimal density and stimulation index following treatment with NAFE, which was almost comparable to that observed with Con-A. This observation also amply adds credence to the potential of NAFE to exhibit immunostimulant activity through splenocytes proliferation.

*Nyctanthes* flowers extract is rich in diterpenoids, nycanthin, nycanthoside, rengolone, astragalin, flavonoids, anthocyanins, d-mannitol, essential oils, glycosides, carotenoids,<sup>11-18</sup> and most of these phytoconstituents possess potent antioxidant and free radicals scavenging activity and overall immunostimulant activity.<sup>17</sup>

**Conclusion**

Therefore, based on the information available in literature and also our findings, *Nyctanthes* flowers seem ideal for formulation of a safe and effective herbal immunomodulator and/or it can even be considered as a constituent of functional foods in modern world as herbal immunomodulators are becoming very popular in the world of natural health as these do not boost immunity, but normalize immunity. However, further studies on immunomobioactive mechanisms, especially the cross-talk mechanism(s) between splenocytes, cytokines, and cells mediating humoral and cell-mediated immunity are warranted.

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