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Original Research Article

Immunomodulatory potential of *Nyctanthes abrortristis* stem bark

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ABSTRACT

Background: Phytotherapeutic modulation of the immune system to mitigate infectious ailments has been in vogue all over the world.
Objective: The present work has been designed to scientifically explore the immunomodulatory potential of *Nyctanthes arbortristis* stem bark using mice models.
Materials & method: Methanolic (MNA) and aqueous (ANA) extracts of *N. arbortristis* stem bark were evaluated for possible modulation in humoral immunity through serum immunoglobulin estimation. The variation in cellular immunity was assessed using neutrophil adhesion test, carbon clearance assay, and cyclophosphamide-induced neutropenia.
Results and discussion: Administration of MNA and ANA (both at 200 mg/kg, p.o.) significantly augmented the levels of serum immunoglobulins (humoral antibody), neutrophil adhesion, and phagocytic index (a measure of carbon clearance). Extracts also guarded the animals against cyclophosphamide-induced leukopenia, especially neutropenia.
Conclusion: Results indicate that cellular and humoral immune responses were aroused by pretreatment of the animal with methanol and aqueous extract of *N. arbortristis*. Thus, the methanol and aqueous extract of *N. arbortristis* stem bark possesses a significant immunostimulant activity and can be used to uplift the immune system in the infectious condition.

1. Introduction

Immunomodulators are the substances used to modulate the response of our immune system and broadly classified according to their effects such as immune stimulators (activation), suppressors (deactivation), and immunoadjuvant (boost efficacy of vaccines) [1–4]. To amend the immune system, several biomolecules such as monoclonal antibodies, synthetic chemical entities, *etc.* are employed [5]. However, these drugs are beyond the reach of poor people because of price constraints, and to the maximum instance accompanying adverse drug responses. Due to the limitation of these synthetic biomolecules, phytoremedies are considered to be the potential candidate to oust them in therapeutic regimens as immune-modulators. Consequently, most of the people exclusively the rural dwellers of the developing countries practice plants as folkloric medicines, owing to their advantages such as safety, effectiveness, accessibility, and low cost [5–9].

Phyto-remedies can modify the immune system and may contribute as a supportive therapy with conventional medicines in immune-compromised patients [10]. Strong immunomodulatory effect has been documented for several secondary plant metabolites such as 14-deoxy-11,12-didehydroandrographolide [11], Shikonin [12], Dibenzyl trisulphide [13], Camptothecin [14], Quercetin [15], Curcumin [16], Resveratrol [17], epigallocatechol-3-gallate [18], capsaicin [5], Genistein [19], *etc.* Recently gathered evidence has suggested that phytoremedies are generating renewed devotion in search and development of immunomodulators drug predominantly in the prevention and treatment of some chronic ailments [20].

Nyctanthes arbortristis Linn., also known as 'Night jasmine' or 'Harsinghar' is a wonderful plant belongs to family Oleaceae. The plant has found widespread use in the indigenous system of medicine [21]. It is native to southern Asian countries such as India, where it grows wild at an altitude of 1500 m mainly in sub-Himalayan regions [6,22]. Folk healers employed this plant as

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anodyne, anti-inflammatory, anthelmintic, bitter tonic, expectorant, digestive diuretic, immunomodulator, laxative, *etc*. It is also used in tribal herbal medicine to cure asthma, arthritis, baldness, hepatic disorder, rheumatism, *etc.* [6,21,23,24].

The plant has several uses in *Ayurveda* and Integrating system of medicine. In *Ayurveda*, leaves of this plant is used as decoction in the treatment of arthritis, malaria, fungal skin infection. Young leaves are used as female tonic in alleviating gynecological problem [25].

The plant is bestowed with several imperative pharmacological activities such as anticholinesterases [26], anti-tumor [27], anti-plasmodium [28], anti-viral [29,30], antimicrobial [24,31,32], tranquilizer, antihistaminic, purgative [33], hepatoprotective [34], CNS depressant [35], anti-oxidant [36], larvicidal [37], wound healing, anti-diabetic [38], antimalarial activity [39].

The plant is enriched with numerous bioactive potent phytoconstituents such as nyctanthin (alkaloids), arbortristosides- A, B [40], C, D, E [41], and β-hydroxyloganin (iridoid glucoside) [42], arborside- A, B, C (benzoic acid loganin) [43], *etc.*

2. Materials and methods

2.1. Collection, identification & procurement of plant material

The stem bark of the plant was collected from the campus of GJUS&T, Hisar, India and was taxonomically identified and characterized as *N. arbortristis* Linn. by Dr. H.B. Singh, Head, Raw Materials, Herbarium, and Museum Division, NISCAIR, New Delhi, vide reference no, NISCAIR/RHMD/CONSULT/2009–10/1282/86, dt. 07/10/09, and a voucher specimen of the same has been preserved in the herbarium for future reference. The gathered stem bark was cleaned, dried under shade, and pulverized with the help of a manual grinder and passed through the sieve (mesh size # 20–30). The plant material was stored at room temperature for further experimentation.

2.2. Preparation of extract and doses

The coarsely powdered stem bark (1 kg) was defatted using petroleum ether and successively extracted with methanol and distilled water utilizing the Soxhlet apparatus (for 72 h) and maceration process (7 days), respectively. The entire bulk was filtered and concentrated to dryness utilizing a rotary evaporator under reduced pressure (Buchi, Switzerland). The extract yielded a yellow-brown, solid methanol extract (MNA; 14.67% w/w) and dark brown, solid aqueous extract (ANA; 10.13% w/w) of *N. arbortristis*. The extracts were stored in a desiccator for subsequent experiments. The doses of methanol and aqueous extract were prepared in 1 %w/v aqueous carboxy methyl cellulose (CMC), which was taken as a control group.

2.3. Chemical and reagents

Leishmann's stain was procured from Sigma Aldrich. WBC diluting fluid was purchased from Nice Chemical, Cochin India, and Indian Ink from Loba Chemie Pvt. Ltd, Mumbai, India. Cyclophosphamide was purchased as Ledoxan Injection from Dabur Pharmaceuticals; India and all additional chemicals and reagents were of analytical grade and procured from a reputed commercial supplier.

2.4. Preliminary qualitative screening of phytochemicals

The methanol and aqueous extracts of the bark were assessed for the presence of phytochemicals using standard procedures [44–49].

2.5. Animal and ethics statement

Swiss Albino mice (either sex), age 6–8 weeks, and weight 25–30 g were employed in the present study. The animals were kept in polypropylene cages at optimum temp (25 ± 2 °C) and humidity (55 ± 5%) under a 12-h light: 12-h dark regime. They were fed with commercially available chow pellet diet (Hindustan Lever Ltd., Mumbai, India) and water *ad libitum*. The experimental research protocol was approved by the Institutional Animals Ethics Committee (IAEC) of GJUS&T, Hisar (Regn. no. 0436/PORe/S/2001). The animals were maintained as per CPCSEA, Govt. of India, guidelines.

2.6. Acute toxicity study

Acute toxicity study of methanol (MNA) and aqueous (ANA) extract of *N. arbortristis* was performed as per the Organization for Economic Co-operation and Development (OECD) 423 guidelines [50]. By random sampling, the Swiss Albino mice (n = 6) were selected and administered with MNA and ANA at the dose of 2000 mg/kg b.*w./p.o.*, respectively. Animals were visually examined perpetually for the first 2 h followed by every hour up to 6 h and daily thereafter fourteen days for any designations or symptoms of morbidity and mortality. The observations of autonomic profile (urination, defecation), neurological changes (touch, pain response, gait, spontaneous activity, reactivity), and behavioral changes (restlessness, fearfulness, alertness, irritability) were recorded [51].

2.7. Effect on serum immunoglobulins [52,53]

Animals were randomly divided into three groups (n = 6). The animals of the first (control) group were administered 1% aqueous CMC suspension orally as a vehicle. The animals of the second (MNA) and third (ANA) group received 200 mg/kg, *p.o.* of methanol, and aqueous extract of *N. arbortristis*, respectively for 21 days (once daily). Six hours, after the last dose, blood was collected by puncturing retro-orbital plexus with a capillary tube, under light ether anesthesia. The collected blood samples were centrifuged to separate the serum, and employed for the valuation of immunoglobulin levels. Serum (0.1 ml) was pipetted out to a plugged test tube containing 6 ml of distilled water (Blank tube) and 6 ml of zinc sulfate solution (Sample tube). The test tube was inverted to allow complete mixing of the content for 1 h at room temperature. The pH of the solution was monitored (7.2) during the experimental period utilizing pH meter. The turbidity developed was measured using a UV–Visible spectrophotometer (Shimadzu, UV-1800, Japan) at wavelength 550 nm and expressed as zinc sulphate units (ZST). The obtained ZST value was converted to g/L immunoglobulin using the following formula

$$\text{Zinc sulphate turbidity (ZST units)} = \text{OD of Z tube} - \text{OD of control tube} \times 10$$

$$\text{Total immunoglobulin (g/L)} = 0.04 + 0.98 \times \text{ZST units}$$

2.8. Neutrophil adhesion test [52–55]

Animals were randomly divided into three groups (n = 6). The first (control) group of animals were administered 1% aqueous CMC suspension orally as a vehicle. Second (MNA) and third (ANA) group of animals received 200 mg/kg, *p.o.* of methanol, and aqueous extract of *N. arbortristis*, respectively for 14 days (once daily). At the end of the 14 days of drug treatment, blood was withdrawn into

heparinized vials by puncturing retro-orbital plexus with a capillary tube, under light ether anesthesia. The collected blood samples were analyzed for total leucocyte count (TLC) and differential leukocyte count (DLC) after mixing the blood smear and staining with the Leishman's stain (Improved Neubauer Chamber, Rohan, India), the result of this initial count was noted down. The blood samples were incubated at 37 °C for 10 min with nylon fibers (80 mg/ml) and once again analyzed for TLC and DLC respectively. Neutrophil index (NI) of blood samples was calculated by multiplying TLC with % neutrophil (NI = TLC × % neutrophil). The % neutrophil adhesion (%NA) was calculated utilizing the below–given equation:

$$\text{Percentage Neutrophil Adhesion}(\%NA) = \frac{NI_u - NI_t}{NI_u} \times 100$$

NI_u is the neutrophil index of untreated blood samples and NI_t is the neutrophil index of nylon treated blood samples.

2.9. Carbon clearance test [56–58]

Animals were randomly divided into four groups (n = 6). The first (normal) and second (control) group of animals were administered with 1% aqueous CMC suspension orally as a vehicle. Third (MNA) and fourth (ANA) group of animals received 200 mg/kg, *p.o.* of methanol, and aqueous extract of *N. arbortristis*, respectively for 5 days (once daily). On 7th day *i.e.* 48 h after the last dose, animals of all group (except normal group) were injected with 10 μl/g b.*w.* of Indian ink *via* the tail vein. Blood was withdrawn at 0 min and 15 min instantly after ink injection into heparinized vials, by puncturing retro-orbital plexus with a capillary tube, under light ether anesthesia. The individual aliquot (50 μl) was lysed with 4 ml of 0.1% sodium carbonate solution and followed by the quantification of absorbance to calculate the optical density of the mixture spectrophotometrically at 660 nm. The phagocytic index *i.e.* rate of carbon clearance (K) was calculated utilizing the below–given equation:

$$\text{Phagocytic Index (K)} = \frac{\text{Log}_e OD_1 - \text{Log}_e OD_2}{15}$$

where OD_1 and OD_2 are the optical densities at the time 0 and 15 min after blood collection, respectively.

2.10. Cyclophosphamide induced immunosuppression [52,53,55,56]

The animals were divided into 4 groups; first (normal) and second (control) group of animals were administered 1% CMC solution for 10 days, whereas third (MNA) and fourth (ANA) group of animals were administered with 200 mg/kg, *p.o.* methanol and aqueous extract of *N. arbortristis* bark, respectively for 10 days. The blood sample was withdrawn through retro-orbital plexus, 2 h after the last dose, and analyzed for total leucocyte count (TLC) and differential leukocyte count (DLC). Whereas, on the 11thday neutropenic dose of cyclophosphamide (200 mg/kg b/w *i.p.* prepared in 1% aqueous CMC) was injected to 2nd, 3rd and 4th group of animals. Treatment of animals with control and test solution was continued for the next 3 days (*i.e.* 11th to 13th day), hereafter the blood samples were again collected from retro-orbital plexus and analyzed for TLC (cells/μL) and DLC (cells/μL). The difference in TLC and neutrophil count (cells/μL) of test sample were calculated and compared with the control and challenge group**.**

2.11. Statistical analysis

Data were expressed as Mean ± SEM and the statistical analysis was carried out by one-way analyses of variance (ANOVA) followed by Dunette's test utilizing Graph Pad Prism software 7. Value of the P less than 0.05 ($p \leq 0.05$) was considered statistically significant.

3. Results

3.1. Preliminary qualitative screening of phytochemicals

The screening divulges the presence of carbohydrates, glycosides, phenolic compounds, flavonoids, protein and free amino acids, tannins in both methanol and aqueous extracts of the bark. However, saponins were found to be present only in aqueous extracts.

3.2. Acute toxicity study

Acute toxicity study data revealed no evident sign of toxicity or mortality at the dose regimen until fourteen days of the study. According to OPPT guidelines [59], the 1/10th of the maximum safe dose (2 g/kg) was selected for *in-vivo* pharmacological evaluation. Hence in the present investigation 200 mg/kg dose of methanol and aqueous extract was selected for further investigation.

3.3. Serum immunoglobulin level

Administration of MNA and ANA triggered a significant ($p \leq 0.01$) boost in serum immunoglobulin levels and the response was 0.6448 ± 0.0639 mg/ml & 0.7354 ± 0.0847 mg/ml respectively, compared to control with 0.1319 ± 0.0248 mg/ml (Fig. 1).

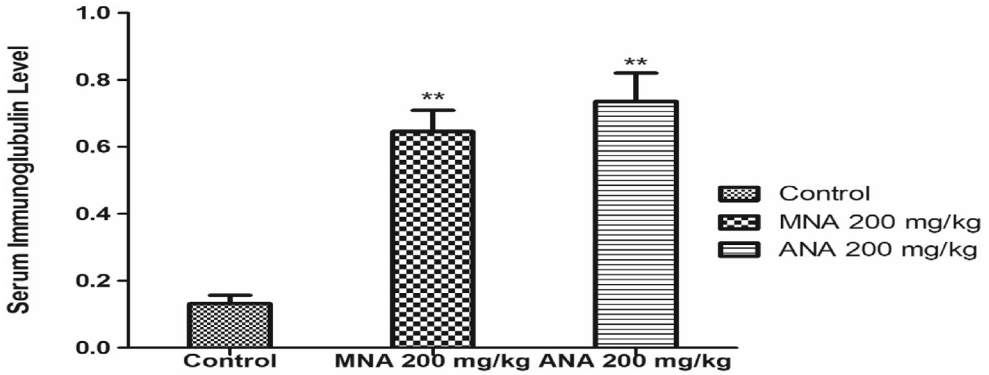

Fig. 1. Effect of MNA and ANA on serum immunoglobulin level. Value indicates mean ± SEM (n = 6). **P ≤ 0.01, significant compared to control group.

Table 1
Effect of MNA and ANA on neutrophil adhesion.

Sr. no.	Treatment	TLC		Reduction of TLC (%)	Neutrophil count (%)		Reduction of neutrophils (%)	Neutrophil index		
		Untreated blood (A)	Treated blood (B)		Untreated blood (C)	Treated blood (D)		Untreated blood (NI_u) (A × C)	treated blood (NI_t) (B × D)	Neutrophil Adhesion (%)
1	Control	7680± 152.97	7220± 124.10	5.98	17.60 ± 0.9274	12.80 ± 0.5831	27.27	135,600 ± 9173.9	92,540 ± 5045.8	31.75
2	Methanolic extract (200 mg/kg)	9120 ± 292.23	8180 ± 270.92	10.30	22.80 ± 1.314	14.00 ± 1.300	38.59	200,360 ± 11,012	115,020 ± 12,642	42.59*
3	Aqueous extract (200 mg/kg)	8700 ± 251.95	7800 ± 242.90	12.64	23.00 ± 1.88	11.80 ± 0.86	48.69	198,600 ± 11,838	92,320 ± 8123.4	53.01**

Value indicates mean ± SEM (n = 6). *$p \leq 0.05$, **$p \leq 0.01$, significant compared to control group.

3.4. Neutrophil adhesion test

Blood samples and nylon fibers incubation caused adhesion of neutrophils to the fibers which lead to a decline in the neutrophil count. Prior therapy of mice with MNA ($p \leq 0.05$) and ANA ($p \leq 0.01$) both at 200 mg/kg evoked a significant growth in neutrophil adhesion with 42.59% and 53.01%, respectively, compared with the control group 31.75% (Table 1).

3.5. Carbon clearance assay

The phagocytic index is the measure of carbon particle clearance from the blood. From the result, we found that animals of the control group engendered a significant ($p \leq 0.01$) decline in the phagocytic index (↓67%) compared with the normal animals. Pretreatment of mice with MNA ($p \leq 0.05$) and ANA ($p \leq 0.01$) aroused significant upsurge in the phagocytic index (K) with ↑66% and ↑116% respectively (Table 2).

3.6. Cyclophosphamide-induced neutropenia

Administration of cyclophosphamide (200 mg/kg *i.p.*) to the control group of animals significantly ($p \leq 0.01$) reduced TLC (72.21%) and neutrophils (86.25%) compared to animals of the normal group. Prior therapy with MNA 200 mg/kg, *p.o.* significantly ($p \leq 0.01$) guarded the animals against cyclophosphamide triggered drop in TLC (51.46%) and neutrophil count (62.79%), compared to animals of the control group. Similarly, the decline in TLC (37.42%) and neutrophil count (55.43%) was significantly ($p \leq 0.01$) shielded by the preliminary administration of ANA 200 mg/kg, *p.o.*, compared to control (Table 3).

4. Discussion

The outcome of present investigation recommends that methanol, as well as aqueous extract of stem bark of N. arbortristis at 200 mg/kg, may kindle cell facilitated immunity, as revealed by the gain in neutrophil adhesion to nylon fibers, escalation in macrophage persuaded phagocytosis in carbon clearance test and drop in cyclophosphamide-initiated neutropenia. The extracts moreover, aroused humoral immunity, demonstrated by an ascension in serum immunoglobulin levels.

Table 2
Effect of MNA and ANA on phagocytic index.

Treatment	Phagocytic index	% change
Normal	0.018 ± 0.003	–
Control	0.006 ± 0.001##	↓ 67%
Methanolic extract (200 mg/kg)	0.010 ± 0.002*	↑66
Aqueous extract (200 mg/kg)	0.013 ± 0.003**	↑ 116

Value indicates mean ± SEM (n = 6).
##$p \leq 0.01$, significant compared to normal group. *$p \leq 0.05$, **$p \leq 0.01$, significant compared to control group.

The level of serum immunoglobulin parallels the serum antibodies level and is the measure of humoral immunity. Immunoglobulins are a serum molecules group formed by β-lymphocytes and secreted from the β-cell receptors. Immunoglobulins, so-called antibodies are formed in an immensely colossal number to contravene the assailment of antigen. Based on the electrophoretic migration rate, globulins are classified as α, β, and γ [60]. The zinc sulfate turbidity test is expeditiously employed to assess the immunoglobulins present in the serum. Zinc sulfate solution lysed with the serum causes immunoglobulins precipitation, and produce turbidity in the solution. The absence of turbidity indicates the absence of immunoglobulins and vice versa [61,62]. MNA, as well as ANA, significantly upsurge the serum immunoglobulin levels.

β_2 Integrins, the leukocyte-specific adhesion molecules are the leukocyte receptor found on the surface of neutrophil, their activation facilitates adhesion of neutrophil across the endothelium that endorses their trafficking at the site of action. β_2 integrins are accountable for assorted neutrophil functions decisive for innate immunity [63]. Neutrophils adhesion towards nylon fibers designates the immigration of neutrophil granulocytes in the blood vessels and the neutrophils number gathering at the site of inflammation. A significant ascent in neutrophil adhesion may be due to the β_2 integrins upregulation, by which neutrophil firmly adheres to the nylon fibers [54]. Therefore, it can be inferred that methanol and aqueous extract of *N. arbortristis* bark may cause the stimulation of neutrophils towards the site of inflammation.

Reticuloendothelial system (RES) is a diffused structure consisted principally of phagocytic cells, which can produce phagocytosis, a vital protagonist in the immunoregulatory network [64]. The main purpose of phagocytosis is to avert pathogenesis of several ailments by amputating foreign invaders together with the abolition of dead, injured, and malignant cells [65]. Exogenously injected antigens such as Indian ink *i.e.* preparation of colloidal carbon particle, are recognized as foreign invading particles and amputate by RES through the process of phagocytosis mainly in the liver and to the small extent in the spleen. Thus, the phagocytic index, the measure of carbon clearance rate is related to the phagocytosis [10,66,67]. Phagocytosis of exogenous stimuli by macrophages excites the innate immune response [68] and thus macrophages are supposed to be the prime target for most of the immunomodulators. In the present work, methanol and aqueous extract of *N. arbortristis* triggered the RES and thus exhibited a significant escalation in the phagocytosis by monocytes and macrophages, consequently presented a significant upsurge in carbon clearance. Thus, extracts may kindle the host defence and make it competent to tackle the infectious organism.

Table 3
Effect of MNA and ANA on cyclophosphamide-induced neutropenia.

Sr. No.	Treatment	TLC			Reduction in TLC (%)	Neutrophil count (%)			Reduction of neutrophils (%)
		Before (A)	After (B)	Reduction (A-B)		Before (C)	After (D)	Reduction (C-D)	
1	Normal	8912 ± 268.47	8789 ± 278.86	123 ± 10.78	01.38	29.67 ± 3.92	28.97 ± 2.89	0.70 ± 0.09	02.25
2	Control	7680 ± 152.97	1875 ± 149.30	5805 ± 103.67	72.21##	17.60 ± 0.92	2.50 ± 0.64	15.18 ± 01.34	86.25##
3	Methanolic extract (200 mg/kg)	9120 ± 292.23	4425.0 ± 513.77	4694 ± 780.78	51.46**	22.80 ± 01.34	8.500 ± 01.25	14.30 ± 0.91	62.79**
4	Aqueous extract (200 mg/kg)	8700 ± 251.95	5425.0 ± 217.47	3275 ± 34.05	37.42**	23.00 ± 01.88	10.250 ± 0.94	12.75 ± 01.12	55.43**

Value indicates mean ± SEM (n = 6).
##$p \leq 0.01$, significant compared to normal group. **$p \leq 0.01$, significant compared to control group

Administration of immunosuppressive drugs such as cyclophosphamide is the superlative method to recognize the intricacy of the immune system. Cyclophosphamide, an alkylating agent of the nitrogen mustard group is generally employed for the treatment of cancer but is often accompanying with a severe adverse effect like leukopenia specifically neutropenia. It also distresses the bone marrow and harms the integrity of M cells which reduced the production of novel blood cells leading to thrombocytopenia and leukopenia [69,70]. Measurement of TLC and DLC before and after the treatment with cyclophosphamide was performed to study the hemopoietic potential of the extracts. Treatment of mice with MNA and ANA produced striking leucocytosis particularly neutrophilia, possibly by stimulating macrophages and secretion of substances such as interleukins, colony-stimulating factors, *etc*. [71,72]. The result of the present investigation opens up elating hematopoietic possibilities of *N. arbortristis* bark to stop unfavorable bone marrow impacts associated with cyclophosphamide.

The above findings indicate that cellular, as well as the humoral immune response, is aroused by pretreatment of the animal with methanol and aqueous extract of *N. arbortristis*.

5. Conclusion

The outcome of the present investigation has opened up new vistas in the field of immune-phytopharmacology. However, it is not always appropriate to speculate the preclinical study with clinical setting, but the exhilarating outcome of the present study indicate that clinical studies are required to assess the plants for their immunomodulatory potential.

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Conflict of interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Author contribution

Hitesh Kumar: Conceptualization, Software, Validation, Data curation, Draft writing, Visualization, Investigation. **Neeru Vasudeva:** Methodology, Supervision, Software, Validation.

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