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An Immuno-Pharmacological Investigation of Indian Medicinal Plant *Nyctanthes arbor-tristis Linn*

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Abstract: The immuno-pharmacological properties of ethanolic extract of *Nyctanthes arbor-tristis Linn.* (NA) have been investigated. After administration of *Nyctanthes arbor-tristis* in doses of 0.25 and 0.5 g/kg body weight (BW) a significant increase in phagocytic index, leukocyte count and spleenic antibody secreting cells were noticed. Stimulation of humoral immune response was further observed with heamagglutination antibody titer. This extract was further submitted to Thin Layer Chromatography (TLC) and High performance liquid chromatography (HPLC) and it confirmed the presence of methoxylated flavonoid quercetin-3,3'-dimethoxy-7-0-rhamnoglucopyranose. The results suggest that bio active compound flavonol glycoside of *Nyctanthes arbor-tristis* influences both humoral as well as cell mediated immune system.

Key words: Phytochemical · Immune-pharmacology · *Nyctanthes arbor-tristis* · Humoral immunity · Cellular immunity · Delayed type hypersensitivity · Herbal immunomodulator

INTRODUCTION

Herbal medicine has become an integral part of standard healthcare, based on a combination of time honoured traditional usage and ongoing scientific research. Burgeoning interest in medicinal herbs has increased scientific scrutiny of their therapeutic potential and safety. Some of the medicinal plants are believed to enhance the natural resistance of the body to infections [1]. The modulation of immune response with the aid of various bioactives in order to alleviate certain diseases is an active area of interest. Apart from being specifically stimulatory or suppressive, certain agents normalize or modulate pathophysiological processes and are hence called 'immunomodulatory' agents [2]. The property of any substance to enhance non-specific resistance of body against pathogens is termed 'adaptogenic'. Most important area in which herbal medicine has not to witness any breakthrough is the development of adjuvants to be used in vaccination programs or immunosuppressants that can be safely exploited in organ transplantation and autoimmune diseases. These fundamental fields of immunomodulators are currently receiving inadequate attention. A number of plant products are being investigated for immune response modifying activity [3]. A plethora of plant-derived materials (proteins, lectins, polysaccharides, etc.) have been shown to stimulate the immune system [4]. Some of the plants with established immunomodulatory activity are *Viscum album*, *Panax ginseng*, *Asparagus racemosus*, *Azadirachta indica*, *Tinospora cordifolia*, *Polygala senega*, *Ocimum santum* [5-8].

Nyctanthes arbor-tristis Linn. belonging to family Oleaceae is a well known medicinal plant. *N. arbor-tristis* Linn is commonly known as "Harsinghar or Night Jasmine". Nyctanthes means 'night flowering' and arbor-tristis mean 'the sad tree' as it loses its brightness during daytime. The plant has been extensively used in Ayurvedic system of medicine for various ailments and is shown to posses significant anti-inflammatory, hepatoprotective, wound healing and antimicrobial properties [9-14]. The present study is aimed at studying the immunomodulatory activity of aqueous extract of *Nyctanthes arbor-tristis* in mice and the phytochemical study of the ethanolic crude extract led to identification of major groups of chemical constituents by TLC and HPLC methods.

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MATERIALS AND METHODS

Reagents: Agarose and bovine serum albumin (BSA) were purchased from Himedia, India. Tetanus toxoid (TT) was obtained from Biological E, India. Tween 20 and ovalbumin (OV) were purchased from Sigma, USA. Goat anti-mice IgG-HRP (horseradish peroxidase) conjugate and tetra methyl benzidine (TMB) was procured from Genei, Banglore, India. All other reagents were of analytical grade unless otherwise mentioned.

Plant Material and Extraction: Healthy plant leaves of *Nyctanthes arbor-tristis* Linn. were collected from South Western Ghats of Tirunelveli range, Tamil Nadu, India. They were collected in early morning and were shade dried for 10 days and powdered mechanically. The collected plant materials were botanically authenticated by Botany Department, VHNSN College, Virudhunagar, India. Large scale extractions were prepared from the selected plants. Dried, ground plant leaves (1000 g) were extracted seven times with 4 liter petroleum ether (60–80°C) for 12 h in total, yielding 120 g crude extract (12%). The same plant material was subsequently extracted with 4.5 liter chloroform and ethanol for 9 h, yielding 215 g crude extract (21.5%). Finally all the extracts were stored at 4°C until use.

Phytochemical Screening: Partitioning of the *Nyctanthes arbor-tristis* extracts, for alkaloid analysis, was performed according to previous report [15]. Dragendorff's reagent and Mayer's reagent were used to indicate the presence of alkaloids by the formation of a precipitate [16]. Extracts were screened for cardiac glycosides by testing for 2-deoxy-sugars using the Keller–Killiani test [17]. The presence of unsaturated lactone rings were tested for by spraying identical TLC plates with Kedde's reagent (for cardenolide detection), chloramine Ttrichloroacetic acid reagent and antimony(III) chloride reagent (for bufadienolide detection). The procedure for testing for tannins was performed using the Gelatin–salt Block test [18].

HPLC Analysis: The assays were performed using a HPLC Hewlett Packard model 1100, with a DAD detector. Column: RP-18 Chromolith 100mm × 4.6mm (Cat. 1.02129.0001/Merck). The eluent was acetonitrile: water (EM Science, AX0142-1), with gradient of 10–90% of acetonitrile in 30 min. Detection: UV spectra at 254 nm. Injection volume: 20 µl. One ml of each sample was evaporated to dryness at less than 50°C, dissolved in 20% acetonitrile, filtered (Minisart RC-15, Sartorius, 0.45 µm) and injected directly in HPLC. The identification of the compounds was carried out by analysis of retention time (Rt) versus the characteristic bands for polyacetylene and flavonoids in UV spectra (Brandão *et al*., 1997). The flavonoid quercetin-3,3'-dimethoxy-7-0- rhamnoglucopyranose, previously reported [19] was used as a standard and showed a Rt of 3.97 min.

Immunomodulatory Studies

Experimental Animals: Swiss male albino mice weighing 35-40 g were used. They were fed a standard pellet diet and tap water ad libitum in animal house facility and maintained under standard laboratory conditions. All protocols were approved by the ethical committee of the Institute for animal handling.

Treatment of Mice: Two groups of mice each consisting of six animals received *Nyctanthes arbor-tristis* extract intraperitonially (i.p.) in doses of 0.25 and 0.5 g/kg body weight (BW) for 5 consecutive days. The control group was treated with 0.5 ml phosphate buffer saline (PBS, pH 7.4), whereas native group was devoid of any treatment. No mortality or any toxic effects were observed in the above mentioned doses of *Nyctanthes arbor-tristis* administered i.p.

Phagocytic Activity: *Nyctanthes arbor-tristis* extract was injected intraperitoneally (0.25 and 0.5 g/kg BW) for 5 consecutive days. Control was given equal volume of phosphate buffer saline (pH 7.4). After 48 h of last dose mice were injected via the tail vein with colloidal carbon (Indian ink), which was diluted with PBS (pH 7.4) to eight times before use (10 µl/g BW). Blood samples were drawn from the retro-orbital plexus at 0, 3, 6, 9, 12 and 15 min. The blood (25 µl) was dissolved in 0.1% sodium carbonate (2 ml) and the absorbance was measured at 660 nm [20]. The phagocytic index, *K*, was calculated by equation:

$$K = \frac{\ln OD1 - \ln OD2}{t2 - t1}$$

where, OD1 and OD2 depict the optical densities at times $t1$ and $t2$, respectively.

Leukocyte Count: Two groups of mice were treated with different doses of *Nyctanthes arbor-tristis* extract for 5 days. On day 6, blood was collected from retro-orbital plexus for white blood cells (WBC) count. The animals

were sacrificed by cervical dislocation and their spleens were harvested for weighing and spleen leukocytes count [21]. The results of these analyses were compared with that of control and native groups.

Heamagglutination Antibody Titer: Animals in two different groups were injected i.p. 0.2 ml of 5×10^9 SRBC on day 0. *Nyctanthes arbor-tristis* extract was administered i.p. 0.25 and 0.5 g/kg BW to animals on -4, -2, 0, 2, 4 days. Control group received equal volume of PBS (pH 7.4). Blood samples were collected from retro-orbital plexus on day 7. Antibody titer was determined by the following procedure reported previously [22]. To two-fold dilutions of serum samples made in 25 µl volumes of normal saline containing 0.1% BSA (BSA saline) in V bottom heamagglutination plates (Tarsons, India) were added 25 µl of 0.1% suspension of SRBC in BSA saline. After thorough mixing SRBC were allowed to settle at room temperature for 90 min until control wells showed small button of cells (negative pattern). The values of the highest serum dilution causing visible haemagglutination were considered as the antibody titer.

Plaque Forming Cell (PFC) Assay: The assay was performed according to the method reported previously [22]. Briefly, the spleen cells of SRBC immunized *Nyctanthes arbor-tristis* extract treated mice were separated in RPMI-1640 medium, washed twice and suspended in same medium (10^6 cells/ml). Glass petridishes were layered with 1.2% agarose in 0.15M NaCl to form bottom layer. A mixture of 2ml agarose (0.6%) in RPMI-1640 medium, 0.1 ml suspension of 20% SRBC and 1×10^6 spleen cells in a volume of 0.1 ml was poured over the bottom layer of agarose followed by an incubation period of 90 min at 37°C. two ml quantity of 1:9 diluted fresh rabbit serum was added to petridish and plate was reincubated for 40 min to allow the formation of plaques. The number of plaques was counted immediately and values are expressed as counts per 10^6 spleen cells.

Delayed Type Hypersensitivity (DTH): Hypersensitivity reaction to SRBC was induced in mice following the method reported previously [23]. Animals were sensitized with 10% SRBC (1×10^8 cells) at day 0 and day 7 subcutaneously (s.c). They were divided into two groups: one group was treated with *Nyctanthes arbor-tristis* extract 0.25 g/kg BW i.p. and other received 0.5 g/kg BW *Nyctanthes arbor-tristis* extract i.p. on days -4,-2, 0, 2, 4, 6, 8, whereas control group was administered with equal volume of PBS (pH 7.4). On day 9, both groups were challenged with 1×10^8 SRBC cells, intradermally into the left footpad of each mouse, while PBS (pH 7.4) was injected into right hind paw. The increase in footpad thickness (FPT) was measured 24 h after SRBC challenge by volume differential meter. The degree of DTH reaction was expressed as the percentage increase in FPT over the control values.

Systemic Anaphylaxis Reaction: Animals were sensitized by 1mg bovine serum albumin subcutaneously in 0.2 ml aluminum hydroxide (15 mg/ml) at day 0 and were shocked by intravenous injection of 1mg BSA in PBS (pH 7.4) on day 14. 1 mg of ovalbumin i.v. in 0.2 ml PBS (pH 7.4) served as negative control. Immunomodulatory activity was examined by injecting *Nyctanthes arbor-tristis* extract 0.25 and 0.5 g/kg BW of mice on alternative days from day 0 to day 14 for seven times before shocking injection. The systemic anaphylactic reaction was observed within 30 min following the shocking injection and rated in following fashion: positive reaction, mortality or animal rendered still for at least 1 min; negative reaction with no change or normal movement [24].

Antibody Response Against Specific Antigen: Conventional marketed alum adsorbed tetanus toxoid was used to generate specific antibody titer. Priming was done on day 0 with booster dose on day 28. *Nyctanthes arbor-tristis* extract was administered i.p. in two doses of 0.25 and 0.5 g/kg BW on day -4, -2, 0, 2, 4, 6, 8. Control group received PBS (pH 7.4) in place of extract, while native group was devoid of any treatment. TT challenge was given to all groups except native animals. Blood sampling was done on day 14 and day 28 to measure antibody titer against TT. Anti-TT antibodies in mice sera were estimated using ELISA protocol. Briefly, 1g of TT in 100µl of PBS (pH 7.4) was coated in each well of 96 well flat bottom polystyrene immunoplate (Tarsons). The plates were washed with PBS-T (0.5% Tween 20 in PBS) and different dilutions of test serum in PBS-T were added to the wells and incubated for 1 h at room temperature. After washing with PBS-T for three times, goat-anti mice antibody-horseradish peroxidase conjugate was added and incubated for 1 h at room temperature followed by addition of tetramethyl benzidine. After 30 min reaction was stopped with 2M H_2SO_4 and absorbance was measured at 450 nm using Labsystems, Finland microplate reader. Simultaneously, standard curve from known quantities of anti-TT antibodies was also prepared.

Statistical Analysis: Data were expressed as the mean ± standard deviation (S.D.) and statistical analysis was performed using Student's *t*-test.

RESULTS

Phytochemical Screening: No alkaloids, cyanogenic glycosides or saponins were detected in any of the extracts from the species tested. In screening for cardiac glycosides, yellow bands were observed in leaf extracts from *Nyctanthes arbor-tristis* after spraying with antimony (III) chloride reagent. These results indicate the possible presence of bufadienolides. Cardenolides were not detected, although they are known to occur in many *Oleaceae* species [25].

HPLC Fingerprint: Plate 1 shows the results obtained by HPLC analysis. The chromatograms for the ethanolic extract of *Nyctanthes arbor-tristis* show peaks for several compounds between Rt 2.17 and 4.73 min, which were identified by their characteristic bands on TLC as flavonoids. A higher peak at Rt 3.97 min corresponded to the methoxylated flavonoid quercetin-3,3'-dimethoxy-7-0-rhamnoglucopyranose, previously characterised in *Bidens pilosa* roots [26].

Phagocytic Activity: Carbon clearance test was conducted to establish phagocytic activity of reticuloendothelial system (RES) after treatment with *Nyctanthes arbor-tristis* extract (Fig. 1). Phagocytic index was significantly increased after the administration of *Nyctanthes arbor-tristis* extract compared to control group ($P < 0.05$). Relatively less difference was recorded between two doses of ethanolic extract, with higher dose (0.5 g/kg BW) showing phagocytic index value of 0.56±0.04 compared to 0.38±0.06 recorded with the dose of 0.25 g/kg BW.

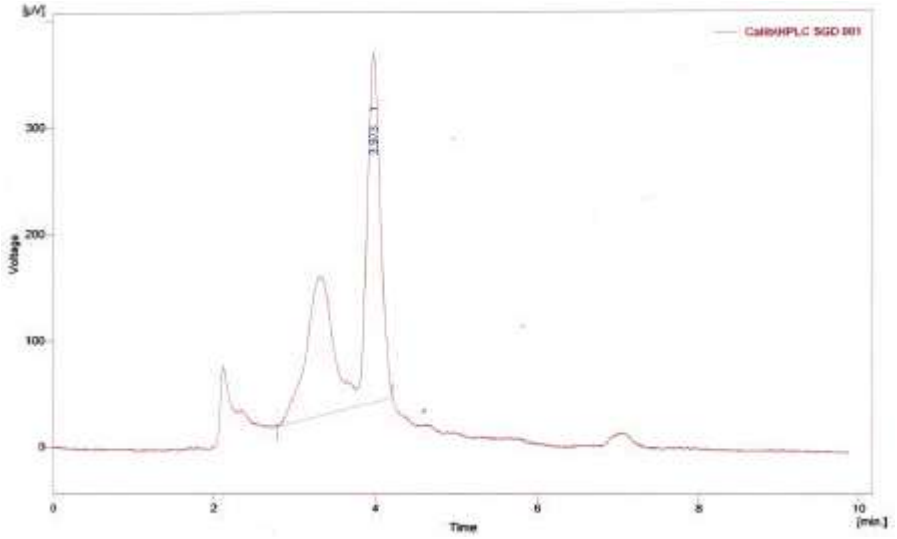

Plate 1: HPLC Profile of the *Nyctanthes arbor-tristis* extract

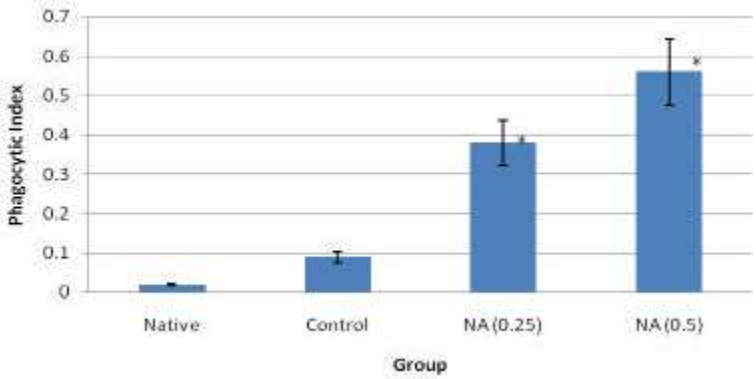

Fig. 1: Effect of Nyctanthes arbor-tristis extract on carbon clearance test
*P<0.05 (compared to control group)

Table 1: Effect of *Nyctanthes arbor-tristis* extract on spleen weight, WBC count and spleen leukocyte count

Group (Treatment)	Spleen weight (g)	WBC count/mm^3	Spleen leukocyte count
Native (Not Treated)	0.545±0.07	1308±4.62	1197±2.1
Control (PBS)	0.428±0.05	1409±3.21	1498±2.65
0.25g/kg BW NA	0.621±0.05*	1421±2.36*	1525±2.6*
0.5g/kg BW NA	0.615±0.01	1498±2.65	1764±3.69

Values are mean±S.D. n of six mice. $P<0.05$ when compared with control

Table 2: Effect of *Nyctanthes arbor-tristis* extract on heamagglutination antibody titer and plaque forming cell

Group (Treatment)	heamagglutination antibody titer	Plaque forming cell/ 10^6 Spleen cells
Control (PBS)	4.07±0.03	1560±120
0.25g/kg BW NA	4.94±0.07	1990±140
0.5g/kg BW NA	5.67±0.04*	2120±140

* Values are mean ± S.D. n of six mice. $P<0.1$ when compared with control

Table 3: Effect of *Nyctanthes arbor-tristis* extract on DTH reaction

Group (Treatment NA)	Percentage of increases in paw volume
Control (PBS)	0.097±0.01
0.25g/kg BW	0.131±0.30[a]
0.5g/kg BW	0.125±0.031[b]

Values are mean ± S.D. mm of six mice. a - $P<0.05$ b - $P<0.01$

Table 4: Effect of *Nyctanthes arbor-tristis* extract on anaphylactic symptoms

			Number of Mice		
Sensitizing injection	Shocking injection	Treatment (NA)	Total Mice	Anaphylactic symptoms	Deaths
BSA (s.c.)	BSA (i.v)	-	6	6	2
BSA (s.c.)	OV (i.v)	-	6	0	0
BSA (s.c.)	BSA (i.v)	0.25g/kg BW	6	4	0
BSA (s.c.)	BSA (i.v)	0.5g/kg BW	6	2	0

Values are expressed as mean±S.D. *P < 0.01 (compared to positive control group). Abbreviations: BSA: bovine serum albumin; OV: ovalbumin; s.c.: subcutaneous; i.v.: intravenous,

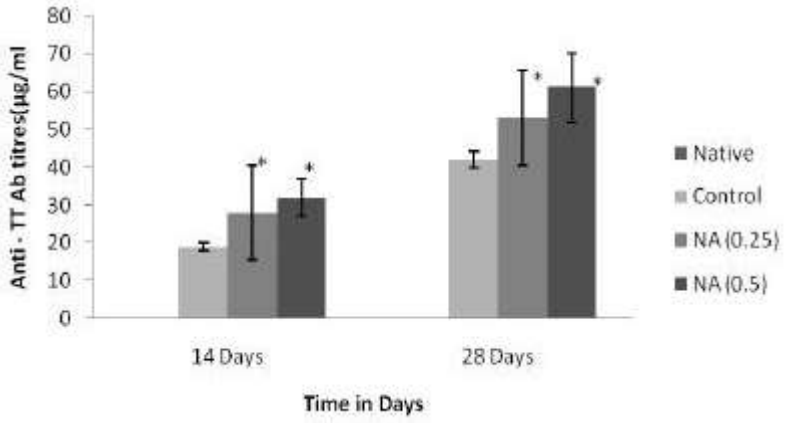

Fig. 2: Specific antibody titer against TT booster dose on 28 day
*P<0.05 (compared to group)

Leukocyte Count: White blood cell count was increased with the treatment of *Nyctanthes arbor-tristis* extract compared to control and native groups. In addition, a positive effect on spleen weight and spleen leukocyte count was also observed (Table 1). However, dose response was not proportionate, i.e. with double the dose of *Nyctanthes arbor-tristis* extract proportionate shoot up in response was not witnessed.

Heamagglutination Antibody Titer: A dose related increase in heamagglutination antibody titer was observed with five doses of *Nyctanthes arbor-tristis* extract (Table 2) compared to control group. However, the response was not doubled on increasing doses of *Nyctanthes arbor-tristis* extract.

Plaque Forming Cell Assay: *Nyctanthes arbor-tristis* extract administration for 5 days beginning from the day of sensitization with SRBC produced 30–35% increase in antibody secreting cells in mouse spleen (Table 2). Effect was significant compared to control group ($P < 0.01$) however, higher dose did not produced corresponding elevation in antibody secreting cells.

Delayed Type Hypersensitivity Reaction: The effect of *Nyctanthes arbor-tristis* extract administration on T-cell mediated DTH reaction is shown in Table 3. A significant enhancement (8–10%) in foot pad thickness of *Nyctanthes arbor-tristis* extract treated mice was observed when compared to control group ($P < 0.05$).

Active Systemic Anaphylaxis: Effect of *Nyctanthes arbor-tristis* extract treatment on active systemic anaphylaxis was shown in Table 4. Results indicate a positive effect on anaphylaxis with the treatment of *Nyctanthes arbor-tristis* extract suggesting a reduction in number of animals presenting anaphylactic symptoms with no death observed. At the other extreme, in the case of positive control group with no *Nyctanthes arbor-tristis* extract treatment, all mice exhibited anaphylactic symptoms with two deaths, whereas negative control group showed no symptoms of anaphylaxis.

Specific Antibody Titer: Anti-TT antibody IgG titer is shown in Fig. 2. Control group exhibited an increased antibody titer with priming as well as booster dose of TT. Treatment of *Nyctanthes arbor-tristis* extract further enhanced the effect of TT injection with a corresponding elevation in anti-TT IgG antibodies, however, level of antibodies was not increased proportionately with dose.

DISCUSSION AND CONCLUSIONS

The prime objective of the study was to investigate the Phytocompounds and immunomodulatory effect of *Nyctanthes arbor-tristis* Linn. Chemical study of the ethanol crude extract of *Nyctanthes arbor-tristis* led to identification of two major groups of chemical constituents, the polyacetylenes and the flavonoids, the most likely active compounds previously [26]. In the present study, the presence of polyacetylene and flavonoids was established using High Performance Liquid Chromatography (HPLC) studies. Previous studies had evinced the presence of iridoids, such as glucosylplumeride, saponins and demethoxyaspidospermine [27] in *Nyctanthes arbor-tristis* were investigated.

There was a growing interest in identifying herbal Immunomodulators ever since their possible use in modern medicine has been suggested [28, 29]. The findings outlined above have demonstrated that *Nyctanthes arbor-tristis* extract possesses potent immunostimulant action. Phagocytosis represents an important innate defence mechanism against ingested particulates including whole pathogenic microorganisms. The specialized cells that are capable of phagocytosis include blood monocytes, neutrophils and tissue macrophages. Once particulate material is ingested into phagosomes, the phagosomes fuse with lysosomes and the ingested material is then digested. Enhanced uptake of particulate matter with the treatment of *Nyctanthes arbor-tristis* is evident from carbon clearance test. However, a dose proportionate response was not observed, since immune response is not always directly related with the immunomodulator concentration. This may be partly due to different constituents present in fraction at different concentrations and saturation of cells responsible for immune response. Some of these constituents may have immunosuppressive activity, whereas other possesses immunostimulant action [30].

The resultant effect of *Nyctanthes arbor-tristis* extract is immunostimulation with a typical plateau effect beyond the dose of 0.25 g/kg BW. A variety of white blood cells, or leukocytes, including neutrophils, lymphocytes, monocytes, eosinophils and basophils participate in the development of an immune response. Of these cells, only the lymphocytes possess the attributes of diversity, specificity, memory and self/non-self recognition, the hallmark of an immune response. All the other cells play accessory roles, serving to activate lymphocytes, to increase the effectiveness of

antigen clearance by phagocytosis or to secrete various immune effector molecules. Among different organs of immune system, spleen represents a major secondary lymphoid organ involved in elicitation of immune response. Unlike lymph nodes, which are specialized to trap-localized antigen from regional tissue spaces, the spleen is adapted to filtering blood and trapping blood-borne antigens and thus can respond to systemic infections. Results revealed an increase in the blood leukocytes count, weight of spleen and spleenic leukocytes count suggesting an uplift of immune status. Number of antibody secreting cells from spleen was determined using plaque forming cell assay. Since spleen contributes immensely to the humoral as well as cellular arms of immune system, its role in generation of antibody secreting cells was studied. Results suggested an enhancement in number of cells secreting antibodies against SRBC, which served as specific antigen. Haemagglutination antibody titer was determined to establish the humoral response against SRBC. At neutral pH, red blood cells possess negative ions cloud that makes the cells repel from one another, this repulsive force is referred to as zeta potential. Because of its size and pentameric nature, Ig M can overcome the electric barrier and get cross-link red blood cells, leading to subsequent agglutination. The smaller size and bivalency of Ig G, however, makes them less capable to overcome the electric barrier. This characteristic may accounted for, Ig M being more effective than Ig G in agglutinating red blood cells [31]. *Nyctanthes arbor-tristis* extract treatment improved the heamagglutination antibody titer reflecting an overall elevation of humoral immune response.

Delayed type hypersensitivity reaction is characterized by large influxes of non-specific inflammatory cells, in which the macrophage is a major participant. It is a type IV hypersensitivity reaction that develops when antigen activates sensitized T_{DTH} cells. These cells generally appear to be a T_{H1} subpopulation although sometimes T_C cells are also involved. Activation of T_{DTH} cells by antigen presented through appropriate antigen presenting cells results in the secretion of various cytokines including interleukin-2, interferon-α, macrophage migration inhibition factor and tumor necrosis factor- α [32]. The overall effects of these cytokines are to recruits macrophages into the area and activate them, promoting increased phagocytic activity vis-a-vis increased concentration of lytic enzymes for more effective killing. Several lines of evidence suggest that DTH reaction is important in host defense against parasites and bacteria that can live and proliferate intracellularly. Treatment of *Nyctanthes arbor-tristis* extract enhanced DTH reaction, which is reflected from the increased footpad thickness compared to control group suggesting heightened infiltration of macrophages to the inflammatory site. This study may be supporting a possible role of *Nyctanthes arbor-tristis* extract in assisting cell-mediated immune response. The lactones, which are isolated from the *Nyctanthes*, have been identified and are known to cause delayed type hypersensitivity [33]. So it might be possible that these sesquiterpene lactones may be present in the *Nyctanthes arbor-tristis* extract. Anaphylatic reaction is mediated by IgE class of antibodies. IgE binds with high affinity to Fc receptors on the surface of tissue mast cells and blood basophils. Such IgE coated mast cells and basophils are said to be 'sensitized.' A later exposure to the same allergen cross-links the membrane bound IgE on sensitized mast cells and basophils causing degranulation of these cells [34]. The pharmacologically active mediators released from the granules exert biological effects like vasodilatation and smooth muscle contraction that may be either systemic or localized depending on the extent of mediator released. There are several probable mechanisms through which *Nyctanthes arbor-tristis* extract could have prevented anaphylactic symptoms, when sensitized and subsequently challenged by BSA. *Nyctanthes arbor-tristis* extract may act by provoking the production of IgG blocking antibodies, which binds the antigen (BSA) thereby preventing it from combining with IgE on the mast cell membrane. It is convincing to assume that the whole extract of *Nyctanthes arbor-tristis* extract must be containing polyacetylenes and the flavonoids, which are reported responsible for the immunostimulatory complexes may presumably be held responsible for stimulation and potentiating of overall immune response. Using an animal model, we were able to measure the immunomodulatory effects of herbal treatment on the production of specific immunoglobulins following primary and secondary exposure to tetanus toxoid, used in clinical practice. To our knowledge, this is the first study that has explored the effect of immunomodulatory agent on the immune status of animal being challenged with a clinical antigen. This simulates the conditions of clinical vaccination and proposed the use of immunostimulant for a possible application in immunocompromised patients to generate sufficient immune response. All animals responded to the TT challenge and exhibited an enhanced IgG titer with booster dose on day 28. In comparison to control group, which received PBS (pH 7.4), *Nyctanthes*

arbor-tristis treated animals showed significant uplift of specific antibodies against TT. In conclusion, the present study has shown the immunostimulatory activity of *Nyctanthes arbor-tristis* and suggests its therapeutic usefulness. *Nyctanthes arbor-tristis* has stimulated both humoral as well as cellular arms of immune system. Further detailed studies are required which might establish a possible use of aqueous extract of *Nyctanthes arbor-tristis* Linn. in immunocomparmised patients and as an adjuvant during vaccination programs in order to reduce number of non-responders to vaccines. However, detailed studies of mechanisms of immunomodulation and probable use in immunocomparmised individuals are still to be investigated.

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