Immunomodulatory activity of *Nerium indicum* through inhibition of nitric oxide and cyclooxygenase activity and modulation of T_H1/T_H2 cytokine balance in murine splenic lymphocytes

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Received: 18 February 2014 / Accepted: 18 November 2014 / Published online: 14 February 2015
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Abstract *Nerium indicum* is a medicinal plant which is used in the treatment of wide range of illness in different ethnopharmacological practices. We have studied the immunomodulatory activity of the 70 % hydro-methanolic extract of *N. indicum* leaves (NILE) by studying plaque forming cell assay, haemagglutination titre, cell proliferation assay, inhibition of nitric oxide expression and cyclooxygenase activity, estimation of IL-2, IFN- γ , IL-4, IL-10, TNF- α and IgM levels. Furthermore, we have characterized NILE with Fourier Transform Infra-red (FTIR) spectroscopy. The results displayed that NILE possessed potent immunomodulatory activity by up-regulating IL-2, IFN- γ , IL-10 expression and down-regulating IL-4, TNF- α expression in vitro. NILE also stimulated the in vivo expression of immunoglobulin level in mouse. FTIR analysis revealed phytochemicals of diverse chemical nature present in NILE. Thus, the potent immunomodulatory activity of *N. indicum* may have serious implications in the treatment of inflammatory diseases in the future.

Keywords Anti-inflammatory · Cytokine · Cyclooxygenase · Immunoglobulin · Immunomodulatory · *Nerium indicum*

Introduction

Herbal immunomodulators have emerged as a mode of alternative and complementary medicine. It is fascinating to note that herbs and polyherbal formulations, once used only by the ethnic people, are gaining popularity in modern medicinal and pharmaceutical science. Today, standardization and proper mechanism based screening of various plants mentioned in the traditional medicinal systems have led to the present state where 25 % of the current drugs are solely derived from plants (Kunle et al. 2012).

Nerium indicum Mill. (syn. *N. oleander* L. and *N. odoratum* Aiton) is one such plant used in Indian (Ghosh 2008; Dastur 1962) and Chinese (Ding et al. 2013; Ji 1999) traditional medicinal system for ages. In ethnomedicine, it is used for the treatment of cardiac illnesses, asthma, corns, cancer, and epilepsy (Duke 1985). In Tunisia, leaves of *N. indicum* are used as anti-gangrenous agent (Leporatti and Ghedira 2009) and the tribes of Mirzapur district of Uttar Pradesh, India, use the leaves and fruits of *N. indicum* against leprosy and swellings (Tiwari and Singh 2012). Detailed phytochemical analysis of different parts of the plant have been performed previously (Dey et al. 2012a).

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Various bioactivities including antibacterial (Hussain and Gorsi 2004; Hadizadeh et al. 2009), antinociceptive (Erdemoglu et al. 2003), anti-anxiety (Singhal and Gupta 2011), neuroprotective (Leung et al. 2006), hepatoprotective (Singhal and Gupta 2012) and anti-oxidant (Dey et al. 2012b; Dey and Chaudhuri 2013) properties of *N. indicum* have already been established. Several extracts and compounds isolated from *N. indicum* have demonstrated potent anticancer activity against various cancer cell lines (Dey and Chaudhuri 2013; Mae et al. 2008).

Therefore, considering its ethnomedicinal use and medicinal properties, we have studied the immunomodulatory activity of the 70 % hydro-methanolic extract of *N. indicum* leaves (NILE). In addition, FTIR analysis of NILE was performed to characterize the major chemical species in the extract.

Materials and methods

Chemicals and solvents

All the reagents were procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), unless otherwise indicated. Nystatin was obtained from Sigma Aldrich (St. Louis, MO, USA). Mouse IL-2, IFN- γ , TNF- α , IL-4 and IL-10 ELISA kits were procured from RayBio (Norcross, GA, USA) and mouse IgM ELISA kit was obtained from MyBiosource (San Diego, CA, USA). COX Activity Assay Kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

Collection of plant material and preparation of the extract

White flower variety of *N. indicum* plant was collected during the month of August from the campus of the University of North Bengal (26.71°N, 88.35°S), India and identified by taxonomist Prof. A. P. Das of the Department of Botany, University of North Bengal. A voucher specimen was stored in the herbarium of the Department of Botany (accession number of 09618). The leaves were separated from the plant and washed properly with distilled water to remove any dirt. The leaves were then shed dried at room temperature for 2 weeks and grinded to powder. The powder (100 g) was mixed with 70 % methanol (1,000 ml) and kept in a shaking incubator overnight (12 h, 37 °C, 160 rpm).

Then the mixture was centrifuged at 5,000 rpm for 15 min. The pellet was mixed with 70 % methanol (1,000 ml) and kept overnight at the shaking incubator and centrifuged. The supernatant liquid was collected from both phases and filtered. The resultant filtrate was concentrated in a rotary evaporator under reduced pressure. The concentrated extract was lyophilized and stored at −20 °C until further use. The extract was previously characterized to contain high phenolic and flavonoid content and demonstrated to possess potent free radical scavenging capacity (Dey et al. 2012b).

Maintenance of animals

Swiss albino mice [30 ± 2 g of body weight (BW)] of 6–8 weeks were used for all the studies. Blood for immunization was collected from sheep by puncturing the jugular vein with a sterile syringe. The blood was diluted with equal volume of Alsever's solution (114 mM dextrose, 27 mM sodium citrate, 71 mM NaCl, pH 6.1) and stored at 4 °C as sheep RBC solution (sRBC) until further use. Blood was collected from a guinea pig by puncturing the heart using a sterile needle and allowed to clot at 4 °C for 60 min to separate the serum. The blood was then centrifuged at 1,000 rpm for 5 min and the clear supernatant serum was collected. This pooled serum was used as guinea pig complement and stored at 0 °C to minimize the complement activity. All animals were maintained under standard laboratory conditions in the animal house of the Department of Zoology, University of North Bengal with food and water *ad libitum* under a constant 12-h photoperiod (temperature 25 ± 2 °C). All experiments were approved by the ethical committee of the Department of Zoology, University of North Bengal (No. 840/ac/04/CPCSEA; date: 15/09/10) and performed in accordance with the legislation for the protection of animals used for scientific purposes.

Plaque forming cell (PFC) assay

A previously described method (Raisuddin et al. 1991) was followed with slight modifications to perform the PFC assay. Briefly, 24 Swiss albino mice were divided into 4 groups (n = 6). Group I (control) received normal saline; group II, NILE extract at 50 mg/kg BW; group III, NILE extract at 100 mg/kg BW and group IV, NILE extract at 200 mg/kg BW for 10 days.

On day 7, mice were immunized with 0.1 ml of 25 % sRBC intravenously through tail vein. On day 4 after immunization with sRBC i.e. 24 h after the last dose of NILE, whole blood was collected from the tail vein initially. Mice were sacrificed under proper anaesthesia and blood was collected from the heart and stored for further use. Single cell suspension (2×10^6 cells/ml) of the spleen was prepared in phosphate buffer saline (PBS). Guinea pig complement (50 μ l) and 50 μ l of 25 % sRBC (prepared in PBS) were mixed with 100 μ l of the cell suspension. The mixture was then charged into Cuningham chambers, prepared using glass slide and double gum tape. The chambers were then sealed with sealing material prepared by mixing paraffin and petroleum jelly at 1:1 ratio and incubated at 37 °C for 3–4 h. After incubation, the plaques were counted under a binocular and expressed as PFC/ 10^6 spleen cells.

Estimation of total IgM

Blood samples from immunised mice of the PFC assay were kept at room temperature for 60 min for separation of serum. The whole blood was then centrifuged at 1,000 rpm for 5 min and the straw coloured supernatant serum was collected in fresh vials. The serum was diluted 10,000 fold and then used to estimate the total IgM level using a commercially available kit (MyBiosource) according to the manufacture's instruction. The final quantity was multiplied by the dilution factor.

Hemagglutination (HA) titre assay

HA titre assay was performed according to a standard method (Karthikumar et al. 2011) with slight modifications. Briefly, Swiss albino mice were fed with different doses of NILE for 10 days and immunized with sRBC as was done in the PFC assay. On day 4 of the immunization, serum was collected from the immunized mice and kept at 56 °C for 45 min in a water bath to inactivate the complement activity. Eight clear Khan tubes were taken and double fold diluted serum was added. In the first tube, 0.1 ml serum and 0.9 ml PBS were added. After thorough mixing, 0.5 ml of the mixture was taken to the subsequent tubes. In this way, serial dilutions were prepared in the ratio of 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, and 1/1,280. Hundred microliters of 10 % sRBC (prepared

in PBS) was added to each tube and mixed thoroughly. The tubes were incubated at 37 °C for 12 h in a humidified atmosphere. After incubation, visible hemagglutination was observed under the binocular. The highest dilution which gave rise to the visible hemagglutination was considered as the optimum concentration of the antibody and the antigen.

Culture of splenocytes

Spleen was removed aseptically from an untreated Swiss albino mouse (not fed with NILE) under proper anaesthesia and splenocyte suspension was prepared in RPMI-1640 using a tissue grinder. The splenocytes were washed thrice using RPMI-1640 (1000 rpm) for 10 min and then resuspended in 1 N NH_4Cl to lyse RBCs. After 5 min ice cold RPMI-1640 was added to stop the reaction. The cells were again centrifuged (1000 rpm) and resuspended in RPMI-1640. Splenocytes were adjusted as 2×10^6 cells/ml with RPMI-1640 (supplemented with 50 U/ml penicillin, 50 μ g/ml streptomycin, 50 U/ml nystatin and 10 % FBS) and seeded into six well culture plates. To this, concanavalin A (con A) was added to get a final concentration of 5 μ g/ml and 100 μ l of different concentrations of NILE (0, 10, 30, 50, 80 μ g/ml) was then added to the wells. The control did not receive con A and NILE. The plates were then covered and incubated under 5 % CO_2 and humidified atmosphere of 90 % air at 37 °C temperature for 48 h.

Cell proliferation assay

The assay was performed using EZcount™ MTT Cell Assay Kit (HiMedia) according to the manufacturer's instructions. In brief, murine splenocytes were cultured in 96 well plate for 48 h as previously described. After the incubation 10 μ l of MTT (5 mg/ml) solution was added to each well. The plate was wrapped with aluminium foil to avoid exposure to light and incubated for 4 h. Then 100 μ l solubilizing reagent was added to each well and the absorbance was measured at 570 nm using Bio-Rad (Hercules, CA, USA) iMark™ microplate absorbance reader.

Measurement of NO inhibition

Murine splenocytes were cultured with varying concentrations of NILE (0–80 μ g/ml) for 48 h and after

incubation, the solutions from each group were centrifuged at $2,850\times g$ for 5 min. The extent of inhibition of NO was estimated from the supernatant using the Griess reagent method (Hibbs et al. 1988) with slight modifications. Briefly, 50 μ l of the supernatant, was mixed with 200 μ l of Griess reagent (1 % sulfanilamide and 0.1 % *N*-(1-naphthyl) ethylenediamine hydrochloride in 2.5 % H_3PO_4). The solution was incubated at 37 °C for 20 min and after the incubation the absorbance was read at 540 nm.

Estimation of cytokine expression

Mouse splenocytes were cultured with different concentrations (0–80 $\mu\text{g/ml}$) of NILE as previously described for 48 h. After incubation, suspensions from each well were centrifuged ($2,850\times g$) for 5 min and the culture supernatants were collected to estimate the levels of IL-2, IFN- γ , IL-4, IL-10 and TNF- α using RayBio ELISA kits according to the manufacturer's instructions. Briefly, 100 μ l culture supernatant from different experimental groups were added to the anti-mouse cytokine (IL-2, IFN- γ , IL-4, IL-10, TNF- α) coated wells of the 96 well ELISA plate. The plate was covered and incubated at room temperature for 2.5 h. The solution was then discarded and the wells were washed 4 times with assay buffer (300 μ l). Next, 100 μ l biotinylated anti-mouse cytokine (IL-2, IFN- γ , IL-4, IL-10, TNF- α) antibody was added to each well and the plate was incubated at room temperature with continuous but gentle shaking. After 1 h, the wells were washed with assay buffer as previously. Then 100 μ l of horseradish peroxidase (HRP)-streptavidin conjugate solution was added and the plate was incubated for 45 min at room temperature with gentle shaking. Next, the wells were washed as previously and 100 μ l 3,3',5,5'-tetramethylbenzidine (TMB) one-step substrate reagent was added followed by 30 min incubation at room temperature in the dark. Then 50 μ l of 0.2 M sulphuric acid was added to each well to stop the reaction. Absorbance was immediately measured at 450 nm using Bio-Rad iMarkTM microplate absorbance reader. The standard was run in parallel to the sample.

Measurement of COX activities

COX 1 and COX 2 activities were measured using COX activity assay kit (Cayman Chemicals Company)

by ELISA method. Briefly, mouse splenocytes were cultured (5 $\mu\text{g/ml}$ Con A at final volume) with varying concentrations (0–80 $\mu\text{g/ml}$) of NILE for 48 h. After incubation, the cell suspensions were centrifuged at $2,850\times g$ for 5 min and the cell pellets were homogenized in cold buffer consisting of 0.1 M Tris-HCl in 1 mM EDTA (pH 7.8). The homogenate was centrifuged ($10,000\times g$ for 15 min) and the resultant supernatants were used for the assay. Then, assay buffer, heme reagent, test samples and background samples were added to their respective wells in the 96 well plate according to the manufacture's instructions. DuP-697 (COX 2 selective inhibitor) and SC-560 (COX 1 selective inhibitor) were added to the respective inhibitor wells. The plate was incubated at 25 °C for 5 min and then 20 μ l *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) was added to each well. Arachidonic acid (20 μ l) was added to each well to start the reaction. The plate was incubated at 25 °C for 5 min and absorbance was read at 590 nm. Ovine COX-1 was run in parallel as standard.

Corrected OD values (ΔA_{590}) were obtained by subtracting the background OD values from their corresponding sample and inhibitor treated sample OD values. COX 1 and COX 2 activities were calculated using the following formula:

$$\text{COX activity (nmol/min/ml)} = (\Delta A_{590} \text{ per 5 min} \div 0.00826 \mu\text{M}^{-1}) \times (0.21 \div 0.04) \div 2$$

where, 2 molecules of TMPD were required to reduce prostaglandin G_2 (PGG_2) to prostaglandin H_2 (PGH_2). Percentage inhibition of COX activity was calculated by the following formula:

$$\text{Percentage of inhibition} = \left[(\text{TCA}_S - \text{TCA}_1 \text{ or } \text{TCA}_2) \div \text{TCA}_S \right] \times 100$$

where, TCA_S = Total COX activity (COX 1 + COX 2) of sample; TCA_1 = COX 1 activity (DuP-697 treated sample); TCA_2 = COX 2 activity (SC-560 treated sample).

FT-IR analysis

FTIR spectrophotometer was used to identify the characteristic functional groups in NILE. Small quantity (<10 mg) of NILE was taken in CaF_2 vessel

and placed in a sample cup of a diffuse reflectance accessory. The IR spectrum was obtained using Shimadzu 8300 FT-IR spectrophotometer at ambient temperature. Background correction was made by taking IR spectrum of de-ionized water as the reference in identical condition. The sample was scanned from 400 to 4,000 cm^{-1} for 16 times to increase the signal to noise ratio.

Statistical analysis

All data are reported as the mean $\pm$ SD of six measurements. The comparisons between the control group and the test group were performed by one-way analysis of variance (ANOVA) test using KyPlot version 2.0 beta 15 (32 bit) for windows. $P < 0.05$ was considered significant. Half-maximal inhibitory concentration (IC_{50}) values were calculated using the formula $Y = 100 \times A1/(X + A1)$, where $A1 = \text{IC}_{50}$; Y = response ($Y = 100\%$ when $X = 0$); X = inhibitory concentration. The linear correlation analysis was performed by Microsoft excel 2010.

Results

Plaque forming cell (PFC) assay

The result of PFC assay is presented in Fig. 1a. The increase in PFC value was highly significant at all the doses ($P < 0.001$) and the increase was dose dependent. At 50, 100 and 200 mg/kg doses, the increase of PFC value was 1.31 ± 0.06 , 1.48 ± 0.10 and 1.57 ± 0.09 fold, respectively, compared to 0 mg/kg group.

Estimation of total IgM

At 0 mg/kg dose, the level of total IgM in serum was 0.21 ± 0.02 mg/ml which significantly ($P < 0.001$) increased to 0.37 ± 0.01 mg/ml (Fig. 1b) at the 200 mg/kg dose. This accounted for 1.77 ± 0.22 fold increase in total IgM level.

Hemagglutination (HA) titre

The highest level of visible agglutination was found in the 200 mg/kg dose, followed by 100 and 50 mg/kg dose. At the highest dose (200 mg/kg) visible agglutination was formed up to a 1/320 dilution whereas, in

the case of 0 mg/kg group, agglutination was visible only up to a 1/40 dilution.

Cell proliferation assay

Figure 1d displays the effect of NILE on the murine splenic lymphocyte proliferation in vitro. Cell viability of the con A stimulated group (0 NILE $\mu\text{g/ml}$) was $121.98 \pm 4.91\%$ when the cell viability of the control group was considered as 100 %. Cell viability at the highest dose (80 $\mu\text{g/ml}$) was $125.15 \pm 6.43\%$ which was 1.24 ± 0.06 fold higher than the control and 1.01 ± 0.06 fold higher ($P > 0.05$) than that of 0 $\mu\text{g/ml}$ group.

Measurement of the inhibition of NO

The NO inhibitory capacity of NILE is depicted in the Fig. 2f in terms of NO expression. The 0 $\mu\text{g/ml}$ group, which was stimulated only with con A displayed $251.16 \pm 19.79\%$ increase in NO expression ($P < 0.001$), when NO expression of control was considered as 100 %. Gradual dose dependent decrease in NO expression was evident and at the highest dose the level of NO expression was $146.49 \pm 13.25\%$ which was significantly ($P < 0.01$) lower than 0 $\mu\text{g/ml}$ group.

Estimation of cytokine expression

The effect of NILE on the expression pattern of various cytokines are displayed in Fig. 2. Expression of all cytokines were increased except $\text{TNF-}\alpha$ and IL-4. The expression of IL-2 was significantly ($P < 0.001$) increased from 3.71 ± 0.73 pg/ml (control) to 39.47 ± 2.70 pg/ml (80 $\mu\text{g/ml}$), accounting for a 10.81 ± 1.43 fold increase. $\text{IFN-}\gamma$ expression was also increased significantly ($P < 0.001$), from 53.29 ± 5.83 pg/ml (control) to 343.33 ± 11.59 pg/ml (80 $\mu\text{g/ml}$). This accounts for a 6.49 ± 0.76 fold increase in $\text{IFN-}\gamma$ level. The expression of IL-4 at 80 $\mu\text{g/ml}$ (1.37 ± 0.15 pg/ml) was decreased 4.00 $\pm$ 1.70 fold compared to the 0 $\mu\text{g/ml}$ group (4.12 ± 0.13 pg/ml). The level of IL-10 was elevated from 190.33 ± 56.76 pg/ml (control) to $4,388.00 \pm 295.48$ pg/ml (80 $\mu\text{g/ml}$), accounting for a 24.06 ± 5.15 fold increase. Decreased expression of $\text{TNF-}\alpha$ was 1.77 ± 0.69 fold. At 80 $\mu\text{g/ml}$ concentration, the expression of $\text{TNF-}\alpha$ was 225.66 ± 11.67 pg/ml,

Fig. 1 **a** Increase in number of plaques in the PFC assay. **b** Dose dependent increase in total IgM expression. **c** Correlation between PFC and IgM. Where, R^2 = coefficient of determination. **d** Effect of NILE on splenic lymphocyte viability. N.S. = non-significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus group 1 (control) and α = non-significant ($P > 0.05$) compared to the 0 $\mu\text{g/ml}$ group

which was significantly lower than 0 $\mu\text{g/ml}$ group (399.66 ± 16.50 pg/ml).

The IFN- γ /IL-4 ratio being a major determinant of immunomodulatory activity was also found to be elevated when increasing the NILE dose from 0 to 80 $\mu\text{g/ml}$. At 0 $\mu\text{g/ml}$ the ratio was 40.27 ± 3.89 whereas, at 80 $\mu\text{g/ml}$ the ratio was increased several folds (252.34 ± 23.56).

Measurement of COX activities

Figure 3 displays the effect of NILE on the COX activity of murine splenic lymphocytes. Activity of both COX 1 and COX 2 was inhibited in a dose

dependent manner with gradual increase in NILE concentration resulting in suppression of total COX activity. At 0 $\mu\text{g/ml}$, the total COX, COX 1 and COX 2 activities were 23.47 ± 1.58 U/ml (nmol/min/ml), 10.46 ± 2.63 U/ml and 12.74 ± 1.00 U/ml, respectively. Highly significant decrease in COX 2 ($P < 0.001$) activity was found at the highest dose (80 $\mu\text{g/ml}$) when compared to the 0 $\mu\text{g/ml}$ group. At 80 $\mu\text{g/ml}$, the total COX, COX 1 and COX 2 activities were 9.75 ± 2.00 , 5.10 ± 1.70 and 4.99 ± 0.27 U/ml, respectively. The percentages of inhibition of COX 1 and COX 2 activity at 80 $\mu\text{g/ml}$ were 39.80 and 47.11 %, respectively in reference to total COX activity.

Fig. 2 Immunomodulatory activity of NILE in vitro. Murine splenic lymphocytes were cultured with Con A (5 μg/ml) in the presence of different concentrations of NILE (0–80 μg/ml). After 48 h of incubation, culture supernatant was collected and the level of NO was estimated by Griess reagent method and

various cytokines were estimated by Enzyme-linked immunosorbent assay (ELISA). **a** IL-2; **b** IFN-γ; **c** TNF-α; **d** IL-4; **e** IL-10 and **f** NO. ** $P < 0.01$, *** $P < 0.001$ versus control and $\alpha =$ non-significant ($P > 0.05$), $\beta = P < 0.05$, $\gamma = P < 0.01$, $\delta = P < 0.001$ versus 0 μg/ml group

Correlation study

Correlation analysis (Fig. 4) revealed that the expression of IL-10 was highly correlated ($R^2 = 0.9812$) with expression of IL-2, where both the cytokines were up-regulated. High negative correlation of IL-2 resided with TNF-α ($R^2 = 0.9781$). Figure 5 displays correlations between the expression of different cytokines and NO, where a highly negative correlation was found between IFN-γ and NO ($R^2 = 0.9881$).

FT-IR analysis

The IR spectrum (Fig. 6) of NILE displayed different peaks (Table 1) corresponding to different functional groups (Silverstein et al. 2007). The $2,923.9 \text{ cm}^{-1}$ peak was formed primarily due to the presence of methylene groups. Presence of two peaks, one close to $2,330 \text{ cm}^{-1}$ and another close to 667 cm^{-1} may correspond to the presence of ketone group such as in some aliphatic moieties. Presence of alkanes was

Fig. 3 Effect of NILE on total COX, COX 1 and COX 2 activity in murine splenocytes. Murine splenic lymphocytes were cultured for 48 h with different concentrations (0–80 $\mu\text{g/ml}$) of NILE in presence of 5 $\mu\text{g/ml}$ Con A. The control did not received NILE and Con A. Culture supernatants were collected after incubation and total COX, COX 1 and COX 2 activities were measured by colorimetric method. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus group 1 (control) and d = $P > 0.05$, c = $P < 0.05$, b = $P < 0.01$, a = $P < 0.001$ versus 0 $\mu\text{g/ml}$ group

detected by the two peaks at $1,460\text{ cm}^{-1}$ and $1,379\text{ cm}^{-1}$. Peak shifts in FTIR analysis are very common phenomenon. Some of the functional groups may thus, be affected by peak shift. The peak at $1,379.0\text{ cm}^{-1}$ may correspond to aromatic amines indicating C–N stretching (Mahitha et al. 2011). The peak at $2,856.4\text{ cm}^{-1}$ may be formed due to the presence of carboxylic acids (Bobby et al. 2012).

Discussion

Majority of the research works on the therapeutic potential of *N. indicum* has been confined in the anti-cancer properties of the plant. There were only few works describing the possible immunomodulatory potential of *N. indicum*. Macrophage-mediated mild cytotoxic activity of the polysaccharide fraction of dried leaves was reported by Muller et al. (1991). Nuclear factor $\kappa\beta$ (NF- $\kappa\beta$) activation inhibitory activity of a glycoside Oleandrin from the leaves was demonstrated by Manna et al. (2000) and Sreenivasan

et al. (2003). Therefore, our study is the first mechanism based report of the immunomodulatory activity of *N. indicum* extract.

PFC assay is a sensitive technique in which complement mediated lysis of foreign antigen (sRBC) takes place in presence of secreted IgM. Initially on activation by foreign antigen, naive B-cells get activated for clonal expansion. Later, when the sensitised B-cells again encounter the same antigen, they evoke IgG and IgM mediated immune response. The sRBC with numerous antigenic determinants then get lysed by the action of complement. Thus, the PFC assay is a simple tool to accurately study the generation of antibody secreting plasma cells from the naive B-cells which were triggered by foreign antigen (Dey et al. 2013). In this present study, the PFC value gradually increased with the increase in dose of NILE. Furthermore, the increase in IgM level detected by ELISA method (Fig. 1b) corroborates the findings of the PFC assay, which is solely dependent on the increase in immunoglobulin expression. The correlation between PFC value and total IgM level (Fig. 1c) was also high ($R^2 = 0.9722$). This demonstrates that NILE stimulates the humoral immune system as evident from the dose dependent increase in the complement mediated lysis of sRBC by IgM. Gradual dose dependent increase in the number of plaques signifies the increase in the IgM formation.

If PFC assay is coined as the qualitative test, the HA titre may be tagged as the quantitative test to study the stimulation of the humoral immune response. Naive B-cells encountering antigen, proliferate to form plasma cells which secrete antibodies. Neutralization of the foreign antigen occur through antigen–antibody binding which forms a cross linking latex. In the living system, later this agglutination complex gets digested by the phagocytic cells (Dey et al. 2013). In the present study, the antibody from the previously sensitized B-cells recognize the sRBC and binds to form agglutination complex. The presence of agglutination complex in higher dilution reflects not only the stimulation of the humoral system but also immunogenicity of the antigen. Higher doses of NILE resulted in visible hemagglutination at higher dilutions of the antiserum. Thus, NILE demonstrated stimulation of murine humoral immunity as evidenced from the significant increase in the humoral antibody titre.

The secondary lymphoid organ, spleen, mediates the immune response against the antigen in the blood

Fig. 4 Correlation between the expression of **a** IL-2 versus IFN- γ ; **b** TNF- α versus IFN- γ ; **c** IL-4 versus IFN- γ ; **d** IL-10 versus IFN- γ ; **e** TNF- α versus IL-2; **f** IL-4 versus IL-2; **g** IL-10 versus IL-2; **h** IL-4 versus TNF- α ; **i** IL-10 versus TNF- α ; **j** IL-10

versus IL-4. Axes 'x' and 'y' denote correlation points of respective cytokines measured at pg/ml at different doses of NILE (0–80 μ g/ml). Where, R^2 = coefficient of determination

stream. The red pulp on the outer space consists of erythrocytes, macrophages and lymphocytes and the inner white pulp is chiefly populated by T-lymphocytes. The plant lectin con A stimulates blastoid differentiation of the T-lymphocytes at an optimal dose of 5 μ g/ml and thus, functions as a T-lymphocyte mitogen (Chaudhuri and Chakravarty 1981). Stimulation with con A therefore, must have stimulated only the T-lymphocyte out of the whole splenocyte population. Though significant increase ($P < 0.01$) in cell proliferation was evident compared to control, the extent of proliferation was insignificant (Fig. 1d) when compared with the 0 μ g/ml group. Therefore, the increase in cell viability may only be due to con A and therefore, NILE may not possess stimulatory effect on the proliferation of lymphocytes.

Bacterial lipopolysaccharide and con A stimulate splenocytes to produce NO (Ramirez and Silva 1997)

possibly through stimulation of inducible NO synthase (iNOS). Various diseases such as multiple sclerosis, arthritis, juvenile diabetes and ulcerative colitis are associated with chronic release of NO (Dey et al. 2012b). In this study, suppression of NO expression by NILE on con A stimulated cells was evident as shown in Fig. 2f. NO is an excellent pro-inflammatory mediator. Expression of NO affects pro- and anti-inflammatory cytokines and vice versa. IFN- γ and TNF- α are well known inducers of NO production (Andrade et al. 1999). But in this case, the inversed expression of NO with IFN- γ ($R^2 = 0.9881$) (Fig. 5a) defines that the inhibition of NO expression by NILE was neither IFN- γ mediated nor cell mediated as the cell viability did not decrease as evident from the cell proliferation assay (Fig. 1d). The in vitro inhibition of the free radical NO by NILE has already been demonstrated (Dey et al. 2012b). In the present study,

Fig. 5 Correlation of NO with **a** IFN- γ ; **b** IL-2; **c** TNF- α ; **d** IL-4 and **e** IL-10 expression under the influence of NILE. Axes 'x' and 'y' denote correlation points of respective cytokines

measured at pg/ml and NO levels at different doses of NILE (0–80 μ g/ml). Where, R^2 = coefficient of determination

Fig. 6 Fourier transform infrared spectra of NILE

Table 1 Fourier transform infrared spectroscopy peak values of NILE

Wave number (cm ⁻¹)	Type of bonds	Functional groups	Type of vibration	Intensities
2,923.9	CH ₂ -O	Alkane (C-H) Aliphatic	Stretching	Strong
2,856.4	CH-pyrrole	Alkane (C-H)	Stretching	Strong
2,362.6	C = C	–	Stretching	
2,333.7	C = O	Aliphatic ketone	–	–
1,560.3	N-O	Nitro	Stretch	Strong
	N-H	Amide	Bending	–
1,460.0	C = C	Aromatic	Stretching	Medium-weak
	CH ₃	Alkane (C-H)	–	–
1,379.0	–C-H	Alkane	Bending	Variable
	N-O	Nitro	Stretching	Strong
1,280.6	C-O	Acid	Stretching	Strong
754.1	=C-H	Alkene	Bending	Strong
675.0	=C-H	Alkene	Bending	Strong
418.5	N-H	Amide	Bending	–

the correlation of NO suppression with IL-10 expression was negative ($R^2 = 0.9651$) besides positive correlation resided between NO and TNF- α expression ($R^2 = 0.9785$). Other groups (Gazzinelli et al. 1992; Oswald et al. 1992) have previously demonstrated that inhibition of iNOS in macrophages occur through TNF- α inhibition by IL-10. Therefore, it may be stated that the inhibition of iNOS was due to NILE mediated inhibition of TNF- α . Under the influence of NILE, the decrease in the expression of IL-4 and NO may be beneficial for asthmatic patients, as both IL-4 and iNOS are associated with asthmatic conditions (Batra et al. 2007).

Immune responses are chiefly regulated by the fine balance between the cytokine expressions induced either by specific antigen or by mitogens. IL-2, IFN- γ and TNF- α are pro-inflammatory cytokines whereas, IL-4 and IL-10 belong to anti-inflammatory category. Antiviral and anti-tumor environment is primarily initiated by IFN- γ (Schroder et al. 2004) apart from activating of macrophage, stimulation of MHC expression and amplification of leukocyte migration.

Body's innate response against microbial infections, proliferation of CD4⁺ and CD8⁺ T-lymphocytes and self/foreign antigen recognition is mediated by IL-2. IL-4 is a potent mediator of allergic inflammation and promotes conditions such as asthma and atopic syndrome. It activates mast cells, eosinophils and basophils and also promotes IgM to IgE class switching in B-lymphocytes. The anti-inflammatory cytokine IL-10 results in secretion of IgG1 and IgG3 upon activation of naive IgD⁺ B-lymphocytes and it is also a Ly-1⁺ B-lymphocyte growth factor (Kumar and Creery 2000). High level of TNF- α has been found to be associated with autoimmune conditions such as rheumatoid arthritis, psoriasis, and Crohn's disease (Scheinfeld 2004) and is responsible for promotion of inflammatory conditions. The present study demonstrated that NILE has the potential to up-regulate IL-2, IFN- γ and IL-10, expression and down-regulate TNF- α and IL-4 expression in the mitogen induced splenic lymphocytes in vitro.

IL-10 possesses inhibitory effects on the T_H1 cytokine expressions. But in this case, increase in the expression of IL-10 had no suppressive effect on IL-2 or IFN- γ expression, even though TNF- α was down-regulated. Further, two major promoters of inflammation, TNF- α and NO (Selvakkumar et al. 2007) were down-regulated by NILE. Previously, Erdemoglu et al. (2003) have demonstrated in vivo anti-inflammatory activity of *N. indicum* leaf and flower extracts on paw edema model. In this study, both TNF- α and NO under the influence of NILE were down-regulated and shared high positive correlation ($R^2 = 0.9785$) in their expression patterns. Thus, this may contribute to the anti-inflammatory activity NILE in addition to the increase in IL-10 level. High negative correlation between IL-10 and TNF- α ($R^2 = 0.9827$) induced by NILE may prove a beneficial target in the conditions such as psoriasis, ulcerative colitis, psoriatic arthritis and Crohn's disease (Cutler and Brombacher 2005). Besides, increase in NILE mediated IL-2 secretion may prove beneficial in autoimmune colitis, rheumatoid arthritis, allograft rejection, as IL-2 is primarily associated with immune tolerance (Lee and Margolin 2011).

At 0 μ g/ml, the increased expression of COX 1 and COX 2 compared to control was attributed to Con A stimulation. Both COX isoforms were down-regulated in a dose-dependent manner in parallel to gradual increase in NILE concentration. The suppression of

COX activity by NILE at 80 µg/ml was 58.75 ± 5.54 , 51.63 ± 9.26 and 59.94 ± 1.60 % for total COX, COX 1 and COX 2 activity respectively, when compared to the 0 µg/ml group. Cyclooxygenase (COX), also known as prostaglandin H synthase is one of the central molecules of inflammatory response which catalyses the conversion of arachidonic acid to PGH₂ (Williams et al. 1999). Among the two COX isoforms, COX 2 is predominantly involved in prostaglandin synthesis during acute inflammatory response (Xie et al. 1991). Therefore, suppression of COX activity, especially COX 2 is considered quintessential in the treatment inflammatory diseases.

Chemical species identified using FTIR analysis had serious implications in the anti-inflammatory activity of NILE. Thirteen different ketones with trifluoromethyl group have demonstrated group IV and VI phospholipases A2 inhibitory activity contributing to down-regulation of arachidonic acid formation (Ghomashchi et al. 1999). Furthermore, various ketone analogues were demonstrated through in vivo models to possess anti-inflammatory and immunosuppressive potential (Hall et al. 1980). Carignan and his group (2013) reported that aliphatic *n*-alkanols target nuclear factor of activated T cells (NFAT) and nuclear factor kappa-β (NF-κβ) situated downstream to the cell membrane. Compounds with active amide linkage such as anandamide, inhibit TNF-α and IL-1β expression in experimental inflammatory periodontitis and stress models (Rettori et al. 2012). In addition, there are numerous compounds possessing active nitro groups with diverse medicinal and therapeutic properties (Kapoor et al. 2003).

Conclusion

NILE displayed potent immunomodulatory activity which may prove beneficial after further exploration. Suppression of NO, TNF-α, COX activity and increase in IL-10 levels are important determinant for anti-inflammatory activity. However, from the present study it is difficult to conclude whether NILE specifically modulates the immune response towards T_H1 or T_H2 phenotype as expression of both classes of cytokines were up- and down-regulated. Besides, the functional nature of inflammatory cytokines differs with different inflammatory models (Cavaillon 2011). Therefore, further study is required on prostaglandins

and nuclear factors to evaluate the immunomodulatory potential of NILE.

Acknowledgments The authors are grateful to Mr. Somit Dutta and Mr. Bijoy Mahanta for their assistance in the experiments.

References

- Andrade JL, Arruda S, Barbosa T, Paim L, Ramos MV, Cavada BS, Barral-Netto M (1999) Lectin-induced nitric oxide production. *Cell Immunol* 194:98–102
- Batra J, Pratap Singh T, Mabalirajan U, Sinha A, Prasad R, Ghosh B (2007) Association of inducible nitric oxide synthase with asthma severity, total serum immunoglobulin E and blood eosinophil levels. *Thorax* 62:16–22
- Bobby MN, Wesely E, Johnson M (2012) FT-IR studies on the leaves of *Albizia lebbek* benth. *Int J Pharm Pharm Sci* 4:293–296
- Carignan D, Desy O, Ghani K, Caruso M, de Campos-Lima PO (2013) The size of the unbranched aliphatic chain determines the immunomodulatory potency of short and long chain *n*-alkanols. *J Biol Chem* 288:24948–24955
- Cavaillon JM (2011) Pro- versus anti-inflammatory cytokines: myth or reality. *Cell Mol Biol* 47:695–702
- Chaudhuri TK, Chakravarty AK (1981) Activation of murine thymocytes *in vivo*. Part 1: study of blastogenesis and DNA synthesis after stimulation with concanavalin A. *J Indian Inst Sci* 63:149–156
- Cutler A, Brombacher F (2005) Cytokine therapy. *Ann NY Acad Sci* 1056:16–29
- Dastur JF (1962) Medicinal plants of India and Pakistan. DB Taraporevala Sons and Co Ltd, Bombay
- Dey P, Chaudhuri TK (2013) Antioxidant capacity of *N. indicum*: a correlation study using principal component analysis and multivariate statistical approach. *Int J Pharm Pharm Sci* 5:931–937
- Dey P, Roy S, Chaudhuri TK (2012a) A quantitative assessment of bioactive phytochemicals of *Nerium indicum*: an ethnopharmacological herb. *Int J Res Pharm Sci* 3:579–587
- Dey P, Chaudhuri D, Chaudhuri TK, Mandal N (2012b) Comparative assessment of the antioxidant activity and free radical scavenging potential of different parts of *Nerium indicum*. *Int J Phytomed* 4:54–69
- Dey P, Roy S, Chaudhuri TK (2013) Stimulation of murine immune response by the tubers of *Dioscorea alata* L. of North-Eastern region of India. *Proc Zool Soc* 67:140–148
- Ding K, Fang JN, Dong T, Tsim KW, Wu H (2013) Characterization of a rhamnogalacturonan and a xyloglucan from *Nerium indicum* and their activities on PC12 pheochromocytoma cells. *J Nat Prod* 66:7–10
- Duke JA (1985) Handbook of medicinal herbs. CRC Press, Boca Raton
- Erdemoglu N, Kupeli E, Yeşilada E (2003) Anti-inflammatory and antinociceptive activity assessment of plants used as remedy in Turkish folk medicine. *J Ethnopharmacol* 89:123–129

- Gazzinelli RT, Oswald IP, James SL, Sher A (1992) IL-10 inhibits parasite killing and nitrogen oxide production by IFN- γ -activated macrophages. *J Immunol* 148:1792–1796
- Ghomashchi F, Loo R, Balsinde J, Bartoli F, Apitz-Castro R, Clark JD, Dennis EA, Gelb MH (1999) Trifluoromethyl ketones and methyl fluorophosphonates as inhibitors of group IV and VI phospholipases A(2): structure–function studies with vesicle, micelle, and membrane assays. *Biochim Biophys Acta* 1420:45–56
- Ghosh A (2008) Ethnomedicinal plants used in west Rarrh region of West Bengal. *Nat Pod Rad* 7:461–465
- Hadizadeh I, Peivastegan B, Kolahi M (2009) Antifungal activity of nettle (*Urtica dioica* L.), colocynth (*Citrullus colocynthis* L. Schrad), oleander (*Nerium oleander* L.) and konar (*Ziziphus spina-christi* L.) extracts on plants pathogenic fungi. *Pak J Biol Sci* 12:58–63
- Hall IH, Hall JE, Mohseni M, Sajadi Z (1980) Anti-inflammatory activity of diazomethyl ketone and chloromethyl ketone analogs prepared from *N*-tosyl amino acids. *J Pharm Sci* 69:1451–1452
- Hibbs JB Jr, Taintor RR, Vavrin Z, Rachlin EM (1988) Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem Biophys Res Commun* 157:87–94
- Hussain MA, Gorski MS (2004) Antimicrobial activity of *Nerium oleander* Linn. *Asian J Plant Sci* 3:177–180
- Ji YB (1999) Pharmacological action and application of anti-cancer traditional Chinese medicine. Heilongjiang Science Press, Heilongjiang
- Kapoor VV, Chadha R, Venisetti PK, Prasanth S (2003) Medicinal significance of nitroimidazoles—some recent advances. *J Sci Ind Res India* 62:659–665
- Karthikumar S, Jegatheesan K, Thangaraja A, Banupriya K, Dhivya T, Malarvizhi JM (2011) Immunomodulatory activity of *Eclipta prostrata* SRBC immunized mice. *J Pharmacog Phytother* 3:52–55
- Kumar A, Creery WD (2000) The therapeutic potential of interleukin 10 in infection and inflammation. *Arch Immunol Ther Exp* 48:529–538
- Kunle OF, Egharevba HO, Ahmadu PO (2012) Standardization of herbal medicines—a review. *Int J Biodivers Conserv* 4:101–112
- Lee S, Margolin K (2011) Cytokines in cancer immunotherapy. *Cancers* 3:3856–3893
- Leporatti ML, Ghedira K (2009) Comparative analysis of medicinal plants used in traditional medicine in Italy and Tunisia. *J Ethnobiol Ethnomed* 5:31
- Leung MY, Liu C, Koon JC (2006) Polysaccharide biological response modifiers. *Immunol Lett* 105:101–114
- Mae SH, Sofia M, Bolhuis RL, Nooter K, Oostrum RG, Subagus W, Ibnu GG (2008) Selectivity of compounds isolated from the leaves of *Nerium indicum* Mill. on various human cancer cell lines. *Med J Malaysia* 63:24–25
- Mahitha B, Raju BDP, Dillip GR, Reddy CM, Mallikarjuna K, Manoj L, Priyanka S, Rao KJ, Sushma NJ (2011) Biosynthesis, characterization and antimicrobial studies of AgNPs extract from *Bacopa monniera* whole plant. *Dig J Nanomat Biostruct* 6:135–142
- Manna SK, Sah NK, Newman RA, Cisneros A, Aggarwal BB (2000) Oleandrin suppresses activation of nuclear transcription factor- κ B, activator protein-1, and c-Jun NH2-terminal kinase. *Cancer Res* 60:3838–3847
- Muller BM, Roskopf F, Paper DH, Kraus J, Franz G (1991) Polysaccharides from *Nerium oleander*: structure and biological activity. *Pharmazie* 46:657–663
- Oswald IP, Wynn TA, Sher A, James SL (1992) Interleukin 10 inhibits macrophage microbicidal activity by blocking the endogenous production of tumor necrosis factor alpha required as a costimulatory factor for interferon gamma-induced activation. *Proc Natl Acad Sci USA* 89:8676–8680
- Raisuddin S, Zaidi SIA, Singh KP, Ray PK (1991) Effect of subchronic aflatoxin exposure on growth and progression of Ehrlich's ascites tumor in mice. *Drug Chem Toxicol* 14:185–206
- Ramirez F, Silva A (1997) Glucocorticoids enhance concanavalin A-induced mitogenic response through the inhibition of nitric oxide production. *Immunology* 90:66–73
- Rettori E, De Laurentiis A, Zorrilla Zubilete M, Rettori V, Elverdin JC (2012) Anti-inflammatory effect of the endocannabinoid anandamide in experimental periodontitis and stress in the rat. *NeuroImmunoModulation* 19:293–303
- Scheinfeld N (2004) A comprehensive review and evaluation of the side effects of the tumor necrosis factor blockers etanercept, infliximab and adalimumab. *J Dermatolog Treat* 15:280–294
- Schroder K, Hertzog PJ, Ravasi T, Hume DA (2004) Interferon- γ : an overview of signals, mechanisms and functions. *J Leukoc Biol* 75:163–189
- Selvakkumar C, Gayathri B, Vinaykumar KS, Lakshmi BS, Balakrishnan A (2007) Potential anti-inflammatory properties of crude alcoholic extract of *Ocimum basilicum* L. in human peripheral blood mononuclear cells. *J Health Sci* 53:500–505
- Silverstein RM, Bassler GC, Morrill TC (2007) Spectrometric identification of organic compounds, 6th edn. Wiley, New York
- Singhal KG, Gupta GD (2011) Anti-anxiety activity studies of various extracts of *Nerium oleander* linn. flowers. *Int J Pharm Pharm Sci* 3:323–326
- Singhal KG, Gupta GD (2012) Hepatoprotective and antioxidant activity of methanolic extract of flowers of *Nerium oleander* against CCl₄-induced liver injury in rats. *Asian Pac J Trop Med* 5:677–685
- Sreenivasan Y, Sarkar A, Manna SK (2003) Oleandrin suppresses activation of nuclear transcription factor- κ B and activator protein-1 and potentiates apoptosis induced by ceramide. *Biochem Pharmacol* 66:2223–2239
- Tiwari M, Singh RH (2012) Ethnomedicinal practices among the tribes of Lalganj block of district Mirzapur, Uttar Pradesh. *Life Sci Leaflets* 10:84–99
- Williams CS, Mann M, DuBois RN (1999) The role of cyclooxygenases in inflammation, cancer, and development. *Oncogene* 18:7908–7916
- Xie WL, Chipman JG, Robertson DL, Erikson RL, Simmons DL (1991) Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc Natl Acad Sci USA* 88:2692–2696