Anti-inflammatory activity of *Nerium indicum* by inhibition of prostaglandin E2 in murine splenic lymphocytes

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**ABSTRACT**

**Objective:** *Nerium indicum* Mill (syn. *N. oleander* L. and *N. odorum* Aiton; family: Apocynaceae) is a medicinal plant, used in the treatment of diverse ailments including various chronic inflammatory diseases in traditional medicine. We have previously demonstrated the immunomodulatory activity of a bioactive fraction of *Nerium indicum* leaf (NILE) by studying up-regulation of interleukin-2 (IL-2), IL-10, interferon-gamma and down regulation of IL-4, tumor necrosis factor-alpha (TNF-α), nitric oxide, cyclooxygenase-1 (COX-1) and COX-2 activities. Therefore, this study aimed to confirm the anti-inflammatory activity of NILE by inhibition of prostaglandin E$_2$ (PGE$_2$) activity in murine splenic lymphocytes in *vitro.*

**Materials and Methods:** Murine lymphocytes were isolated from spleen and stimulated with 5 μg/mL concanavalin A in RPMI-1640, supplemented with 50 U/mL penicillin, 50 U/mL streptomycin, 50 U/mL nystatin and 10% fetal bovine serum. Different concentrations (0–80 μg/mL) of NILE were added and the cells were cultured for 48 h. The culture supernatants were thereafter collected by centrifugation and assayed for expression of PGE$_2$ level. The data were analyzed statistically.

**Results:** The results demonstrated a 2.26-fold inhibition of PGE$_2$ level at 80 μg/mL of NILE. Half maximum inhibitory concentration (IC$_{50}$) was calculated to be 44.95 ± 0.45 μg/mL. Linear correlation analysis of the dose-dependent PGE$_2$ inhibition with other pro- and anti-inflammatory mediators demonstrated high inter-correlation between the parameters.

**Conclusions:** Thus, the present study remains in accordance with our previous report and confirms the anti-inflammatory claim of *N. indicum*, mentioned in the traditional medicine.

**KEYWORDS:** Anti-inflammatory, cyclooxygenase, lymphocyte, nerium, prostaglandin

Introduction

The fascinating medicinal potential of *Nerium indicum* in the treatment of diverse immunological conditions such as inflammation, has been mentioned in Indian[1,2] and Chinese[3,4] traditional medicinal systems. However, in *vitro* and *in vivo* studies of the therapeutic potentiality of *N. indicum* was only confined within the anti-cancer properties of the plant.[5,6] Study confirming the anti-inflammatory activity of *N. indicum* has not yet been done by investigating the terminal prostaglandins (PG) responsible for the sensitization phase of inflammation. Previously, Müller *et al.,* demonstrated the stimulation of macrophage mediated cytotoxic activity by polysaccharides of *Nerium indicum* leaves (NILE). Moreover, very recently we have showed that a bioactive hydro-methanolic fraction of NILE up-regulates interleukin-2 (IL-2), IL-10, interferon-gamma and down-regulates tumor necrosis factor-alpha (TNF-α), IL-4, nitric oxide, cyclooxygenase-1 (COX-1) and COX-2 activities. Therefore, this study aimed to confirm the anti-inflammatory activity of NILE by inhibition of prostaglandin E$_2$ (PGE$_2$) activity in murine splenic lymphocytes in *vitro.*

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To remove dirt and shade dried at room temperature for 20 days, followed by grinding to powder. The powder (50 g) was mixed with 100% methanol (500 mL) with continuous shaking (160 rpm) for 20 h at 37°C. The mixture was filtered and then centrifuged at 2850 × g for 15 min. The pellet was again mixed with 100% methanol (500 mL) and re-processed as previously described. The supernatants were collected from both the phases, filtered and the filtrate was concentrated under reduced pressure in a rotary evaporator.

**Materials and Methods**

**Chemicals**

All reagents were procured from HiMedia Laboratories Pvt., Ltd., (Mumbai, India), otherwise indicated. Nystatin and concanavalin A (Con A) were obtained from Sigma-Aldrich (USA). PGE₂ enzyme immuno-assay kit was purchased from Cayman Chemical Company (An Arbor, MI, USA).

**Plant Material and Extract Preparation**

White flower variety of *N. indicum* plant was collected from the University of North Bengal Campus, India (26.71°N, 88.35°S) and identified by taxonomist Prof. A. P. Das of Department of Botany, University of North Bengal. A voucher specimen was stored in the herbarium of Department of Botany (accession number of 09618).

The leaves were washed with double distilled water to remove dirt and shade dried at room temperature for 20 days, followed by grinding to powder. The powder (50 g) was mixed with 100% methanol (500 mL) with continuous shaking (160 rpm) for 20 h at 37°C. The mixture was filtered and then centrifuged at 2850 × g for 15 min. The pellet was again mixed with 100% methanol (500 mL) and re-processed as previously described. The supernatants were collected from both the phases, filtered and the filtrate was concentrated under reduced pressure in a rotary evaporator. The resultant NILE extract was lyophilized and stored at −20°C until further use.

**Maintenance of Animals**

Swiss albino mice (30 ± 2 g of body weight) of 6–8 weeks were used for the following studies. 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). Animal Ethical Committee approval of University of North Bengal (No. 840/ac/04/Committee for the Purpose of Control and Supervision of Experiments on Animals) was taken prior to the experiments.

**Culture of Splenocytes**

Spleen was removed aseptically from Swiss albino mice under proper anesthesia and washed with RPMI-1640 to remove blood. The spleen was passed through the mesh of a tissue grinder and then splenocyte suspension was prepared in RPMI-1640. The splenocytes were washed (200 × g) thrice using RPMI-1640 for 10 min and resuspended in 1N NH₄Cl to lyse red blood cells. After 5 min ice cold RPMI-1640 was added to stop the reaction. The cells were again centrifuged (200 × g) and resuspended in RPMI-1640. Splenocytes were adjusted as 2 × 10⁶ cells/mL with RPMI-1640, supplemented with 50 U/mL penicillin, 50 U/mL streptomycin, 50 U/mL nystatin and 10% fetal bovine serum and seeded into 6-well culture plates. Con A was added to get a final concentration of 5 μg/mL and 100 μL of different concentrations of NILE (0–80 μg/mL) was then added to the wells. Control did not receive Con A and NILE. The plates were then covered and incubated under 5% CO₂ and humidified atmosphere of 90% air at 37°C temperature for 48 h.

After incubation, culture supernatants from each well were centrifuged at 2850 × g for 10 min. The supernatants were used to assay PGE₂ levels.

**Measurement of Prostaglandin E₂ Level**

PGE₂ was measured by competitive binding between PGE₂ and PGE₂-acetylcholineesterase (PGE₂-AChE) for restricted amount of PGE₂ monoclonal antibody using PGE₂ EIA Kit (Cayman) according to the manufacturer’s instructions. In brief, 50 μL cell culture supernatants were added to the respective wells of a 96-well plate, precoated with goat polyclonal anti-mouse IgG, followed by 50 μL of PGE₂-AChE tracer and 50 μL PGE₂ monoclonal antibody. The plate was incubated for 18 h at 4°C and then washed five times with wash buffer. Ellman’s reagent (200 μL) was added and incubated in dark for 60 min with constant mild shaking. Then, absorbance was read at 415 nm using Bio-Rad iMark™ microplate absorbance reader. Standard curve of PGE₂ was prepared in parallel to the samples.

**Data Analysis and Statistics**

All data are reported as the mean ± standard deviation of six measurements. Comparisons between the control group and the test groups were performed by one-way analysis of variance and group comparisons for percentage of inhibition were performed by paired t-test using KyPlot version 2.0 (KyensLab Inc.,) beta 15 (32 bit) for windows. *P < 0.05 was considered significant. Linear correlation analysis were performed using Microsoft Excel 2010 (Microsoft Corporation).

**Results**

**Inhibition of Prostaglandin E₂**

This study demonstrated that NILE has the potentiality to inhibit PGE₂ synthesis in murine splenic lymphocytes [Figure 1]. PGE₂ level was increased to 649.30 ± 44.6 pg/mL (4.6-fold increase compared to control) due to stimulation with Con

![Figure 1: Levels (pg/mL) of prostaglandin E₂ under different doses (0–80 μg/mL) of Nerium indicum leaf. Data are represented as mean ± standard deviation of six observations. ***P < 0.001 compared to control. β = P < 0.01 and α = P < 0.001 versus 0 μg/mL group](image-url)
A, which upon NILE treatment at 80 μg/mL, was significantly down-regulated (P < 0.001) to 286.06 ± 26.75 pg/mL (2.26-fold decrease compared to 0 μg/mL). The percentage of inhibition (inhibitory concentration was 44.95 ± 0.45 μg/mL) of PGE₂ is presented in Figure 2. This demonstrated in vitro dose-dependent activity of NILE to inhibit PGE₂ levels.

Correlation Analysis

The linear correlation analysis between the PGE₂ level with different pro- and anti-inflammatory signals are demonstrated in Figure 3. High positive correlation between PGE₂ level with COX-1 (R² = 0.6238) and COX-2 activities (R² = 0.6014). The highest degree of correlation resided between PGE₂ and IL-2 level (R² = 0.895).

Discussion

The complex scenario of chronic inflammation is primarily governed by the fine balance between pro- and anti-inflammatory cytokines and formation of arachidonic metabolites through the enzymatic cascade of COX. COX-1 is constitutively expressed, whereas COX-2, the inducible isoform, is predominantly involved in chronic inflammation. PG are metabolites of arachidonic acid, generated through a enzymatic cascade of COX and PG synthase, which leads to the cardinal signs of inflammation, that is, redness due to increase in blood flow, swelling due to vasodilation and pain due to induction of peripheral sensory neurons. PG are the terminal mediators of hyperalgesia, generation of fever, increase in vascular permeability as well as primarily responsible for vascular diseases and angiogenesis. COX inhibitors are therefore routinely used as therapeutics to inhibit PG synthesis to eliminate inflammation in a wide range of diseases.

The plant lectin Con A originated from Canavalia brasiliensis is a potent T-lymphocyte polyclonal activator and also a potent mediator of chronic inflammation through janus kinase/signal transducers and activators of transcription three pathway. In the present study, murine splenic lymphocytes were stimulated using an optimum dose of Con A (0 μg/mL) to generate a local plethora of inflammatory mediators such as PGE₂. As a result, the PGE₂ level was increased 4.60-fold from nonstimulated control to Con A stimulated 0 μg/mL. Dose-dependent decrease in PGE₂ was evident with the increase in the NILE concentration, which resulted in 2.26-fold decrease at 80 μg/mL compared to 0 μg/mL group. The pattern of dose-dependent PGE₂ inhibition directly corroborates the inhibition of COX-1 and COX-2 activities as demonstrated in our previous study.

PGE₂ is a potent inhibitor of IL-2 without any killing of IL-2 expressing cells. Wolf et al., reported that PGE₂ inhibits

![Figure 2: Percentage of inhibition of prostaglandin E₂ under different doses (0–80 μg/mL) of Nerium indicum leaf. Data are represented as mean ± standard deviation of six observations. ***P < 0.001 compared to 0 μg/mL group](image)

![Figure 3: Correlation of prostaglandin E₂ level with the expression of different pro- and anti-inflammatory mediators. Axes “x” and “y” denotes correlation points of respective parameters measured at pg/mL at different dose of Nerium indicum leaf (0–80 μg/mL). Where, R² = coefficient of determination](image)
IL-2 expression only at sub-optimal stimulation by Con A (<5 μg/mL). However, the dose-dependent negative-linear correlation analysis in the present study demonstrated very high correlation (R² = 0.895) between IL-2 and PGE₂ level [Figure 2] reflecting on the fact that PGE₂ level had serious implications on the IL-2 expression in the lymphocytes. Besides, the high positive correlation of PGE₂ with the pro-inflammatory mediators such as NO (R² = 0.7866) and TNF-α (R² = 0.8797) and anti-inflammatory cytokine IL-10 (R² = 0.876) reflect the anti-inflammatory effect of NILE.

There are numerous reports on the anti-cancer activities of various fractions and compounds from N. indicum. However, there were only few studied demonstrating the immunomodulatory activity of the plant in spite of the fact that the plant is extensively used in traditional medicine. Müller et al.,[7] demonstrated the macrophage-mediated mild cytotoxic activity of a polysaccharide fraction of NILE. A glycoside oleandrin, isolated from the leaves showed potent nuclear factor κB activation inhibitory activity.[16,17] Thus, in continuation to our previous study, here we demonstrate the potent anti-inflammatory activity of N. indicum by the inhibition of PGE₂ expression in Con A stimulated murine lymphocytes, which is possibly due to the inhibition of COX activities responsible for production of PGE₂.

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Conflicts of Interest
There are no conflicts of interest.

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