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COMPARATIVE PHYTOCHEMICAL PROFILING AND EFFECTS OF *NERIUM OLEANDER* EXTRACTS ON THE ACTIVITIES OF MURINE PERITONEAL MACROPHAGES

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Abstract: *Nerium oleander* is a medicinal plant. Apart from its ethnopharmacological uses, pharmacognostic studies have revealed several of its bioactivities. Previously we demonstrated that the phenolic and flavonoid rich extracts of oleander leaf, stem and root possess potent antioxidant and free radical scavenging activities. Moreover, the leaf extract actively modulates the Th1/Th2 cytokine balance and exerts anti-inflammatory activities on murine splenic lymphocytes. Therefore, the present study was designed to evaluate the effect of oleander leaf, stem and root extracts on phagocytosis and the free radical-related activities of murine peritoneal macrophages. In addition, phytochemical profiling was performed using gas chromatography-mass spectrometry (GC-MS). The results demonstrated that the increase in phagocytosis and decrease in myeloperoxidase (MPO) were in the order of leaf>root>stem. The inhibition of cell adhesion, nitric oxide (NO) and elevation of respiratory burst activity was in the order of leaf>stem>root. However, the bioactivities of the leaf extract were much high than those of the stem and root extracts. Phytochemical analysis also revealed the presence of several bioactive constituents in oleander extracts. Therefore, the present study demonstrated that oleander possesses the capacity to modulate macrophage activities and the bioactivities are attributed to the numerous phytochemicals identified in oleander extracts.

Key words: GC-MS; immunomodulatory; macrophage; *Nerium oleander*; nitric oxide; phagocytosis

INTRODUCTION

Oleander is extensively used for the treatment of diverse ailments in the traditional medicine of different parts of the world, especially in India and China [1]. Recent pharmacognostic studies have demonstrated diverse bioactivities such as antioxidant, hepatoprotective, analgesic, anti-ulcer, anticancer immunomodulatory and antidiabetic activities associated with oleander [1]. Previously, we demonstrated that *N. oleander* possesses potent *in vitro* antioxidant and free radical scavenging activities [2]. Some free radicals such as superoxide ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), peroxynitrite anion ($ONOO^-$), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2) and nitric oxide (NO) are an integral part of macrophage bioactivities, especially in inflammatory conditions. In addition, the potent immunomodulatory activities of oleander leaf in the modulation of the Th1/Th2 balance and inhibition of cyclooxygenase levels and associated prostaglandin were also demonstrated recently [3].

It is interesting to note that most of the pharmacognostic studies of oleander are rooted in traditional phytotherapies. However, just like other medicinal plants, most of the studies have concentrated only on oleander leaf extracts or compounds isolated from the leaves, in spite of the fact that traditional therapies mention the use of other parts of oleander as well. Thus, the possible bioactivities of oleander stem and root remain unexplored. Most phytochemical studies have also concentrated only on oleander leaf. We therefore decided to investigate the effects of oleander leaf, stem and root on the phagocytosis and free radical-related activities of murine macrophages. In addition, this is the first report of complete phytochemical profiling of the major parts of oleander.

MATERIALS AND METHODS

Chemicals and solvents

All reagents were procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), unless otherwise indicated. Freund's incomplete adjuvant, nystatin, poly-L-lysine, lipopolysaccharide (LPS) and zymosan were obtained from Sigma Aldrich (USA). Milli-Q® ultrapure water from the departmental facility was used for all the experiments. High performance liquid chromatography (HPLC)-grade solvents were used for GC-MS analysis, and procured from Merck India Pvt. Ltd.

Plant material

Leaves, stems and roots of fresh and disease-free white-flowered oleander were collected from the garden of the University of North Bengal (26.71°N, 88.35°S), West Bengal, India. The plant material was identified and authenticated by Prof. Abhaya Prasad Das, senior Taxonomist of the Department of Botany, University of North Bengal. A voucher specimen was stored at the herbarium of the Department of Botany, University of North Bengal (accession no. 09618).

Extract preparation

The plant material was twice washed with double-distilled water to remove any dirt. The stems and roots were chopped into 1-1.5-cm pieces. The material was then shade-dried at room temperature (RT). After 14 days, the dried parts were ground to powder using a blender (Lords® Hummer 1100). The resultant powder (100 g) was mixed with 1000 mL of 7:1 methanol: water (v/v) and kept at 37°C in a shaking incubator (160 rpm) for 18 h. The mixture was then centrifuged at 5000 rpm for 15 min. The supernatants were filtered using a vacuum pump and stored separately. The remaining pellet was again mixed with 1000 mL of 7:1 methanol: water (v/v) and once more kept in a shaking incubator (160 rpm) for 18 h. The supernatant was filtered and mixed with the stored filtrate of the previous phase. The final filtrate was concentrated under reduced pressure in a rotary evaporator (Buchi Rotavapour®), lyophilized (SJIA-10N) and stored at -20°C until further use. The hydromethanolic extracts of *N. oleander* leaf, stem and root were designated as NOLE, NOSE and NORE, respectively.

Animals

Swiss albino mice 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 University of North Bengal (No. 840/ac/04/CPCSEA) and performed in accordance with the legislation for the protection of animals used for scientific purposes.

Acute toxicity study

OECD guidelines (test 423: Acute oral toxicity – Acute toxic class method; 2002) were followed to study the acute toxicity profile of NOLE, NOSE and NORE and for dose selection [4]. Animals were divided into different groups (n=6) and fasted overnight prior to the experiment. The plant extracts were administered orally in increasing doses up to 2000 mg/kg body weight (BW). Thereafter, all the groups were carefully observed for the development of any clinical or toxicological symptoms at 30 min and then at 2, 4, 8, 24 and 48 h.

Carbon clearance test

The carbon clearance test was performed according to a standard method [5] with minor modifications. Different doses (50 and 200 mg/kg) of NOLE, NOSE and NORE were administered orally for 14 days to Swiss albino mice and a control group received water. On day 16 (48 h after the last dose), 0.1 mL of Indian ink was injected into the tail vein and then 25 µL of blood were drawn from the orbital vein at 0, 5, 10 and 15 min after injection and mixed with 2 mL of 0.1% Na_2CO_3. The absorbance was immediately read at 650 nm to estimate the extent of carbon clearance, i.e. the rate of carbon elimination from the blood.

Peritoneal macrophage quantification

Different sets of mice were fed orally with NOLE, NOSE and NORE (50, 100 and 200 mg/kg) for 14 days. A separate group, used as control, was not treated with any extracts. Twenty-four to 36 h prior to the experiment, 0.5 mL of Freund's incomplete adjuvant was injected [6,7] into the peritoneum. RPMI-1640 (2 mL) was injected intraperitoneally prior to the experiment. Under anesthesia, a midline incision was made in the abdomen and the peritoneum was carefully washed with RPMI-1640. The peritoneal exudate cells were collected and centrifuged at 1000 rpm at 4°C, for 5 min [8]. The pellets were resuspended in RPMI-1640 and incubated for 45 min at 37°C in Petri dishes. After incubation, the supernatants were removed and the Petri dishes were washed with chilled PBS and centrifuged at 1000 rpm for 5 min. The pellets were resuspended in PBS and the solutions were mixed with an equal volume of neutral red and charged on the hemocytometer to count macrophages under a phase-contrast microscope.

Phagocytic activities

Phagocytic activities were assessed according to the standard method [9] with small modifications. Animals were treated with oleander extracts and peritoneal macrophages were subsequently collected as previously described. Cell suspensions (100 µL) from each group were mixed with 100 µL RPMI-1640 medium containing 20% fetal bovine serum (FBS) and 100×10^6 cells/mL of heat-treated (inactivated) yeast cells. The mixtures were incubated at 37°C for 60 min with occasional shaking. After incubation, 50 µL of the mixtures were smeared onto a glass slide, air-dried and stained with Wright-Giemsa stain. The slides were observed under light microscope using an oil immersion and the cells were counted. The phagocytic activity was expressed as phagocytic capacity (PC), and the phagocytic index (PI) was calculated using the following formula: PI=A × B, where, A is the percentage of yeast-ingesting phagocytes and B is the number of yeast ingested per phagocyte. PC is the mean percentage of cells that engulfed ≥4 yeast cells.

MTT cell viability assay

Cell viability was measured using an EZcountTM MTT Cell Assay Kit (HiMedia) according to the manufacturer's instructions.

Cell adhesion property

Cell adhesion was examined according to the previously described method [10] with some modifications. Murine peritoneal macrophages were isolated as previously described. The cells were then seeded in a 96-well plate with different concentrations (0-100 µg/mL) of NOLE, NOSE and NORE for 60 min. After incubation, the wells were gently washed with RPMI and then 100 µL of 0.5% crystal violet (dissolved in 12% neutral formaldehyde) and 10% ethanol were added to each well and incubated for 4 h at 37°C in a humidified chamber. After incubation, the wells were washed with RPMI-1640 and air-dried for 30 min. To each well, 100 µL of 1% SDS (dissolved in RPMI-1640) were added and the absorbance was measured at 570 nm. The change in the adherence property was measured using the following formula: % inhibition of adherence=$[(A_o-A_1)\div A_o] \times 100$, where A_o was the absorbance of the control and A_1 was the absorbance in the presence of the plant extracts.

Respiratory burst activity

Respiratory burst activity was examined according to a previously standardized protocol with some modifications [11]. Murine peritoneal exudate macrophages were collected in RPMI-1640 as previously described and seeded into a 96-well plate, which was pre-coated with 0.2% poly-L-lysine along with various concentrations (0-100 µg/mL) of *N. oleander* extracts. To this, 0.1% zymosan (in 100 µL of RPMI-1640) was added and the plate was incubated for 30 min at 37°C in a humidified chamber. The zymosan was discarded and the cells were washed thrice with RPMI-1640 followed by staining with 100 µL of nitro blue tetrazolium (NBT, 0.3%) at RT. After 30 min, the NBT solution was discarded and the reaction was stopped by the addition of 100 µL of absolute methanol. The formazan that

was generated was dissolved in 120 µL of 2M KOH and 140 µL of DMSO. Absorbance was immediately read at 630 nm. The dose-dependent increase in OD demonstrates an increase in respiratory burst activity.

Myeloperoxidase release

Myeloperoxidase release was examined according to a standard method [12] with minor modifications. Murine peritoneal macrophages (2×10^6 cells/mL) were seeded into 96-well culture plates with 100 ng/mL LPS. To this, varying concentrations (0-100 µg/mL) of *N. oleander* extracts were added and the cells were incubated at 37°C in a humidified chamber. After incubation, the solutions from each well were centrifuged at 13000 rpm for 10 min and the supernatants were removed. To the cell-pellet, 0.01% of SDS in RPMI-1640 was added to lyse the cells. The solutions were centrifuged at 13000 rpm for 10 min and the supernatant was collected. Supernatants (100 µL) from each group were mixed with 100 µL of substrate buffer (ortho-phenylenediamine) and kept at 37°C. After 20 min, the reaction was stopped using 100 µL of 2N H_2SO_4. Absorbance was read at 492 nm.

Lipopolysaccharide-induced nitric oxide production

The standard Griess reagent method [13] was used, with some modifications, to estimate the change in NO level. Peritoneal macrophage cells were collected as previously described. A cell suspension (2×10^6 cells/mL) was prepared in RPMI-1640 (containing 50 U/mL of penicillin, 50 U/mL of streptomycin and 50 U/mL of nystatin) supplemented with 10% FBS and 200 µl of the cell suspension was added with 100 µl of different concentrations (0-80 µg/mL) of NOLE, NOSE and NORE (dissolved in RPMI-1640) to each well of the 96-well plate. To each well, 20 µg/mL of LPS suspension was added, the plates were covered and incubated for 24 h under 5% CO_2 and a humidified atmosphere of 90% air at 37°C. After incubation, the solutions from each well were centrifuged at 5000 rpm for 5 min. The supernatants were used to determine the NO level.

Briefly, 50 µl of the supernatants were mixed with 200 µl of Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine hydrochloride in 2.5% H_3PO_4) in each well of the 96-well plate. The solution was incubated for 20 min at room temperature and the generated purple azo dye was detected at 540 nm. The percentage inhibition of NO generated was calculated using the following formula: % of inhibition= $[(A_0\text{-}A_1) \div A_0] \times 100$, where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample.

Gas chromatography-mass spectrometry analysis

N. oleander leaf, stem and root were bifractionated by methanol (polar) and n-hexane (non-polar) and passed through anhydrous Na_2SO_4 and activated charcoal (2:1; w/w) to remove any trace of moisture and color. The samples were analyzed using a Thermo Scientific Trace 1300 gas chromatograph (GC) attached to a Thermo Scientific ISQ QD single quadrupole mass spectrophotometer (MS). The GC was equipped with a TG-5MS column (30 m×0.25 mm×0.25 µm). The inlet temperature was maintained at 250°C. The initial temperature was set at 60°C (solvent delay 5 min) with a hold of 2 min, followed by a ramp of 5°C to 290°C with a hold of 6 min (54 min program). Samples (1 µL) were injected into a splitless mode (split flow 50 mL/min) with splitless time of 0.80 min, using a Thermo Scientific AI-1310 auto-sampler. The carrier gas was helium, with a constant flow of 1 mL/min. the MS transfer line temperature was set at 290°C with an ion source temperature of 230°C (electron ionization). The individual samples were analyzed at 70 eV. The mass analyzer range was set to 50-650 amu. All samples were analyzed three times.

Data analysis

MS data analysis was performed by Automated Mass Spectral Deconvolution and Identification System (AMDIS) version 2.70. The major and essential compounds were identified by mass fragmentation patterns using the database of the National Institute of Standards and Technology (NIST) with an MS library

version 2011. All data are presented as the means±SD of six measurements. Comparisons between the control group and test group were performed by one-way analysis of variance (ANOVA) 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 $A1=IC_{50}$; Y=response (Y=100% when X=0); X=inhibitory concentration. The linear correlation analysis was performed using Microsoft Excel 2010.

RESULTS AND DISCUSSION

The present study was designed to evaluate the immunomodulatory activities of oleander leaf (NOLE), stem (NOSE) and root (NORE) extracts on the activities of murine peritoneal macrophages. The overall results demonstrated that the bioactivities of NOLE were much higher than those of NOSE and NORE. Furthermore, phytochemical analysis revealed the presence of several bioactive compounds that may be responsible for the pharmacognostic activities of oleander.

Oleander is an ethnopharmacological plant used in traditional medicine for the treatment of several diseases [1]. Recent pharmacognostic studies have demonstrated several pharmacological activities associated with oleander extracts. However, reports exist on the toxic effect of oleander on clinical and pathological features *in vivo* [14]. Therefore, an acute toxicity study was performed for safety evaluation and dose selection for the *in vivo* experiments. Results demonstrated that no signs of mortality were present in the experimental animals up to the highest dose of 2000 mg/kg. Therefore, 0.025, 0.05 and 0.1 of the maximum dose (2000 mg/kg) were considered for the *in vivo* studies.

The murine peritoneal cavity is primarily colonized with macrophages. These *in situ* nonadherent cells have higher expression of inducible NO synthase and IL-12 compared to the macrophages of splenic origin [15]. Based on the morphology and surface molecular characteristics, peritoneal macrophages are highly mature and possess greater phagocytic capacity than splenic macrophages. Moreover, these cells are characterized by significantly lower expression of CD-80, CD-86, CD115, Gr-1 and high expression of the B7-H1 marker compared to other macrophage subsets [15]. Orally administered NOLE, NOSE and NORE increased the total peritoneal macrophage population in mice (Fig. 1a). The number of cells at 0 mg/kg group was $3.33\pm0.51 \times 10^6$ cells/mL. At the 200 mg/kg dose, the number was altered to 5.66±0.81, 3.50±0.54 and $4.00\pm0.89 \times 10^6$ cells/mL for NOLE, NOSE and NORE, corresponding to 1.70-, 1.05- and 1.20-fold increases, respectively. A previous study demonstrated the potential of oleander leaf extract to upregulate IFN-γ levels without any effect on lymphocyte cellularity [3]. IFN-γ is a potent activator of peritoneal macrophages and thus, the increase in macrophage population may be attributed to the oleander-induced expression of endogenous IFN-γ. The result also corroborates the previous findings of Muller et al. [16] who demonstrated the mild mitogenic activity of a polysaccharide fraction of oleander on murine macrophages.

The effect of oleander extracts on the reticuloendothelial system comprising of mononuclear mobile and fixed-tissue macrophages was evaluated by carbon-clearance test (Fig. 1b). These phagocytes play an

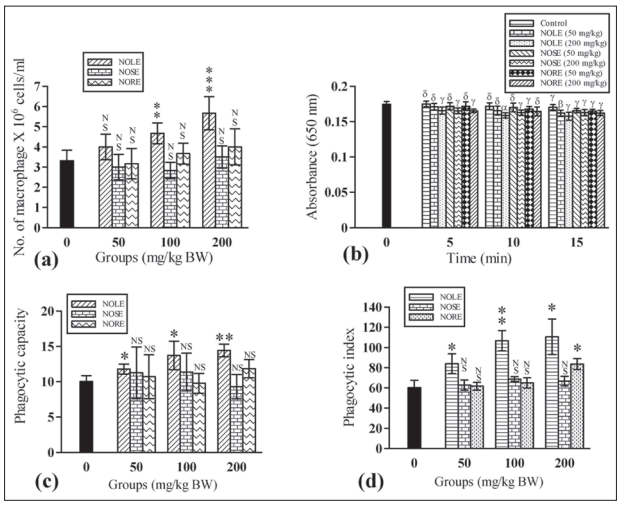

Fig. 1. Immunomodulatory activities of oleander leaf, stem and root extracts. The effects on **(a)** the total macrophage count; **(b)** carbon-clearance test; **(c)** phagocytic capacity; **(d)** phagocytic index. NS= P>0.05, *= P<0.05, **= P<0.01, ***= P<0.001 vs. 0 mg/kg group. δ= P>0.05, γ= P<0.05, β= P<0.01, α= P<0.001 vs. control.

important role in the clearance of non-specific foreign particulates (carbon particle in Indian ink) from the systemic circulation. The gradual decrease in absorbance at 650 nm with time indicated the rate of carbon clearance from the systemic circulation. NOLE at a dose of 200 mg/kg demonstrated the highest level of carbon-clearance activity at 15 min compared to the control group. Similarly, only NOLE at 200 mg/kg demonstrated a significant (P<0.01) increase in phagocytic capacity, which was 1.43-fold higher than the control in the phagocytic assay (Fig. 1c). Similarly, a significant (P<0.05) dose-dependent increase in the phagocytic index of NOLE and NORE at the highest dose was also observed (Fig. 1d). In a preliminary study, Bor et al. [17] demonstrated that oleander extract induces phagocytosis in canine neutrophils and they predicted that the extract may promote healing through efficient phagocytic process. Previously Muller et al. [16] showed a stimulation in phagocytic activity by a polysaccharide-rich fraction from the aqueous extract of the oleander leaves. The present study is therefore in agreement with the previous findings that oleander extracts exert stimulatory activity on phagocytes.

The recognition and phagocytosis of invading bacteria is the primary function of macrophages. Inside the phagosome, highly reactive hypochlorous acid (HOCl) is generated through the myeloperoxidase reaction and a plethora of oxygenated radicals are produced due to respiratory burst activity. Respiratory burst activity was measured at 630 nm, where an increase in absorbance signifies an increase in respiratory burst activity. A statistically significant increase (P<0.01) in respiratory burst was observed only in the case of NOLE (Fig. 2a). The extent of increase in respiratory burst at 100 µg/mL for NOLE, NOSE and NORE were 1.70-, 1.32- and 1.31-fold, respectively. However, the MPO level was reduced significantly (P<0.001) for all the extracts (Fig. 2b). At 100 µg/mL, the amount of reduction in MPO for NOLE, NOSE and NORE was 16.00±1.64%, 7.17±1.68% and 10.64±0.83%, respectively.

It is of interest to note that in the present study the increase in phagocytosis and respiratory burst activities was not accompanied by MPO release, which might appear paradoxical (Fig. 3). However, it has

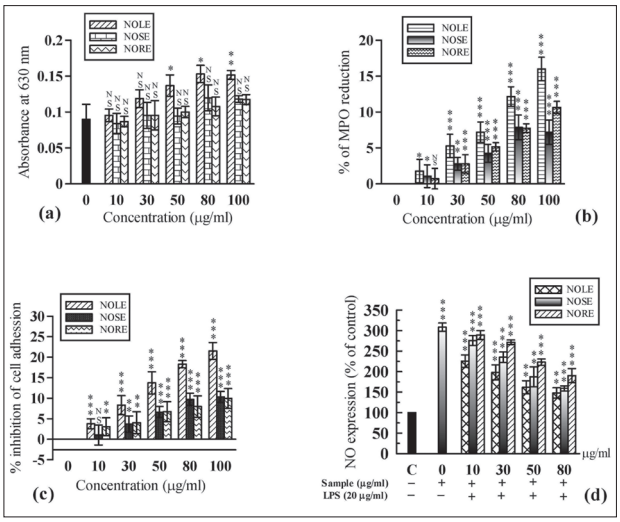

Fig. 2. The effect of oleander leaf, stem and root extracts on murine peritoneal macrophages. The effects on **(a)** respiratory burst activity; **(b)** myeloperoxidase (MPO) release; **(c)** cell-adhesion property; **(d)** nitric oxide (NO) release. NS= P>0.05, *= P<0.05, **= P<0.01, ***= P<0.001 vs. 0 µg/mL or control (C).

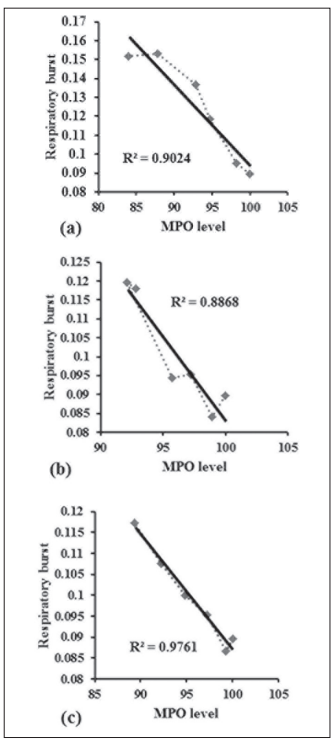

Fig. 3. Dose-dependent correlation analysis between respiratory burst activity and myeloperoxidase (MPO) level. The effects of **(a)** NOLE, **(b)** NOSE and **(c)** NORE. Axes 'x' and 'y' denote correlation points of respective MPO release and respiratory burst at different doses of oleander extract (0-100 µg/mL). R2=coefficient of determination.

previously been demonstrated that phagocytic activity elevates in MPO-deficient granulocytes [18-20]. It has been hypothesized [20] that phagocytic activity in MPO-deficient cells can be enhanced due to the increased expression of complement 3b- or Fc-receptors, which are translocated from the intracellular pool to the cell surface, the process being comparatively easier in MPO-deficient cells [21]. It was demonstrated [19] that the extent of complement 3b- and Fc-receptor-mediated phagocytosis is decreased in zymosan-activated MPO-deficient cells when induced with extracellular MPO. Furthermore, it is essential to note that NOLE, NOSE and NORE also demonstrated potent superoxide radical, H_2O_2 and hypochlorous acid scavenging activities, all of which are key elements of MPO reaction during respiratory burst activity [1]. Thus, the direct inhibition of MPO reaction by means of free radical scavenging may have contributed to the gradual reduction in MPO level.

During localized inflammation, circulatory macrophages are recruited to the site of inflammation and enter the target tissue by adhering to and passing between the endothelial cell lining of the blood vessels in an innate immune response termed as extravasation. P- and M-selectins and their carbohydrate counter ligands initially mediate rolling and tethering of the macrophages [22]. Thereafter, the integrins and their ligands mediated firm cell adhesion. In this process, various mediators such as IL-8 and macrophage inflammatory protein (MIP-1b), TNF, IL-1β and different chemokines play a vital role in activating the integrins on the surface of the macrophage [23,24]. Murine peritoneal macrophages, under stimulation with NOLE, NOSE and NORE demonstrated significant ($P<0.001$) inhibition of cell-adhesion properties. At 100 µg/mL, the percentage of inhibition of cell adhesion for NOLE, NOSE and NORE were 21.52±2.06%, 10.32±1.32% and 9.92±2.41%, respectively (Fig. 2c). The present study demonstrated the inhibition of cell adhesion properties due to *N. oleander* extracts, which is in accordance with similar studies. Previous reports suggested that plant extracts with immunomodulatory or anti-inflammatory properties possess the potential to downregulate the cell-adhesion properties in phagocytes by inhibiting the expression of vascular cell adhesion protein-1 (VCAM-1) or P-selectin [7,25,26]. The cell-adhesion inhibitory activity of oleander extracts may have resulted due to its TNF inhibitory activity [3], because TNF is a potent inducer of cell-adhesion activity in phagocytes.

NO is released from activated macrophages and functions as a marker for inflammatory progression and cytotoxic activity. Even though NO itself possess bactericidal activity, as a result of its coupling with the superoxide radical generates the highly reactive peroxynitrite radical. Therefore, the suppression of NO release during chronic inflammatory diseases has been a central idea behind the functioning of anti-inflammatory drugs. Dong et al. [27] previously showed that a polysaccharide fraction from *N. oleander* flower stimulates NO production in RAW264.7 cells. However, the present study demonstrated the potent activity of *N. oleander* extracts in inhibiting the expression of NO in LPS-stimulated macrophages (Fig. 2d). The extent of NO inhibition at 80 µg/mL in the case of NOLE, NOSE and NORE was around 0.47-, 0.51- and 0.61-fold, respectively, compared to the 0 µg/mL group. The IC_{50} values of NOLE, NOSE and NORE were 66.25±6.66, 142.83±17.58 and 142.83±17.58 µg/mL, respectively. The lowering of NO was not cell-viability-mediated as the extracts had negligible effect on macrophage viability, as demonstrated by the MTT method (Fig. 4). The percentage of viable cells at 100 µg/mL for leaf, stem and root was 97.44±0.80, 94.35±1.12 and 96.68±0.55, respectively. The similar

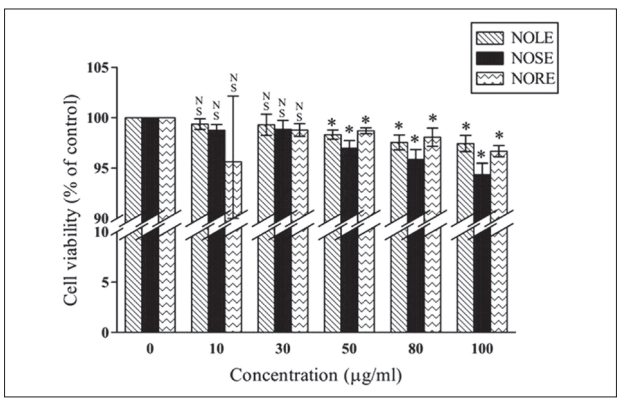

Fig. 4. The effect of NOLE, NOSE and NORE on macrophage viability measured by the MTT method. $^{NS}P>0.05$ and $^{*}P<0.05$ Vs 0 µg/mL group.

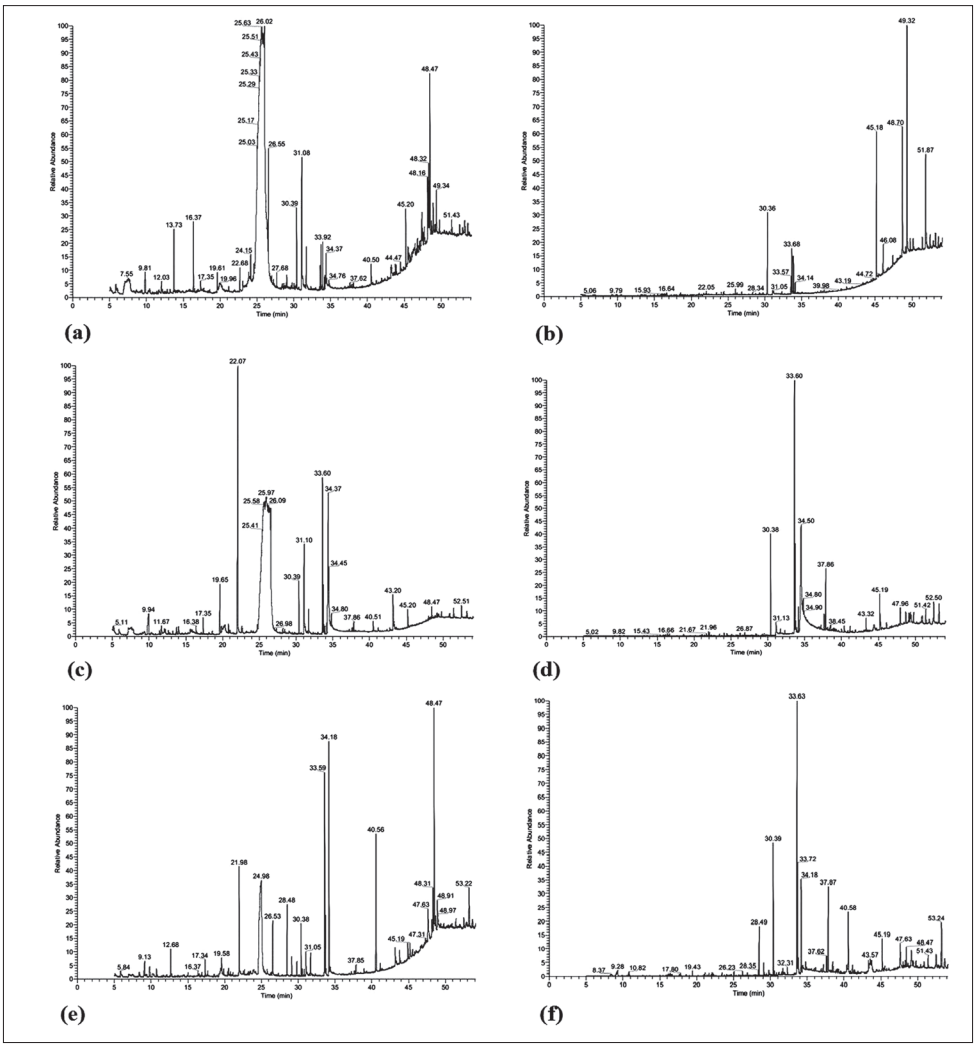

Fig 5. GC chromatogram of various parts of *N. oleander*. **(a)** NOLE methanol fraction; **(b)** NOLE n-hexane fraction; **(c)** NOSE methanol fraction; **(d)** NOSE n-hexane fraction; **(e)** NORE methanol fraction; **(f)** NORE n-hexane fraction. The identified compounds corresponding to the figs are enlisted in Table 1-6.

NO inhibitory activity of oleander leaf extract has also been demonstrated on murine splenic lymphocytes stimulated with concanavalin A [3). In fact, the 70% hydromethanolic extracts of oleander demonstrated the *in vitro* free radical NO scavenging capacities, with NOSE demonstrating greater activity than NOLE and NORE [1]. The NO inhibitory activity of oleander may prove beneficial in the case of inflammatory diseases such as multiple sclerosis, arthritis, juvenile diabetes and ulcerative colitis, which are associated with the chronic expression of NO [1].

The present results of anti-inflammatory activities of oleander by the inhibition of cell-adhesion properties and NO release also correlate with the study of Sreenivasan et al. [28], who demonstrated that the chief cardiac glycoside of oleander suppresses the progression of inflammation in a wide variety of cells, including macrophages, by inhibition of the activation of the transcription factors nuclear factor-κβ and activator protein-1, both of which are primary regulators of inflammation. Moreover, inhibition of inflammatory progression by oleander leaf extract was

also demonstrated by the inhibition of NO, COX activates, prostaglandin levels and modulation of pro- and anti-inflammatory cytokines in murine lymphocytes [3,29].

NOLE, NOSE and NORE were further subfractionated using a bi-solvent system and subjected to GC-MS analysis to reveal the phytochemical constituents in oleander (Fig 5. and Table 1-6). Results

Table 1. Compounds identified in NOLE methanol subfraction. Corresponds to Fig 5a.

Sl No	Compound name	Formula	Retention Time
1.	6-Oxa-bicyclo[3.1.0]hexan-3-one	$C_5H_6O_2$	5.84
2.	Diglycerol	$C_6H_{14}O_5$	7.55
3.	D-Fructose, diethyl mercaptal, pentaacetate	$C_{20}H_{32}O_{10}S_2$	8.23
4.	3-O-Benzyl-d-glucose	$C_{13}H_{18}O_6$	8.62
5.	β-D-Glucopyranose, 4-O-β-D-galactopyranosyl-	$C_{12}H_{22}O_{11}$	9.35
6.	Cyclohexanamine, N-3-butenyl-N-methyl-	$C_{11}H_{21}N$	9.81
7.	7-Methyl-Z-tetradecen-1-ol acetate	$C_{17}H_{32}O_2$	10.24
8.	trans-13-Octadecenoic acid	$C_{18}H_{34}O_2$	10.41
9.	DL-Arabinose	$C_5H_{10}O_5$	11.65
10.	2,5-Octadecadiynoic acid, methyl ester	$C_{19}H_{30}O_2$	12.03
11.	4-Benzoyloxy-1-morpholinocyclohexene	$C_{17}H_{21}NO_3$	12.15
12.	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	$C_6H_6O_4$	12.80
13.	1,3-Benzenediol (syn. Resorcinol)	$C_6H_6O_2$	13.20
14.	Benzofuran, 2,3-dihydro- (syn. Coumaran)	C_8H_8O	13.73
15.	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	16.37
16.	Phenol, 2,6-dimethoxy- (syn. Pyrogallol 1,3-dimethyl ether and syringol)	$C_8H_{10}O_3$	17.35
17.	2-Myristynoyl pantetheine	$C_{25}H_{44}N_2O_5S$	17.78
18.	Phenol, 2,3,5-trimethyl- (syn. Isopseudocumenol)	$C_9H_{12}O$	19.61
19.	3-tert-Butyl-4-hydroxyanisole (syn. 3-BHA)	$C_{11}H_{16}O_2$	22.68
20.	d-Mannose	$C_6H_{12}O_6$	23.88
21.	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	26.55
22.	5,5,8a-Trimethyl-3,5,6,7,8,8a-hexahydro-2H-chromene	$C_{12}H_{20}O$	27.68
23.	Ethanone, 2-(benzoyloxy)-1-[1,1'-biphenyl]-4-yl-	$C_{21}H_{16}O_3$	28.42
24.	1-Heptatriacotanol	$C_{37}H_{76}O$	28.65
25.	Pentadecanoic acid	$C_{15}H_{30}O_2$	29.05
26.	8,11,14-Eicosatrienoic acid, (Z,Z,Z)-	$C_{20}H_{34}O_2$	29.77
27.	11,13-Dihydroxy-tetradec-5-ynoic acid, methyl ester	$C_{15}H_{26}O_4$	8.67
28.	Cyclopropanedodecanoic acid, 2-octyl-, methyl ester	$C_{24}H_{46}O_2$	30.31
29.	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	30.39
30.	n-Hexadecanoic acid (syn. Palmitic acid)	$C_{16}H_{32}O_2$	31.08
31.	Estra-1,3,5(10)-trien-17β-ol	$C_{18}H_{24}O$	31.20
32.	5,6-Dimethoxyphthalaldehydic acid (syn. Opianic acid)	$C_{10}H_{10}O_5$	31.69
33.	9,12-Octadecadienoic acid, methyl ester, (E,E)- (syn. Linolelaidic acid, methyl ester)	$C_{19}H_{34}O_2$	33.59
34.	9-Octadecenoic acid (Z)-, methyl ester (syn. Oleic acid, methyl ester)	$C_{19}H_{36}O_2$	33.70
35.	Phytol	$C_{20}H_{40}O$	33.92
36.	Heptadecanoic acid, 16-methyl-, methyl ester	$C_{19}H_{38}O_2$	34.17
37.	trans-13-Octadecenoic acid	$C_{18}H_{34}O_2$	34.37
38.	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	37.62
39.	Cholestan-3-one, cyclic 1,2-ethanediyl aetal, (5β)-	$C_{29}H_{50}O_2$	38.08
40.	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester (syn. Dipalmitin)	$C_{35}H_{68}O_5$	40.50
41.	Squalene	$C_{30}H_{50}$	45.20
42.	Pregn-5-en-20-one, 3-(acetoxy)-16,17-epoxy-6-methyl-, (3β,16α)-	$C_{24}H_{34}O_4$	48.47
43.	Vitamin E	$C_{29}H_{50}O_2$	49.34

Table 2. Compounds identified in NOLE n-hexane subfraction. Corresponds to Fig 5b.

Sl No	Compound name	Formula	Retention Time
1.	4-Cyclopropyl carbonyloxytridecane	$C_{17}H_{32}O_2$	9.79
2.	1-Dodecanol, 3,7,11-trimethyl-	$C_{15}H_{32}O$	9.91
3.	10-Heptadecen-8-ynoic acid, methyl ester, (E)-	$C_{18}H_{30}O_2$	10.74
4.	2-Myristynoyl pantetheine	$C_{25}H_{44}N_2O_5S$	13.18
5.	2-Hexadecanol	$C_{16}H_{34}O$	14.96
5.	Octadecane, 6-methyl-	$C_{19}H_{40}$	15.93
7.	1-Hexadecanol, 2-methyl-	$C_{17}H_{36}O$	16.12
8.	Cyclohexane, 1,3,5-trimethyl-2-octadecyl-	$C_{27}H_{54}$	18.42
9.	Tetradecane	$C_{14}H_{30}$	18.55
10.	Phenol, 3,5-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	21.37
11.	Cyclopropanedodecanoic acid, 2-octyl-, methyl ester	$C_{24}H_{46}O_2$	21.66
12.	Tetradecane, 2,6,10-trimethyl-	$C_{17}H_{36}$	22.05
13.	Ethanol, 2-(octadecyloxy)-	$C_{14}H_{30}$	23.42
14.	17a-Ethyl-3β-methoxy-17a-aza-D-homoandrost-5-ene-17-one	$C_{22}H_{35}NO_2$	23.71
15.	Dodecanoic acid, 1-methylethyl ester	$C_{15}H_{30}O_2$	24.05
16.	3-O-Methyl-d-glucose	$C_7H_{14}O_6$	24.40
17.	1,7-di-iso-propylnaphthalene	$C_{16}H_{20}$	25.36
18.	Tetradecanal	$C_{14}H_{28}O$	25.99
19.	Tetradecanoic acid, 12-methyl-, methyl ester	$C_{16}H_{32}O_2$	28.34
20.	Ethanol, 2-(9-octadecenyloxy)-, (Z)-	$C_{20}H_{40}O_2$	28.74
21.	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	30.36
22.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	31.05
23.	Hexadecanoic acid, 14-methyl-, methyl ester	$C_{18}H_{36}O_2$	32.29
24.	Methyl 9-cis,11-trans-octadecadienoate	$C_{19}H_{34}O_2$	33.57
25.	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	33.68
26.	Phytol	$C_{20}H_{40}O$	33.90
27.	Methyl stearate	$C_{19}H_{38}O_2$	34.14
28.	Cholestan-3-one, cyclic 1,2-ethanediyl aetal, (5β)-	$C_{29}H_{50}O_2$	38.05
29.	Squalene	$C_{30}H_{50}$	45.18
30.	Heptacosane	$C_{27}H_{56}$	48.70
31.	α-Tocopherol	$C_{29}H_{50}O_2$	49.32

Table 3. Compounds identified in NOSE methanol subfraction. Corresponds to Fig 5c.

Sl No.	Compound name	Formula	RT
1.	6-Oxa-bicyclo[3.1.0]hexan-3-one	$C_5H_6O_2$	5.88
2.	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	$C_6H_8O_4$	7.16
3.	Diglycerol	$C_6H_{14}O_5$	7.47
4.	3-Allyloxy-1,2 propanediol	$C_6H_{12}O_3$	7.57
5.	Glycerin	$C_3H_8O_3$	7.69
6.	2,5-Piperazinedione	$C_4H_6N_2O_2$	8.78
7.	Thymine	$C_5H_6N_2O_2$	9.94
8.	1-Dodecanol, 3,7,11-trimethyl-	$C_{15}H_{32}O$	10.34
9.	Methyl 6-oxoheptanoate	$C_8H_{14}O_3$	10.49
10.	Eicosanoic acid, phenylmethyl ester	$C_{27}H_{46}O_2$	10.85
11.	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (syn. Pyranone)	$C_6H_8O_4$	11.67
12.	Hexanediamide, N,N'-di-benzoyloxy-	$C_{20}H_{20}N_2O_6$	12.21
13.	5-Methoxypyrrolidin-2-one	$C_5H_9NO_2$	21.61

Table 3. continued

Sl No.	Compound name	Formula	RT
14.	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl- (syn. 5-Hydroxymaltol)	$C_6H_6O_4$	12.82
15.	Catechol	$C_6H_6O_2$	13.20
16.	Cystine	$C_6H_{12}N_2O_4S_2$	13.65
17.	Benzofuran, 2,3-dihydro- (syn. Coumaran)	C_8H_8O	13.73
18.	5-Hydroxymethylfurfural (syn. HMF)	$C_6H_6O_3$	13.99
19.	Methyl 6-oxoheptanoate	$C_8H_{14}O_3$	15.59
20.	Desulphosinigrin	$C_{10}H_{17}NO_6S$	15.82
21.	2-Methoxy-4-vinylphenol (syn. p-Vinylguaiacol)	$C_9H_{10}O_2$	16.38
22.	d-Glycero-d-ido-heptose	$C_7H_{14}O_7$	16.52
23.	Phenol, 2,6-dimethoxy- (syn. Syringol And syn. Pyrogallol 1,3-dimethyl ether)	$C_8H_{10}O_3$	17.35
24.	Methyl 3,4-tetradecadienoate	$C_{15}H_{26}O_2$	18.11
25.	Vanillin lactoside	$C_{20}H_{28}O_{13}$	18.85
26.	Ethanone, 1-(2-hydroxyphenyl)-	$C_8H_8O_2$	19.65
27.	Phenol, 2-methoxy-4-(1-propenyl)- (syn. Isoeugenol)	$C_{10}H_{12}O_2$	19.90
28.	Apocynin	$C_9H_{10}O_3$	20.80
29.	Ethanone, 1-(2,4-dihydroxyphenyl)-	$C_8H_8O_3$	22.07
30.	Benzoic acid, 4-hydroxy-3-methoxy- (syn. Vanillic acid)	$C_8H_8O_4$	22.61
31.	2,5-Dihydroxy-4-isopropyl-2,4,6-cycloheptatrien-1-one	$C_{10}H_{12}O_3$	22.69
32.	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	23.09
33.	3-O-Methyl-d-glucose	$C_7H_{14}O_6$	25.78
34.	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	26.57
35.	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl) methyl ester, cis-	$C_{28}H_{44}O_4$	27.70
36.	Benzoic acid, 4-hydroxy-3,5-dimethoxy- (syn. Syringic acid)	$C_9H_{10}O_5$	28.20
37.	Ethanone, 2-(benzoyloxy)-1-[1,1'-biphenyl]-4-yl-	$C_{21}H_{16}O_3$	28.43
38.	Pentadecanoic acid	$C_{15}H_{30}O_2$	29.05
39.	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	30.39
40.	n-Hexadecanoic acid (syn. Palmitic acid)	$C_{16}H_{32}O_2$	31.10
41.	5,6-Dimethoxyphthalaldehydic acid (syn. Opianic acid)	$C_{10}H_{10}O_5$	31.71
42.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	33.60
43.	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	33.71
44.	trans-13-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	33.81
45.	Phytol	$C_{20}H_{40}O$	33.94
46.	Methyl stearate	$C_{19}H_{38}O_2$	34.17
47.	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	34.37
48.	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	14.51
49.	9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester	$C_{21}H_{38}O_4$	43.20
50.	Squalene	$C_{30}H_{50}$	45.20
51.	γ-Sitosterol	$C_{29}H_{50}O$	52.51

Table 4. Compounds identified in NOSE n-hexane subfraction. Corresponds to Fig 5d.

Sl No.	Compound name	Formula	Retention Time
1.	10-Heptadecen-8-ynoic acid, methyl ester, (E)-	$C_{18}H_{30}O_2$	5.14
2.	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis-	$C_{28}H_{44}O_4$	6.86
3.	2-Myristynoyl pantetheine	$C_{25}H_{44}N_2O_5S$	7.61
4.	cis-13-Eicosenoic acid	$C_{20}H_{38}O_2$	8.89
5.	1-Hexadecanol, 2-methyl-	$C_{17}H_{36}O$	9.82

Table 4. continued

Sl No.	Compound name	Formula	Retention Time
6.	1-Dodecanol, 3,7,11-trimethyl-	$C_{15}H_{32}O$	10.27
7.	2,7-Diphenyl-1,6-dioxopyridazino[4,5:2',3']pyrrolo[4',5'-d]pyridazine	$C_{20}H_{13}N_5O_2$	11.96
8.	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	$C_{26}H_{54}$	12.19
9.	9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol, (3β,5Z,7E)-	$C_{27}H_{44}O_3$	12.97
10.	Dodecane	$C_{12}H_{26}$	13.20
11.	Octadecane, 1-(ethenyloxy)-	$C_{20}H_{40}O$	13.36
12.	Octadecane, 6-methyl-	$C_{19}H_{40}$	14.44
13.	Tetradecane, 2,6,10-trimethyl-	$C_{17}H_{36}$	14.98
14.	Dodecane, 2,7,10-trimethyl-	$C_{15}H_{32}$	15.43
15.	2,4-Decadienal, (E,E)-	$C_{10}H_{16}O$	15.80
16.	3-Eicosene, (E)-	$C_{20}H_{40}$	16.13
17.	1-Octadecyne	$C_{18}H_{34}$	16.38
18.	Dodecane, 2,6,11-trimethyl-	$C_{15}H_{32}$	16.66
19.	Tetradecane	$C_{14}H_{30}$	15.95
20.	Nonanoic acid, 9-oxo-, methyl ester	$C_{10}H_{18}O_3$	19.44
21.	Hexadecane	$C_{16}H_{34}$	21.07
22.	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	21.38
23.	Tetradecanoic acid, 12-methyl-, methyl ester	$C_{16}H_{32}O_2$	21.67
24.	Ethanone, 1-(2,4-dihydroxyphenyl)-	$C_8H_8O_3$	21.96
25.	Hexadecane, 2,6,11,15-tetramethyl-	$C_{20}H_{42}$	22.07
26.	Ethanol, 2-(octadecyloxy)-	$C_{20}H_{42}O_2$	22.33
27.	Nonadecane	$C_{19}H_{40}$	23.34
28.	17-Octadecenal	$C_{18}H_{34}O$	23.73
29.	Dodecanoic acid, 1-methylethyl ester	$C_{15}H_{30}O_2$	24.07
30.	3-O-Methyl-d-glucose	$C_7H_{14}O_6$	24.44
31.	Methyl tetradecanoate (syn. Myristic acid, methyl ester)	$C_{15}H_{30}O_2$	26.23
32.	2-Hexadecanol	$C_{16}H_{34}O$	27.18
33.	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	28.35
34.	1-Hexadecanol	$C_{16}H_{34}O$	21.45
35.	9,12-Hexadecadienoic acid, methyl ester	$C_{17}H_{30}O_2$	29.73
36.	9-Hexadecenoic acid, methyl ester, (Z)- (syn. .Methyl palmitoleate)	$C_{17}H_{32}O_2$	29.97
37.	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	30.38
38.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	31.13
39.	Oleic Acid	$C_{18}H_{34}O_2$	31.96
40.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (syn. Linoleic acid, methyl ester)	$C_{19}H_{34}O_2$	33.60
41.	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	33.71
42.	Methyl stearate	$C_{19}H_{38}O_2$	34.16
43.	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	34.50
44.	12-Methyl-E,E-2,13-octadecadien-1-ol	$C_{19}H_{36}O$	38.08
45.	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	39.99
46.	Heptacosane	$C_{27}H_{56}$	40.35
47.	Oleic acid, 3-(octadecyloxy)propyl ester	$C_{39}H_{76}O_3$	44.38
48.	Squalene	$C_{30}H_{50}$	45.19
49.	Octadecanal, 2-bromo-	$C_{18}H_{35}BrO$	45.26
50.	17-Pentatriacontene	$C_{35}H_{70}$	47.96
51.	Vitamin E	$C_{29}H_{50}O_2$	49.33
52.	Desmosterol	$C_{27}H_{44}O$	49.77

Table 4. continued

Sl No.	Compound name	Formula	Retention Time
53.	Stigmasterol	$C_{29}H_{48}O$	51.42
54.	γ-Sitosterol	$C_{29}H_{50}O$	52.50
55.	β-Amyrin	$C_{30}H_{50}O$	53.22

Table 5. Compounds identified in NORE methanol sub-fraction. Corresponds to Fig 5e.

Sl No.	Compound name	Formula	Retention Time
1.	1,2-Cyclopentanedione	$C_5H_6O_2$	5.84
2.	Desulphosinigrin	$C_{10}H_{17}NO_6S$	6.95
3.	Butanoic acid, 2-ethyl-, 1,2,3-propanetriyl ester	$C_{21}H_{38}O_6$	8.37
4.	Z-3-Methyl-2-hexenoic acid	$C_7H_{12}O_2$	9.13
5.	Octanoic acid	$C_8H_{16}O_2$	10.75
6.	12-Methyl-E,E-2,13-octadecadien-1-ol	$C_{19}H_{36}O$	10.81
7.	1-Octene, 3-(methoxymethoxy)-	$C_{10}H_{20}O_2$	12.68
8.	Cyclohexan-1,4,5-triol-3-one-1-carboxylic acid	$C_7H_{10}O_6$	13.18
9.	Cyclopropanedodecanoic acid, 2-octyl-, methyl ester	$C_{24}H_{46}O_2$	13.64
10.	5-Octadecenal	$C_{18}H_{34}O$	14.45
11.	8-Hydroxy-2-octanone	$C_8H_{16}O_2$	15.02
12.	Acetamide, N-methyl-N-[4-(3-hydroxypyrrolidinyl)-2-butynyl]-	$C_{11}H_{18}N_2O_2$	15.82
13.	2-Methoxy-4-vinylphenol (syn. p-Vinylguaiacol)	$C_9H_{10}O_2$	16.37
14.	1,3,5-Pentanetriol, 3-methyl-	$C_6H_{14}O_3$	16.89
15.	Phenol, 2,6-dimethoxy- (syn. Syringol and Pyrogallol 1,3-dimethyl ether)	$C_8H_{10}O_3$	17.34
16.	γ-Dodecalactone	$C_{12}H_{22}O_2$	17.64
17.	n-Butyric acid 2-ethylhexyl ester	$C_{12}H_{24}O_2$	17.72
18.	2-Myristynoyl pantetheine	$C_{25}H_{44}N_2O_5S$	17.64
19.	Vanillin lactoside	$C_{20}H_{28}O_{13}$	18.57
20.	d-Glycero-d-ido-heptose	$C_7H_{14}O_7$	19.45
21.	Ethanone, 1-(2-hydroxyphenyl)-	$C_8H_8O_2$	19.58
22.	Phenol, 2-methoxy-4-(1-propenyl)- (syn. Isoeugenol)	$C_{10}H_{12}O_2$	19.88
23.	Oleic Acid	$C_{18}H_{34}O_2$	20.53
24.	1,4-Dimethoxy-2,3-dimethylbenzene	$C_{10}H_{14}O_2$	20.78
25.	Ethanone, 1-(2,4-dihydroxyphenyl)- (syn. β-Resacetophenone)	$C_8H_8O_3$	21.98
26.	Benzoic acid, 4-hydroxy-3-methoxy- (syn. Vanillic acid)	$C_8H_8O_4$	22.54
27.	3-tert-Butyl-4-hydroxyanisole (syn. 3-BHA)	$C_{11}H_{16}O_2$	22.67
28.	1-Dodecanol, 3,7,11-trimethyl-	$C_{15}H_{32}O$	23.96
29.	3-O-Methyl-d-glucose	$C_7H_{14}O_6$	24.98
30.	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	$C_{10}H_{12}O_3$	26.53
31.	Acetic acid, 3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octa-hydronaphthalen-2-yl ester	$C_{17}H_{26}O_3$	28.48
32.	Nootkaton-11,12-epoxide	$C_{15}H_{22}O_2$	29.12
33.	Isoaromadendrene epoxide	$C_{15}H_{24}O$	29.81
34.	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	30.38
35.	1-Methylcarbazole	$C_{13}H_{11}N$	30.80
36.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	31.05
37.	Hexadecanoic acid, 14-methyl-, methyl ester	$C_{18}H_{36}O_2$	32.31
38.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (syn. Linoleic acid, methyl ester)	$C_{19}H_{34}O_2$	33.59
39.	9-Octadecenoic acid (Z)-, methyl ester (syn. Oleic acid, methyl ester)	$C_{19}H_{36}O_2$	33.70
40.	Murrayafolin a	$C_{14}H_{13}NO$	34.18

Table 5. continued

Sl No.	Compound name	Formula	Retention Time
41.	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	34.79
42.	11-Eicosenoic acid, methyl ester	$C_{21}H_{40}O_2$	37.20
43.	Cholestan-3-one, cyclic 1,2-ethanediyl aetal, (5β)-	$C_{29}H_{50}O_2$	38.08
44.	1H-Indole, 3-t-butyl-1-methyl-2-phenyl-	$C_{19}H_{21}N$	40.56
45.	Oleanolic acid	$C_{30}H_{48}O_3$	47.63
46.	Pregn-5-en-20-one, 3-(acetyloxy)-16,17-epoxy-6-methyl-, (3β,16α)-	$C_{24}H_{34}O_4$	48.47
47.	β-Amyrin	$C_{30}H_{50}O$	53.22

Table 6. Compounds identified in NORE n-hexane subfraction. Corresponds to Fig 5f.

Sl No.	Compound name	Formula	Retention Time
1.	17-Octadecynoic acid	$C_{18}H_{32}O_2$	7.39
2.	Butanoic acid, 2-ethyl-, 1,2,3-propanetriyl ester	$C_{21}H_{38}O_6$	8.37
3.	2-Pentenoic acid, 2,3-dimethyl-	$C_7H_{12}O_2$	9.28
4.	17-Octadecynoic acid	$C_{18}H_{32}O_2$	9.73
5.	1-Dodecanol, 3,7,11-trimethyl-	$C_{15}H_{32}O$	9.82
6.	Tetradecane, 2,6,10-trimethyl-	$C_{17}H_{36}$	10.46
7.	3-Methylpentan-3-yl propyl carbonate	$C_{10}H_{20}O_3$	10.82
8.	2-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	10.88
9.	1-Octene, 3-(methoxymethoxy)-	$C_{10}H_{20}O_2$	12.66
10.	Dodecane	$C_{12}H_{26}$	13.20
11.	Hexadecane, 1,1-bis(dodecyloxy)-	$C_{40}H_{82}O_2$	13.36
12.	Octadecane, 1-(ethenyloxy)-	$C_{20}H_{40}O$	13.60
13.	Octadecane, 6-methyl-	$C_{19}H_{40}$	14.44
14.	2-Myristynoyl pantetheine	$C_{25}H_{44}N_2O_5S$	14.98
15.	Decane, 2,3,5,8-tetramethyl-	$C_{14}H_{30}$	15.42
16.	1,2-15,16-Diepoxyhexadecane	$C_{16}H_{30}O_2$	15.79
17.	Tridecane	$C_{13}H_{28}$	15.95
18.	2-Isopropyl-5-methyl-1-heptanol	$C_{11}H_{24}O$	16.13
19.	1-Hexadecanol, 2-methyl-	$C_{17}H_{36}O$	16.37
20.	2(3H)-Furanone, dihydro-5-pentyl-	$C_9H_{16}O_2$	16.52
21.	Pentanoic acid, 4-oxo-, methyl ester	$C_6H_{10}O_3$	17.80
22.	Tetradecane	$C_{14}H_{30}$	18.57
23.	Nonanoic acid, 9-oxo-, methyl ester	$C_{10}H_{18}O_3$	19.43
24.	Oxiraneoctanoic acid, 3-octyl-, methyl ester, cis-	$C_{19}H_{36}O_3$	21.00
25.	Phenol, 2,4-bis(1,1-dimethylethyl)- (syn. Antioxidant No. 33)	$C_{14}H_{22}O$	21.39
26.	Methyl 9-methyltetradecanoate	$C_{16}H_{32}O_2$	21.67
27.	4-Acetyl-2-hydroxyphenyl hexopyranoside	$C_{14}H_{18}O_8$	21.96
28.	Dodecane, 2,6,11-trimethyl-	$C_{15}H_{32}$	22.07
29.	Nonanedioic acid, dimethyl ester	$C_{11}H_{20}O_4$	22.22
30.	Ethanol, 2-(octadecyloxy)-	$C_{20}H_{42}O_2$	23.42
31.	Tetradecanal	$C_{14}H_{28}O$	23.73
32.	Dodecanoic acid, 1-methylethyl ester	$C_{15}H_{30}O_2$	24.06
33.	Dodecanoic acid, 2,3-bis(acetyloxy)propyl ester	$C_{19}H_{34}O_6$	24.42
34.	β-Eudesmol	$C_{15}H_{26}O$	24.66
35.	Tetradecane, 2,6,10-trimethyl-	$C_{17}H_{36}$	25.68
36.	Methyl tetradecanoate	$C_{15}H_{30}O_2$	26.23

Table 6. continued

Sl No.	Compound name	Formula	Retention Time
37.	Eicosane, 2-methyl-	$C_{21}H_{44}$	26.86
38.	2-Hexadecanol	$C_{16}H_{34}O$	27.88
39.	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	28.02
40.	Ethanol, 2-(9-octadecenyloxy)-, (Z)-	$C_{20}H_{40}O_2$	28.17
41.	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	28.35
42.	Acetic acid, 3-hydroxy-6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octa-hydronaphthalen-2-yl ester	$C_{17}H_{26}O_3$	28.49
43.	Nootkaton-11,12-epoxide	$C_{15}H_{22}O_2$	29.11
44.	1-Eicosanol	$C_{20}H_{42}O$	29.45
45.	(-)-Spathulenol	$C_{15}H_{24}O$	29.80
46.	9-Hexadecenoic acid, methyl ester, (Z)-	$C_{17}H_{32}O_2$	29.96
47.	9H-Carbazole, 9-methyl- (syn. N-Methylcarbazole)	$C_{13}H_{11}N$	30.79
48.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	31.10
49.	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	31.70
50.	cis-10-Heptadecenoic acid	$C_{17}H_{32}O_2$	31.83
51.	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	32.31
52.	Octadecanoic acid, 3-hydroxy-, methyl ester	$C_{19}H_{38}O_3$	33.24
53.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (syn. Linoleic acid, methyl ester)	$C_{19}H_{34}O_2$	33.63
54.	9-Octadecenoic acid (Z)-, methyl ester (syn. Oleic acid, methyl ester)	$C_{19}H_{36}O_2$	33.72
55.	11-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	33.80
56.	Murrayafolin a	$C_{14}H_{13}NO$	34.18
57.	Nonadecanoic acid, methyl ester	$C_{20}H_{40}O_2$	35.58
58.	cis-11-Eicosenoic acid, methyl ester	$C_{21}H_{40}O_2$	37.20
59.	Eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$	37.62
60.	9,12-Octadecadienoyl chloride, (Z,Z)-	$C_{18}H_{31}ClO$	37.87
61.	Heptacosane	$C_{27}H_{56}$	40.36
62.	1H-Indole, 3-t-butyl-1-methyl-2-phenyl-	$C_{19}H_{21}N$	40.58
63.	α-Amyrin	$C_{30}H_{50}O$	43.57
64.	Lup-20(29)-en-3-ol, acetate, (3β)-	$C_{32}H_{52}O_2$	43.78
65.	Squalene	$C_{30}H_{50}$	45.19
66.	Allocholesterol	$C_{27}H_{46}O$	49.15
67.	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	49.37
68.	Desmosterol	$C_{27}H_{44}O$	49.77
69.	Campesterol	$C_{28}H_{48}O$	50.86
70.	Stigmasterol	$C_{29}H_{48}O$	51.43
71.	γ-Sitosterol	$C_{29}H_{50}O$	52.51
72.	β-Amyrin	$C_{30}H_{50}O$	53.24

revealed the presence of several compounds that possess the potential to modulate the activation, proliferation, phagocytosis, extravasation, NO release, respiratory burst and MPO level of murine macrophages. A wide variety of phenolic and flavonoid compounds were identified which are known to possess potent immunomodulatory activities [30-34]. Phenol, 2,6-dimethoxy-(pyrogallol derivative), tocopherol, isoeugenol, γ-Sitosterol, palmitic acid, oleic acid, amyrin, myristic acid, desmosterol, stigmasterol, p-vinylguaiacol, β-resacetophenone, vanillic acid, 3-tert-butyl-4-hydroxyanisole, murrayafolin, oleanolic acid, β-eudesmol, spathulenol, linoleic acid, campesterol, apocynin, syringic acid, catechol, 5-hydroxymethyl-

furfural, phytol and vanillin are the major bioactive constituents identified, which directly correlates with the macrophage modulatory activities of oleander.

CONCLUSIONS

The present study demonstrated that oleander extracts possess the potential to modulate murine macrophages by stimulating phagocytosis and related activities. The bioactivities of oleander leaf were found to be superior to those of the stem and root. Phytochemical investigations revealed the presence of several bioactive constituents, the synergistic and additive activities of which may be attributed to the immunomodulatory activity of oleander.

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